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Characterisation of genes encoding peritrophins from the peritrophic matrix of Diptera : Drosophila and Glossina.

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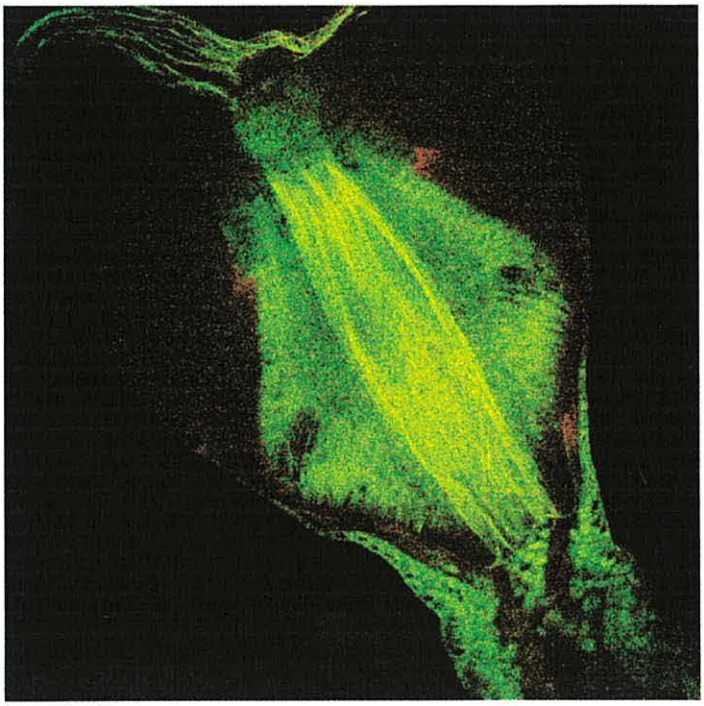

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Abstract

Our aim was to improve our molecular understanding of the type 2 peritrophic matrix (PM) in Diptera. This was achieved by identifying novel chitin binding proteins (peritrophins) in *Drosophila* adult and larval PMs upon isolating cardia-specific cDNAs. A differential library screening strategy yielded ten cardia-specific clones.

A (mostly Fluorescent) *In Situ* Hybridisation approach was used to accurately determine the site of peritrophin transcription in wholemount cardia. The images obtained provide evidence of clear-cut zones of peritrophin expression within the cardia epithelium, used to infer the particular PM layer into which these proteins are inserted, and thus predict their distribution within the mature PM.

Attempts to set up PM-compromised fly lines in which specific peritrophins were knocked down using RNA interference were confounded by redundancy. The deficiency *Df(3L)vin3*, causing maximal disruption to larval PM integrity, served to test the protective function of the PM. Mortality was three-fold greater in deficient compared to wild-type larvae infected with pathogenic bacteria.

Quantitative Reverse Transcription-PCR studies were performed to assess the modulation of peritrophin transcription in response to physiological fluctuations. Despite two days starvation, peritrophin transcription levels in older flies are three times higher than in newly eclosed flies. peritrophin transcription was upregulated following *per os* bacterial infection in larvae only.

In *Glossina*, eight putative peritrophins were identified with the aid of bioinformatics, five of which share similarities with known PM proteins. Transcripts were preferentially expressed in the cardia of *G. morsitans morsitans*, *G. pallidipes* and *G. palpalis*, although they were not modulated in response to trypanosome infection. The homologs showed little variation in the hypervariable regions between conserved cysteine and aromatic regions characteristic of chitin-binding domains.

The results demonstrate the potential link between peritrophins and PM structure, and thus function. A number of remaining questions, worthy of future investigation, are suggested.

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Abbreviations

Standard SI units of measurement have been used throughout this work. Standard DNA and RNA IUPAC codes were adopted, and product sizes are given in either kilobases (kb) or base pairs (bp).

aa	amino acid(s)
$A_{260/280}$	Absorption at 260/ 280 nm
AMP	antimicrobial peptide
AP	Alkaline phosphatase
APES	3-aminopropyltriethoxy-silane
APR	anterior to the PM secreting ring
BCIP	5-bromo-4-chloro-3-indoyl-phosphate
BSA	Bovine serum albumin
CBD	Chitin Binding Domain
CBM_14	Chitin Binding Motif 14
CBP	Chitin Binding Protein
cDNA	copy DNA
CFU	colony forming unit
CG	*Drosophila* coding gene
CHS	chitin synthase
ChtBD2	Chitin Binding Type 2 Domain
CIP	calf intestinal phosphatase
cpm	counts per minute
CRDs	cysteine rich domains
cRNA	recombinant RNA
C_T	threshold cycle
CTLMA	C-type lectin with mannose specificity
Cy-	indocarbocyanine
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
Df	Deficiency
DIC	Differential Interference Contrast Microscopy
DIG	digoxigenin-11-UTP
DmPro	*Drosophila melanogaster* Proventriculus
DNase	Deoxyribonuclease, an enzyme degrading DNA
dsRNA	double-stranded RNA
E	oesophagus
EDTA	disodium ethylene diamine tetraacetate
ECML	extracellular membrane layers
ECPS	Ectoperitrophic space
ENPS	Endoperitrophic space
EST	Estimated sequence tag
EtBr	Ethidium bromide
FG	foregut
Fig.	Figure
(F)ISH	(Fluorescent) *in situ* hybridisation
FITC	fluorescein isothiocyanate
Fuc	Fructose
GAG	Glycosaminoglycan
Gal	Galactose

GalNAc	N-acetyl-galactosamine
GlcNAc	N-acetyl-glucosamine
GmPro	*Glossina morsitans* Proventriculus
GS	glutamine synthase
HKG	House keeping gene
HMT	hindgut and Malpighian tubule
HS	hybridisation solution
IR	inverted-repeat
ISH	*in situ* hybridisation
L	PM layer
L^{-1}	per litre
LB	Luria Bertani
LM	Light Microscope
MG	midgut
MPL	mucin/ peritrophin-like
mRNA	messenger RNA
MUC-	mucin
NBT	4-Nitroblue tetrazolium chloride
nt	nucleotide
ORF	open reading frame
P-	peritrophin
PAS	Periodic acid-Schiff reagent
PBM	post blood meal
PBS	Phosphate buffered saline
PBT	PBS, 0.1% tween 20
pC	pCaSpeR construct
PCR	Polymerase Chain Reaction
PE	peritrophic envelope
PG	proteoglycan
pH	hydrogen ion potential
PL	peritrophin like
PM	peritrophic matrix
PM1	Type 1 PM
PM2	Type 2 PM
PPR	posterior to the PM secreting ring
PR	PM secreting ring
PTSBs	proline, threonine and serine residues
pW	pWIZ construct
QRT-PCR	Quantitative RT-PCR
RC	Reverse complement
RNAi	RNA interference
RNase	Ribonuclease, an enzyme degrading RNA
R.T.	Room temperature
RT-PCR	Reverse transcriptase-PCR
S	Sense
SDS	Sodium dodecyl sulphate
SDW	sterile distilled water
S.E.M.	Scanning Electron Microscopy
SH	Subtractive Hybridisation
siRNA	small interfering RNA
SSC	Saline sodium citrate
SSH	Suppression Subtractive Hybridisation

SV	stomodeal valve
TE	Tris-EDTA
TEM	Transmission Electron Microscope (Microscopy)
T_m	melting temperature
UPW	Ultra pure water
UTR	Untranslated region
V	ventriculus
v/v	Volume added to volume
w/v	Weight added to volume

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Preface and Acknowledgements

Over twenty five years ago A. G. Richards brought to our attention the disparity between morphological and 'modern' sources of knowledge on the peritrophic matrix (PM): "It is time for PM workers to become knowledgeable in current methods of cell biology and to apply such methods". Today, with the completion of the *Drosophila* genome project, the potential for molecular biology to reveal novel concepts about the PM is at its height. The type 2 PM deserves much more attention than it receives at present, since those haematophagous insects with a type 2 PM, e.g. tsetse, are often more refractory to infection compared to mosquitoes (type 1 PM) for example, which carry so many of the debilitating parasites of man and his domesticated animals. It has been my hope that this study may help answer one of the open questions about the PM: its protective function, and thus make a valuable contribution towards clearing up some of the remaining uncertainty about this structure. When I embarked on this doctoral project in autumn 2000, the molecular characterisation of PM proteins was just starting to gain the attention it deserves. In the years that ensued over a dozen PM proteins from four species have been identified and studied.

This PhD thesis has had a far from trivial impact on my life, at least in part due to the Persistence, Hard work and Determination that has gone into its realization. Only now, at the penultimate stage can I fully appreciate the full meaning of the saying 'what you reap is what you sow'. In addition, it has been rewarding in a different sort of way, as each of the genes characterised during this doctoral research project has become sufficiently familiar to me to be regarded as old friends, with whom I may be reunited in the foreseeable future.

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Chapter 1. General Introduction

1.1. Abstract

Peritrophic matrices (or peritrophic membranes) (PMs) are discrete acellular polysaccharide rich matrices that typically line the midgut of most insects at one or more stages of the life cycle. They are common in members of all animal phyla, particularly the phylum Arthropoda. Traditionally thought to simply act as an inert sieve, this hypothesis is under revision as more of the molecular characteristics of PM proteins are discovered. The PM is believed to have important roles in the facilitation of digestion and protection of the insect from ingested toxins and invasion by pathogens and parasites. This review summarises the extensive literature available on the PM of insects, with particular attention to Diptera. Both long-standing evidence and new findings about PMs are presented, with emphasis on the potential of molecular biology to clarify speculative hypotheses about the protective role of the PM.

1.2. Perspectives and Overview

Balbiani (1890) first coined the appropriate name "peritrophic membrane" (surrounding the food) over 100 years ago, as 'a sort of membranous sac which directly surrounds the food in the lumen of the intestine' ; although, such structures were observed as early as 1762 by Lyonet in *Cossus cossus* larvae (Lepidoptera) (Lyonet, 1762). Today the term peritrophic matrix more accurately describes the PM, since a membrane has been re-determined as a lipid bilayer (Ramos *et al.*, 1994). The term now covers any membranous layer that is secreted by midgut epithelial cells, not necessarily associated with food (Peters, 1992). An earlier proposal by Peters to substitute the term "membrane" with "envelope", thus encompassing those PMs composed of numerous layers, was largely ignored except in studies of Lepidoptera (Peters, 1992).

The success of the class Insecta is largely derived from their exploitation of divergent ecological niches; this ability being attributed in part to their diverse gut morphology and function, which almost certainly involves variation in the PM (Peters, 1992). Since the PM has evolved to form numerous divergent structures and functions, including cocoon fabrics (Kenchington, 1976; Tristram, 1977), faecal pellet coverings (Turner and Ferrante, 1979) and the apron of endoparasitic female Strepsiptera larvae (Myrmecolacidae) (Kathirithamby, 2000).

PMs are often thought to be restricted to the Phylum Arthropoda or the Class Insecta (Mason and Gilbert, 1954). However, this is not the case since they have been identified in members of more than eight animal phyla (Table 1) (Mason and Gilbert, 1954; Wharton and

Brody, 1972; Peters, 1976; Peters, 1992; Walters *et al.*, 1995) but are not present in all species. Thus, for example the PM seems to be absent in many lice and fleas (Peters, 1992).

It has become clear during the writing of this review that there is still much to be elucidated about the PM. In particular its protective role needs to be clarified by identifying those components that are essential for its barrier function and determining what happens when this barrier breaks down. This review should help to consolidate our current knowledge of the PM, whilst attempting to identify where more research is needed. In particular details about how some pathogens and parasites evade the PM barrier are given, followed by an account of previous attempts to use a number of PM proteins to create vaccines against medical and veterinary important insects.

1.3. What is the PM? Identification, Origin, Types and Occurrence

1.3.1. Identification

The PM is an acellular secretion product, more analogous to insect cuticle than plasma membrane, that is distinct from the insect integument, since it is secreted by both ecto- and endodermal cells of the foregut and midgut respectively (Kenchington, 1976). The PM was once argued to be an artefact; simply a condensation product resulting from the disturbance of the gut contents during dissection (Freyvogel and Jaquet, 1965; Freyvogel and Staubli, 1965), or a manifestation of the interface between proteolytic enzymes and the blood meal (Gemetchu, 1972). Richards and Richards (1977) proposed a number of characteristics that are indicative of PMs, which lead to the working definition of the PM as 'any solid or seemingly solid secretion of the midgut epithelium'. A more precise definition is presently impossible due to the enormous diversity of PM types; whilst, an agreed upon working definition of a PM is just as lacking. Transmission Electron Microscopy (T.E.M.) has traditionally provided the majority of data about the PM in a particular species, biochemical analysis not being extensively applied to PM research until the 1980s. For many years the detection of chitin has remained the only, generally accepted identifier for the PM despite a reliance on a single criterion being far from ideal (Waterhouse, 1953a).

1.3.2. Origin of the PM

According to a speculative model of the origin and evolution of the PM, the midgut of ancestral insects was lined with mucus, similar to that found in vertebrates. Human gastrointestinal mucus is a gel-like substance composed of mucins (Allen *et al.*, 1993). It is proposed that during evolution the PM was derived from gastrointestinal mucus, facilitated by the evolution of

peritrophins from mucins due to the acquisition of chitin binding domains and the accompanying evolution of chitin secretion by midgut epithelial cells for the formation of the chitin meshwork of the PM (Shen and Jacobs-Lorena, 1999). This hypothesis is supported by the presence of mucin-like domains in the amino acid sequence of some peritrophins (Tellam, 1996; Tellam *et al.*, 1999). Assuming that this hypothesis is correct, the PM would originally have been synthesised by the entire midgut epithelium and had properties of mucus. The evolution of a protein-chitin meshwork would have resulted in PM formation. The restriction of PM synthesis to particular zones of the midgut occurred more recently in evolutionary time (Peters, 1992). Thus, PM formation by the whole midgut epithelium is considered as the ancestral condition; and conversely PM production by small groups of cells or its complete absence, derived conditions. Indeed the ancestral origin of the PM is indicated by its presence in a wide number of animal phyla, and the presence of a PM 1 in Onychophorans (Table 1), since Waterhouse (1953b) believed Onychophorans to represent the only extant 'missing-link' between the phyla Annelida and Arthropoda.

1.3.3. Types of PM

PMs are usually classified into two types, categorised by their method of production, the secretion of PM 1 occurring over a more diffuse area than the discrete site of formation of PM 2 (Wigglesworth, 1930). Classification is traditionally based on Wigglesworth (1929), and it is this notation that is what I shall adopt (Wigglesworth, 1930; Wigglesworth, 1972). He designated the two alternative modes of PM formation as type 1 and 2; in contrast to Waterhouse (1953b) who, confusingly, defines them in the opposite sense to Wigglesworth. PM types vary amongst insect taxa and developmental stages (larvae versus adult). Lists of insects that posses and lack the different PM types have been composed by several authors (Waterhouse, 1953a and 1953b; Wigglesworth, 1972; Richards and Richards, 1977; Peters, 1992) a summary of which is given in table 2, section 1.3.4.

However, many variations exist that are based upon these two basic methods of formation (Peters, 1992), which may reflect the evolution of many variations from a single ancestral form. Some species produce a PM only from the posterior or middle midgut epithelium zones, such as the weevil *Cionus,* and the beetle *Ptinus* respectively (Rudall and Kenchington, 1973). Moreover, a composite PM is produced by certain species, such as *Apis* and *Liponeura* (Blepharoceridae) that secrete a PM 2 from the cardia, which is supplemented with PM 1 proteins as it moves through the midgut (Wigglesworth, 1930). In a few species the PM does not belong to either group, such as in a number of Hemipterans, including *Rhodinius prolixus.* They instead produce a non-chitinous, extracellular membrane with some similarities

to PM 1, in that it is secreted by the entire midgut epithelium following feeding (Perrone and Spielmann, 1988).

Many species produce a specific PM type at different feeding stages. For example, mosquitoes commonly secrete a PM 1 as adults, and PM 2 as larvae, reflecting the variation in the nature of their diet. Even when the same PM type is secreted by the different life stages, such as in blowflies, the PMs have different biochemical properties (Stamm *et al.*, 1978; Tellam, 1996). This suggests that the PM has evolved to closely match the food type, supported by the change from PM 2 in larval mosquitoes to PM 1 in adults. The expression of genes encoding individual components of the PM spatially reflects the type of PM formed in that insect. Schorderet *et al.* (1998) demonstrated that P-48 is expressed exclusively by the cardia of *Lucilia cuprina* larvae, in accordance with its PM 2 status (Schorderet *et al.*, 1998). Conversely, the peritrophin Ag-Aper1, from adult female *Anopheles gambiae* (Shen and Jacobs-Lorena, 1998) and the mucin IIM from *Trichoplusia ni* (Tellam *et al.*, 1999) are expressed exclusively by the midgut epithelium of these PM 1 insects.

1.3.3.1. Formation of the PM by the whole midgut (PM 1)

The two types of PM have distinct properties, PM 1 are thick (commonly 2-20 μm), extracellular matrices, secreted by the entire midgut epithelium (Perrone and Spielmann, 1988) in response to feeding (Wigglesworth, 1930). PM 1 are the most common type of PM in haematophagous insects, including mosquitoes (Perrone and Spielmann, 1988), blackflies and sandflies (Gemetchu, 1972).

Synthesis of the PM is not synchronised across the whole midgut epithelium, but is restricted to smaller areas, forming patches of PM that delaminate from the microvilli to become intermingled with the gut contents, before consolidating into a single PM. In contrast, almost the whole midgut acts as the formation zone for PMs in the earthworm *Lumbricus terrestris* (Peters and Wiese, 1986). In Decapods the diverticula, caeca and midgut gland also contribute to the PM secreted by the midgut epithelium (Peters, 1992). By contrast, harvestmen (Opilionida) secrete a PM from both the anterior and posterior midgut 2-4 hours post ingestion (Peters, 1969). Hemiptera produce extracellular membrane layers (ECML), which are formed by the entire midgut epithelium, that resemble the PM, but are just 10 nm thick and have an unknown function, but are probably the functional equivalent of a PM (Billingsley and Downe, 1983). In Culicidae only adult females produce a PM, since the males feed on nectar instead of blood. The whole midgut epithelium forms the PM, which is voided with the remainder of the blood meal after digestion has occurred (Perrone and Spielmann, 1988).

The midgut caeca of insects may also contribute to the PM secreted by the midgut

epithelium, as is the case in *Locusta migratoria, Schistocerca gregaria* and *Periplaneta americana*, resulting in the formation of a PM (Baines, 1978; Bernays, 1981). A much thinner caecal membrane is secreted by the caeca of *Aedes aegypti* and *Anopheles stephensi* in addition to the PM (Volkmann and Peters, 1989a). It is believed to function as a barrier to prevent the escape of cellular debris from the caeca and maintain a pH gradient across the midgut-caecal boundary. In insects where PMs are formed by both the midgut epithelium and caeca the PM could be classed as both PM 1 and PM 2, and may represent the missing link during the evolution of PM 2 from PM 1.

A PM 1 may be produced continuously, as in locusts that feed constantly; or transiently, in response to feeding, as in adult female mosquitoes which feed intermittently (Freyvogel and Staubli, 1965). PM 1 are less structurally differentiated compared to PM 2 (Berner *et al.*, 1983). The different layers of the PM may be formed by sequential secretion of the different PM components or their differential aggregation following secretion (Lehane, 1997). A peritrophic envelope (PE), consisting of many concentric overlapping PMs is found in the locust *Locusta migratoria* and the cockroach *Periplaneta americana* (Peters, 1976; Peters, 1992). Whereas a single PM 1 of increasing thickness is secreted by blackflies and adult mosquitoes in response to feeding (Reid and Lehane, 1984; Richardson and Romoser, 1972).

There are fundamental differences in the mode of secretion of the PM 1 in adult anopheline and culicine mosquitoes. PM components are secreted *de novo* by posterior midgut epithelial cells of culicine mosquitoes. This mode of formation has been experimentally determined in *Aedes aegypti* by the use of α amanitin, a potent inhibitor of PM production (Rudin and Hecker, 1979). PM precursors are secreted by continuous delamination within 30 min post blood meal, forming successive PMs in the brush border of the midgut. In contrast, anopheline mosquitoes secrete a finite amount of PM precursors, previously stored within numerous apical secretion vesicles in midgut epithethial cells prior to the blood meal, as shown in *Anopheles stephensi* (Berner *et al.*, 1983) or *An. gambiae* (Hecker, 1977). The vesicle contents coalesce and condense before being released into the ECPS (Billingsley and Rudin, 1992). A recent study by Devenport *et al.* (2004) clarified the role of apical vesicles in *An. gambiae*, with the co-localisation of trypsin and PM precursors to the same secretory vesicles, prior to their secretion by exocytosis. They also further demonstrated how depleted stores of Ag-Aper1 are not replensished until sufficient time has elapsed for their re-synthesis and accumulation.

1.3.3.2. Formation of the PM by specialised areas of the midgut (PM 2)

In comparison to PM 1, PM 2 are thin (usually 0.1-2 μm thick), and are continuously produced as a single or small number of unbroken sleeves. Production is restricted to part of the midgut; commonly a small zone of specialised cells (Richards and Richards, 1971) called the formation zone, encased in the vulva cardiaca, which forms an organ termed the cardia. The cardia is commonly (and incorrectly) termed the "proventriculus" since the true proventriculus is part of the foregut (Peters, 1976; Peters, 1992). PM 2 are found in insects irrespective of their state of food ingestion. PM 2 are found in most adult and larvae of higher Diptera (Lehane, 1997), and a number of blood-sucking insects, including adult stablefly and tsetse fly (Lehane, 1976a; Lehane and Msangi, 1991), larval mosquitoes, blackflies and sandflies (Miller and Lehane, 1993a).

The PM is formed by the posterior midgut in Copepoda, the praying mantis, *Mantis religiosa,* larvae of the ant lion, *Myrmeleon europaeus*. In larvae of Ptinidae (Coleoptera) including *Ptinus techus* and *Gibbium psylloides*, secretion of the PM starts in the latter part of the anterior midgut, with subsequent material being added in the posterior midgut (Tristram, 1977).

Secretion of a PM by the cardia represents the highest specialization of the formation zone (Rizki, 1956), since just a small zone of secretion, consisting of 4 or more cell types is involved (Billingsley, 1990) amounting to only 300-400 cells in *A. aegypti* larvae. The formation zone consists of a large fold that occurs at the foregut-midgut junction (Fig. 1), and therefore encompasses both types of epithelium in its construction. The PM is already fully formed before passing through the vulva cadiaca, in terms of possessing a uniform thickness and form. Its movement posteriorly out of the formation zone is thought to be due to the pressure exerted by subsequent secretion of the PM rather than extrusion through the cardia press, as evident in the common earwig, *Forficula auricularia* (Wigglesworth, 1930; Peters *et al.*, 1979). It is believed that the cardia merely forms an ordered series of cell zones that secrete the components of the PM and a suitable extracellular environment in which they can be assembled. Since the cardia may function to prevent the ingress of food particles and microbes into the formation zone, which could potentially disrupt PM formation (Richards and Richards, 1969).

The structure of PM 2 varies widely in Diptera due to convergent evolution in species, specialisation of food intake, and similar differences between adults and larvae. The PM is produced by the cardia at a rate of production that varies with food intake. There is commonly an extensive accumulation of PM in the anterior midgut, which is believed to act as a reservoir of excess PM, to be utilised in situations when the demand for PM exceeds supply. This is preferable to the alternative of a sudden demand for PM disrupting its secretion in the formation zone. The number of concentric PMs can be increased from 1 up to 10, normally 2 or 3, by further folding of the epithelium in the formation zone. Even the primitive suborder Nematocera

produces several PMs, suggesting that the production of multiple PMs is not a derived character. Asymmetry is also observed in the type of PM produced at different instars, for example in the mosquito, and *Anisopus cinctus*. The PM of Brachycera Muscomorpha are not structurally diverse, as the PMs only vary in thickness. Adults commonly secrete 2-3 PMs, with *Drosophila* marking the start of the evolution of PM 2.

1.3.4. Occurrence of the PM

PMs are found in most arthropods (Tellam, 1996; Peters, 1992; Lehane, 1997), and the great majority of insects, even the exceptions Homoptera, Hemiptera and Thysanoptera, which lack true PMs, have instead perimicrovillar membranes (PEs) which are probably functionally equivalent to the PM. PMs appear to be absent from members of the orders: Diploglossata, Megaloptera, Phthiraptera, Psocoptera, Rhaphidioptera, Strepsiptera, Thysanoptera and Zoraptera (Peters, 1992). However, negative reports should be interpreted cautiously, since PM 1 are secreted only in response to ingestion (Richards and Richards, 1977) and can be solubilised by fixation. Waterhouse (1953b) suggested that the distribution of PMs across the entire kingdom Animalia has no discernable phylogenetic pattern, since PM 1 are found in adult Nematocera, Tabanomorpha and Asilomorpha; conversely, PM 2 are common in Dipteran larvae and adult Brachycera and Muscomorpha (Table 2).

It is widely accepted that PMs occur exclusively in larval and/or adult stages of insects, a theory supported by Richards and Richards (1977), who found that the pupae of *Aedes aegypti* lacked a PM. Similarly, Romoser (1979) identified membranous remnants in the gut of newly eclosed mosquitoes. This structure is believed to be the sloughed larval midgut epithelium, which becomes associated with the meconium during the pupal stage, termed the meconial PM (MPM) due to its location, acellular nature and positive chitosan test (Romoser *et al.*, 2000). A theory which has recently been supported by Moll (2001) who found that bacterial count in the midgut during metamorphosis in *Culex pipiens*, *Aedes aegypti* and *Anopheles punctipennis* indicated that any remaining larval gut bacteria are effectively confined by the MPM, before being eliminated in newly eclosed adults. This mechanism should aid the transition from larvae to adults in holometabolus insects where larvae are generalist feeders, potentially ingesting bacteria that are pathogenic to the adult.

1.4. Formation of the PM

Despite a statement by Neville (1975) that 'endodermis has not been proved capable of chitin secretion', midgut epithelial cells are of endodermal origin (Hecker, 1977), and yet have been

shown to secrete chitin both indirectly, through the localisation of chitin synthase expression (Zimoch and Merzendorfer, 2002) and directly by the binding lectins with an affinity to GlcNAc, such as WGA (Peters and Latka, 1986; Rudin and Hecker, 1989; Harper and Hopkins, 1997; Matsuo *et al.*, 2003). Furthermore, there is evidence that WGA strongly interferes with PM synthesis when present at even relatively low concentrations (Harper *et al.*, 1998; Hopkins and Harper, 2001).

Within the same species, the PM may be absent in some stages of development, whilst present in others. For example, the larvae, but not the adults of many Lepidoptera produce a PM (Waterhouse, 1953b). Whereas, a PM can be found in all the feeding stages, and even the non-feeding pupa of some species (Romoser and Rothman, 1979; Romoser *et al.*, 2000; Moll *et al.*, 2001).

The cellular aspects of PM synthesis and secretion have not been studied extensively. Richards and Richards (1977) proposed that the differential expression of PM types at different developmental stages of insects indicate that hormone levels, such as ecdysone may control PM production. A theory supported by the finding that ecdysone increases the rate of PM production by adult *Calliphora erythrocephala* (Becker, 1978).

Table 1. Occurrence of PMs: Animal phyla in which PM has been demonstrated. Adapted from Peters (1992). Key: +, present; -, absent; ?, unsure.

Phylum	Class	PM present/absent	chitin detected
Plathelminthes		-	
Nemertini		-	
Nemathelminthes		-	
Priapulida		+	+
Sipunculida		+	?
Echiurida		-	
Mollusca	Polyplacophora	+	+
	Gastropoda	+	+
	Scaphopoda	-	
	Bivalva	-	
	Cephalopoda	-	
Annelida	Polychaeta	+	+
	Archiannelida	+	+
	Myzostomida	+	?
	Oligochaeta	+	+
	Hirudinea	-	
Onychophora		+	+
Arthropoda	Merostomata	+	+
	Arachnida	+	+
	Pantopoda	-	
	Crustacea	+	+
	Chilopoda	+	+
	Diplopoda	+	+
	Insecta	+	+
Tentaculata	Bryozoa	+/-	-
	Brachiopoda	+	+
Hemichordata		+	-
Echinodermata	Holothuroidea	+	-
	Echinoidea	+	-
Chordata	Tunicata	+	+
	Acrania	+	?
	Agnatha	+	?
	Vertebrata	+	-

Table 2. The occurrence of PMs in adult Diptera.
Adapted from Peters (1976). Key: Reported by a, Waterhouse (1953b); b, Zhuzhikov (1963) and c, Wigglesworth (1929). Type: 1, PM 1; 2, PM 2; ?, PM type not recorded.

Suborder	Infraorder	Family	Reported by	Type
Nematocera	Tipulomorpha	Tipulidae	a	1
	Psychodomorpha	Psychodidae	a	1
	Culicomorpha	Culicidae	a	1
		Simuliidae	a	1
	Bibiomorpha	Bibionidae	a	1
Brachycera	Tabanomorph	Tabanidae	a/b	1
		Erinnidae	b	?
		Stratiomyidae	a	1
	Asilomorpha	Asilidae	a/b	1
		Therevidae	a	1
		Apioceridae	a	1
		Bombyliidae	a/b	1
		Dolichopodidae	a	1
	Muscomorpha	Musidoridae	b	?
		Syrphidae	a	2
		Trypetidae	b	?
		Ortalidae	a	2
		Sepsidae	b	?
		Trichoscelidae	b	?
		Clypselidae	b	?
		Sapromyidae	a	2
		Sciomyzidae	b	?
		Helomyzidae	b	?
		Dryomyzidae	b	?
		Drosophilidae	a/b	2
		Cordyluridae	b	?
		Muscidae	a/b	2
		Hipposboscidae	a	2
		Nycteribiidae	a	2
		Calliphoridae	a	2
		Sarcophagidae	a	2
		Oestridae	a	?
		Tachinidae	a	2
		Glossinidae	c	2

Distension of the midgut is the only prerequisite for PM formation in most insects possessing a PM 1. This was demonstrated by Freyvogel and Jaquet (1965) who induced PM secretion in culicine mosquitoes in response to an enema of water, saline or air, indicating that the response is purely due to the stimulation of stretch receptors. However, not all insects with a PM 1 produce the matrix solely in response to mechanical stimulation, as shown by damselfly larvae, that continue to produce a PM even when starved (Richards and Richards, 1977). There remains some disagreement between authors about the time of the first appearance of a distinct PM after feeding (Waterhouse, 1953b; Richardson and Romoser, 1972); the consensus being that it is produced following ingestion. During digestion of the blood meal, PM 1 become increasingly fragile, due to small faults occurring in the matrix (Adang and Spence, 1981), leading to the eventual disintegration of the PM (Stohler, 1957). The time lag between taking a blood meal and the first appearance of the PM is species specific in mosquitoes, which influences their vectoral capacity for *Plasmodium* parasites. The timings vary greatly, from 5 - 8 h post blood meal (PBM) in *Aedes aegypti*, 13 h in *An. gambiae* and 32 h in *An. stephensi* (Freyvogel and Staubli, 1965). Only female blackflies, *Samulium damnosum* produce a PM, which is produced by the entire midgut epithelium in response to a blood meal (Reid and Lehane, 1984). Sandflies, *Phlebotomus longipes* produce a PM 30 minutes PBM, which is subsequently reinforced as late as 48 h PBM, so that the mature PM is 1-5 μm thick, before being voided post digestion along with the residue of the blood meal 5 days later (Gemetchu, 1972).

Wigglesworth (1929; 1930) proposed from the structural studies of PM 2 from a number of insect species, that PM precursor material is secreted in the cardia, into the space bounded by the formation zone and the apposed foregut, known as the vulvula cardiaca, where it is pressed into a uniform layer. In this way the cardia was believed to act as an extrusion press, moulding the PM before it passes into the midgut lumen (Wigglesworth, 1930). Richards and Richards (1971) and Peters (1976) dismiss this hypothesis, suggesting instead that the PM has already matured on reaching the press in *Aedes aegypti* larvae and adult *Forficula*. They proposed that the true function of the vulvula cardiaca is a valve, used to exclude the contents of the midgut lumen from the vulnerable PM formation zone (Richards and Richards, 1971) in addition to aiding the transport of completed PM into the midgut and production of a lubricant. A theory supported by Becker (1977) who found that the PM accumulates as an amorphous mass when the structure of the vulva cardiaca is destroyed. Either way, the cardia acts as an ordered series of cell zones that sequentially secrete PM components, which assemble before reaching the putative mould. At best the structure of the cardia may physically transform the PM from a semi-fluid secretion into a solid matrix. *A. aegypti* larvae produce a single PM 2 (Zhuzhikov, 1966 and 1970). Whilst Becker (1978) showed that as many as three separate PMs could be formed in PM

2 producing Diptera, as a result of folding of the formation zone in the cardia. *L.cuprina* produce three PMs, that are spatially separate in the gut lumen and have different structures (Peters, 1992), whereas the two individual PMs produced by *S. calcitrans* converge in the midgut to form a composite PM, composed of five distinct layers (Lehane, 1976b). The PM of *Drosophila melanogaster* consists of four layers, each formed by a different region of the folded epithelial walls of the cardia to form a single multilayered PM (King, 1988).

The cardia occurs throughout the muscoid (higher) flies of the order Diptera (King, 1989). The phylogenetic analysis of cellular organization within the cardia conducted by King (1991) indicated that the cardia originated within Diptera from ancestrally undifferentiated tissues through a series of simple tissue transformations. Moreover, King (1991) hypothesized that the evolution of the specialised cell types obligatory for the formation of the cardia organ would only have been possible through the differentiation of cell function and localized gene expression. Genes encoding structural proteins and their regulatory components would in turn have been essential for the emergence of such cellular specialization. The expression of genes encoding PM chitin binding proteins exclusively within the cardia epithelium (Appendix) indicates that the cardia structure took form in order to localize the previously generalized region of PM synthesis.

There are a number of studies that document the known abnormalities in cardia development that result from mutations in *Drosophila*. Including BR-C mutants, in which defects in cardia structure and location occur in addition to the abnormal development of other regions of the gut in adults. These abnormalities are brought about by aberrant metamorphosis during pupation that result from a deficiency in programmed cell death (Restifo and White, 1992). More severe defects in cardia development are displayed by mutants with defective genes encoding *decapentaplegic* (*dpp*), *hedgehog* (*hh*) and *wingless* (wg^{ts}) that code for signalling molecules, *myospheroid* (*mys*) that codes for a β subunit of integrins (Pankratz and Hoch, 1995) and the gene *defective proventriculus* (*dve*) in *dve* mutants (Nakagoshi *et al.*, 1997; Fub and Hoch, 1998), since such mutations are lethal for homozygous individuals. For example, in homozygous *dve* mutants lethality of 1 day old L_1 larvae is due to the collapse of the cardia, which in turn blocks the gut, resulting in starvation (Fub and Hoch, 1998). Similarly, embryos of *dpp*, *hh* and wg^{ts} mutants display a very similar phenotype in homozygotes, namely, a defect in the development of the cardia, termed the 'cardiac arrest' phenotype (Pankratz and Hoch, 1995). This implies that these genes are required to properly coordinate cell movement during development of the cardia, particularly the endodermal part, so that the ectodermal portion of the cardia can invaginate normally, resulting in the formation of a functional organ. Indeed, the diagram in Fig. 1 shows that the *Drosophila* cardia is normally composed of 3 layers; the outer

layer, derived from the anterior midgut, the middle from the foregut and the inner from the oesophagus, it is the latter that fails to form in *dve*, *dpp*, *hh* and wg^{ts} homozygous mutant larvae. By contrast, the cardia phenotype of *mys* mutant embryos is believed to arise as a result of a defective integrin causing the improper accumulation of extracellular matrix material, in which the cardia is normally rich (King, 1988). Indeed, many cell movements are coordinated through interactions with the extracellular matrix (Pankratz and Hoch, 1995).

1.4.1 PM production rate

PM production rates are documented mainly for PM 2 insects since it is more easily measured in PM 2 producing insects. The rate is consistently slower than the rate at which ingested food passes through the alimentary tract (Waterhouse, 1954a). The precise rate varies greatly between species: Waterhouse (1954a) recorded rates that ranged from 5-10 mm per hour (hr^{-1}) for the PM 2 of third instar *L. cuprina* larvae. Adult *C. erythrocephala* form a PM 2 at a rate of 3.5 mm hr^{-1} which slows with time post emergence (Lehane, 1991). Harmsen (1973) found that adult tsetse flies produce PM 2 at 1 mm hr^{-1}. In many cases PM 2 producing insects are capable of sustaining PM production over long periods of time (East and Eisemann, 1993; East *et al.*, 1993). The formation rate of the PM can be determined by the incorporation of the labels: ^{14}C glucose, ^{35}S methionine, ^{35}S cysteine (Peters *et al.*, 1973) or congo red (Zhuzhikov, 1966; Peters *et al.*, 1973). Furthermore, this technique often results in the visualisation of a number of periodic units, representing labelled and unlabelled crossbands, that are composed of macro- (50-80 μm) and micro-units (10-14 μm).

Induction of PM formation upon feeding suggests that it is regulated for particular physiological reasons, such as DDT intoxication (Becker, 1978). Similarly, the rate of PM formation in some adult blood-sucking insects varies greatly. As a distinct PM appears just 6 h post blood meal in *Aedes aegypti* compared to 32 h in *Anopheles stephensi* (Freyvogel and Staubli, 1965). The reason for this difference is still unknown. Moreover, although PM production is a continuous process from emergence in most PM 2 producing species, the formation rate varies depending on the physiological state of the fly.

1.4.2 How are rapid PM production rates achieved?

The *in vitro* culture of PM from the cardia of Diptera was first demonstrated by Zimmermann *et al.* (1973) by the culture of cardia from adult *Calliphora erythrocephala* in appropriate media. The factors affecting PM formation rates have since been examined and a number of studies have demonstrated that PM synthesis occurs *in vitro* when pH, osmolarity and temperature are within certain parameters, indicating that critical enzymatic steps are involved (Peters *et al.*,

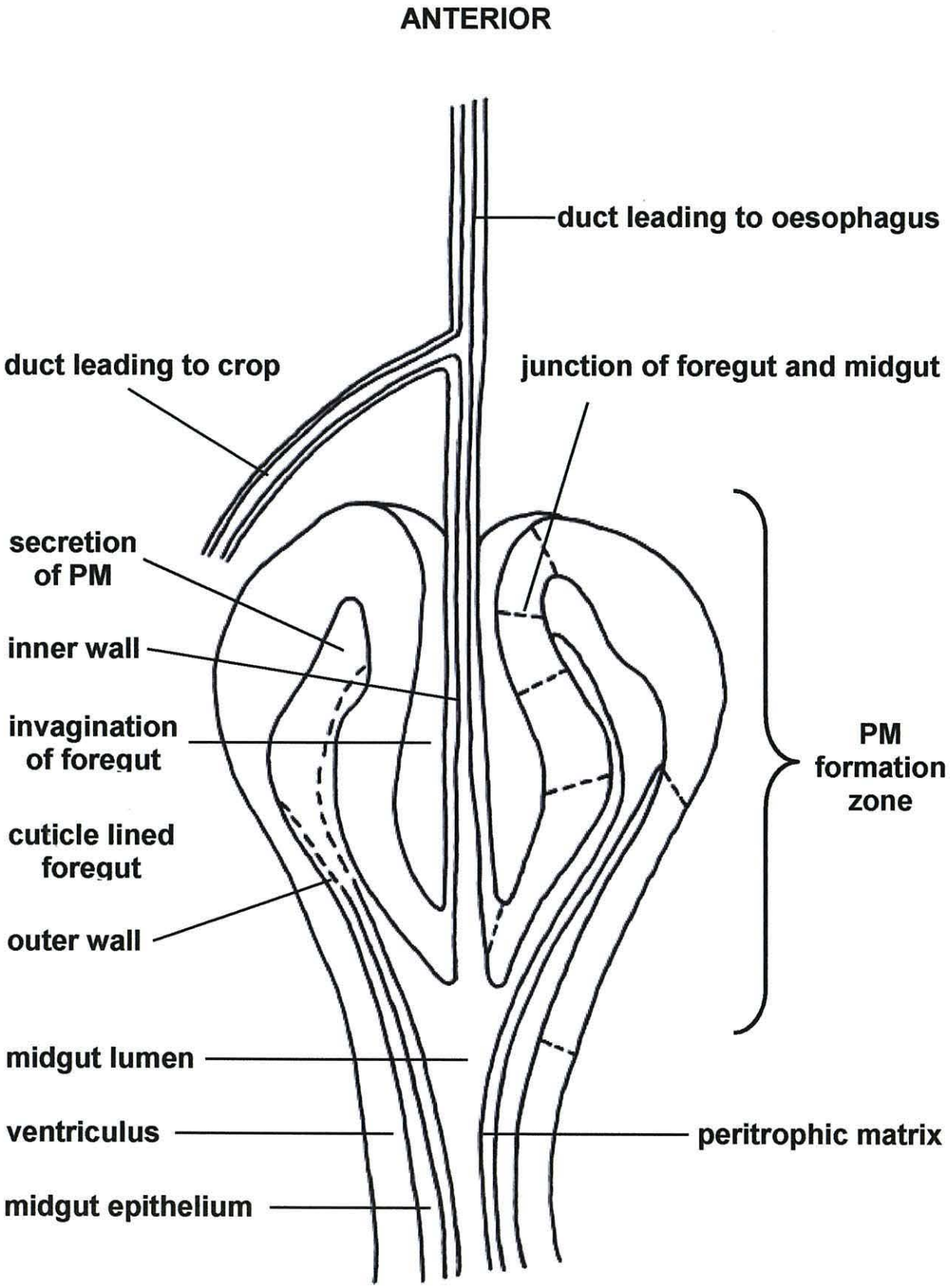
1973). In *vivo* studies have shown that PM production rate is also dependent on the fed or unfed state of the fly, as damselfly larvae produce 3 PM 1s per day (d^{-1}) when fed, compared to just 1 d^{-1} when starved (Waterhouse, 1954a). It may be expected that PM protein synthesis needs to be correspondingly rapid in order to achieve such quick formation rates. This is made possible, in part by the co-expression of a number of functionally identical PM proteins, the genes for which possess strong promoters (Schorderet *et al.*, 1998).

1.5. Structure of PMs

Mercer and Day (1952) first investigated PM structure, utilising the electron microscope to show that PMs are differentiated in structure. They are generally composed of two or more discrete layers or laminae, the number of which varies greatly between species (Ramos *et al.*, 1994). For example, the PM of the Douglas Fir tussock moth, *Orgyia pseudotsugata* is trilaminate (Brandt *et al.*, 1978). Furthermore, the PM of blackfly and mosquito larvae contain 5-6 layers (Peters, 1979); whilst the single PM 1 of *Simulium* species contains between 4-15 laminae (Reid and Lehane, 1984). PMs possessing a laminate substructure commonly have alternating electron dense and electron opaque layers (Peters, 1976; Peters, 1992). The electron dense layer (EDL) commonly represents a palisade pattern due to a number of rod-like structures lying perpendicular to the PM face; the electron-opaque layer being composed of granular or fibrillar material (Peters, 1976). The PM 2 of Diptera including *Drosophila, Culex, Lucilia* and *Chironomus* larvae and *Glossina* adults are genuinely complex multi-layered PMs (Peters, 1976). The EDLs within the laminate substructure have been shown to be rich in mucopolysaccharides; in contrast to the opaque layers, which consist mostly of chitin. The layers are believed to be formed sequentially by successive zones of secretion within the cardia (Lehane, 1976a), thus the layer facing the gut lumen must, by definition be secreted first, and subsequent layers added its epithelial side (Lehane, 1997). The luminal side of the PM is commonly lined by an EDL; one may also be present on the epithelial surface of the PM, but is much less discrete in comparison. The regular structure and constant thickness of PMs may be due to their regular macromolecular structure, thought to have arisen from the highly ordered molecular structure individual components coupled with their ordered synthesis in the cardia (Spence, 1991; Peters, 1992).

Spence (1991) proposed that chitin microfibrils provide the substructural network of the PM, onto which a superstructural proteoglycan matrix is added (Fig 2). This hypothesis is supported by evidence that the PM of larvae of the European corn borer consists of a chitin meshwork that is embedded in a proteinaceous matrix, formed by the microvilli (Harper and Hopkins, 1997; Harper *et al.*, 1998).

Fig. 1. Diagram of the longitudinal section through the *Drosophila melanogaster* proventriculus. Note: PM, peritrophic matrix; 1-6, six distinct zones of formation within the cardia epithelium. Adapted from King (1988).



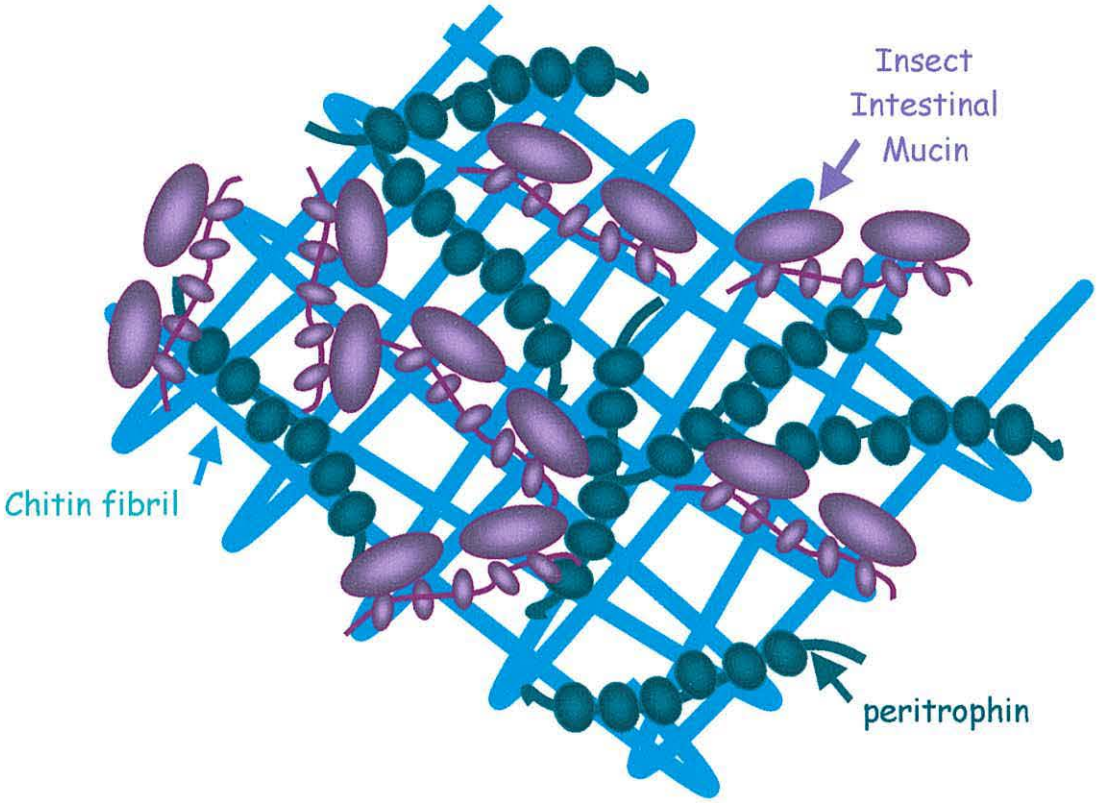
A mature PM forms once secreted proteoglycans hydrate in the gut lumen to form the bulk of the matrix and the other glycoprotein components of the PM (peritrophins) bind the different layers together through tight association. In some cases chitin microfibrils can be detected in the electron-opaque layers of the PM, embedded in an amorphous protein matrix, that are particularly developed in the PM 2 of Dipteran larvae (Peters, 1992). The arrangement of microfibrils was identified by Peters (1976) as belonging to one of three general types:

1. Random, or felt-like texture;
2. Hexagonal, or honeycomb texture;
3. Orthogonal, or grid-like network.

However, the mechanism behind the regular microfibril network of chitin still remains a contentious issue. Mercer and Day (1952) proposed that the arrangement of the microvilli in the midgut epithelium gave rise to PM texture by imprinting on the arrangement of the chitin microfibrils (Peters, 1992). This mold theory is supported by studies of the formation of the PM in *Drosophila* larvae (Dimitriadis, 1985) and *Trichoplusia ni* (Adang and Spence, 1981) in which the secretion of fibrous material between microvilli subsequently aggregates into a chitin meshwork. Conversely, a more plausible explanation is that the microvillar spacing acts as a determinant for the location of chitin synthase, which in turn creates regular patches devoid of chitin (Cohen, 1991). However, Neville (1975) believed that the classification of PMs according to the chitin microfibril architecture is meaningless, since such patterns merely reflect different arrangements of the microvilli.

A model of the basic PM unit has since been proposed for PM 2 (Schorderet *et al.*, 1998), in which the PM components: chitin, glycoproteins (peritrophins) and proteoglycans interact to dictate the properties of the PM (Peters, 1992; 1996). peritrophins, being evenly distributed throughout the PM, form cross-links with chitin microfibrils and other components of the matrix via chitin binding domains, leading to the stable structure of the PM (Fig. 2).

Fig. 2. Diagrammatic representation of PM structure.
Adapted from Wang and Granados (2001).



1.6. PM composition

As discussed earlier, the PMs of arthropods are typically composed of a meshwork of chitin microfibrils embedded in a proteinaceous matrix. Virtually nothing is known about the composition of PM 1, and only limited knowledge exists about PM 2 (Richards and Richards, 1977; Peters, 1992). The matrix consists of glycoproteins and proteoglycans (mucopolysaccharides) (Spence, 1991; Peters, 1992). The components usually make up the following proportions of the PM: chitin 3-13%; protein 20-60% and carbohydrate ~ 15% (Adang and Spence, 1981; Zimmermann and Peters, 1987). Although, as Richards and Richards (1977) state, the sum of all the components for those PMs measured fails to exceed 75%, highlighting the flaws in such measurements. The marked difference in the proportion of proteins and glycoproteins between adult and larval PMs of *Calliphora vicina* (Stamm *et al.*, 1978) indicates that the components of the PM change during development, suggesting that these differences may reflect varied functions. PM composition is known to change post secretion since it is disintegrated by the action of digestive enzymes (Richards and Richards, 1977).

1.6.1. Polysaccharide Terminology

There is a wealth of confusing terminology in glycobiology which needs to be clarified before discussing PM components in further depth. Glycosylation modifications to proteins are ubiquitous in biological systems since they provide a means to expand the information provided by a relatively concise genome, through the synthesis of numerous glycoforms. Polysaccharide containing molecules (PCMs) are required for many complex processes in eukaryotes. Glycoforms have variable bioactivities, allowing PCMs to perform many elaborate tissue-specific functions.

There are two basic types of glycosylation: O- and N-linked. O-linked glycosylation creates a vast array of highly complex structures through the variable glycoprotein side chains, of which there are usually five, namely N-acetyl-glucosamine (GlcNAc), N-acetyl-galactosamine (GalNAc), Galactose (Gal), Fructose (Fuc) and sialic acids, coupled with other modifications such as sulphation. N-linked glycosylation is the more ancient and common form, found in a wide variety of organisms. Normally 70-90% of potential N-glycosylation sites are occupied by N-linked glycans. N-linked glycans may serve the functions. Firstly, they are involved in protein trafficking, such as the quality control checks for protein folding carried out by the calnexin-calreticulin cycle in the cell, that involves endoplasmic reticulum (ER) lectins that interact with N-linked glycans. Secondly, a structural role may be attributed to these large and versatile molecules, increasing protein stability, e.g. within the PM through restricting the flexibility in conformation without compromising the net entropy of the system. Lastly, as ligands for carbohydrate receptors glycoproteins may constitute many growth factors and hormones, such as Erythropoietin (EPO), and equine luteinizing hormone (eLH) and corticogonadotropin (eCG).

Table 3. Definitions of glycosylated molecules relevant to the PM:

Chitin:	A homopolymer of poly-β-(1,4)-N-acetyl-D-glucosamine, forming the simplest glycosaminoglycan.
Glycolipid:	A polymer in which the core is formed from a lipid-like molecule, to which oligo- or polysaccharide side chains are attached. There is little evidence that PMs contain glycolipids.
Glycoprotein:	A polymer containing between 1-60% carbohydrate by weight, in the form of numerous, short, branched oligosaccharide side chains attached to a polypeptide core. In addition, mucin-type glycosylation may occur at single positions on glycoproteins (see below).
Proteoglycan (PG):	A molecule containing approximately 95% carbohydrate by weight, mostly in the form of GAG chains (see below), covalently linked to a protein core (5%) via a β-O-link to serine. The GAG component is the

main determinant of the physiological and chemical properties of PGs. PGs have potentially limitless heterogeneity, since the core peptide ranges from 10,000-600,000 Da in molecular weight and varies greatly in the nature of the attached GAGs.

Glycosaminoglycan (GAG): GAGs are structures that are commonly attached to the protein core of PGs. They are long (typically ~ 80 sugar residues), unbranched polysaccharide chains in the form of repeating disaccharide units, one of which is always the hexosamine GlcNAc or GalNAc, normally paired with a uronic acid residue of either D-glucuronic or iduronic acid. The molecule is hydophilic and has an extended conformation in solution due to the mutually repulsive negative charges on sulphate and carboxylate groups of uronic acid residues. As a consequence GAGs impart high viscosity on PGs.

Mucin-type glycosylation: The most common form of O-linked glycosylation, where GalNAc is α-O-linked to either serine or threonine residues on the protein core. Mucins are highly O-glycosylated proteins (>20 monosaccharides per glycan) forming an antler shaped surface coating which forces the protein core to adopt an extended conformation. Mucin-type glycosylation is characteristic of glycoproteins on cell surfaces, where they function in cell interactions, since the glycans extend beyond the glycocalyx.

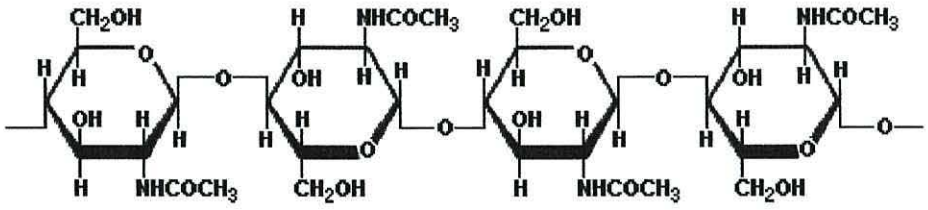
1.6.2. Chitin

The occurrence of chitin in the PM is now widely accepted (Peters, 1992). Until recently there was no direct evidence in support of this; only substantial indirect evidence existed (Peters, 1992; Rudin and Hecker, 1989), the most tenuous evidence being provided by histochemical techniques that cannot discriminate chitin from glycosylated proteins such as peritrophins (East *et al.*, 1993; Tellam *et al.*, 1999) and mucopolysaccharides (Lehane, 1997). For example the lectin wheat-germ agglutinin (WGA) since it also binds to GlcNAc units in glycosylated proteins (East *et al.*, 1993; Casu *et al.*, 1997). Nonetheless, the Chitosan test, first used by Wigglesworth (1930) to demonstrate the chitin content of the PM, still remains the most widely used method. Chitin detection by the more reliable chitinase and X-ray diffraction tests (Peters, 1976; Peters, 1992) has led to the acceptance that microfibrils within the PM do contain chitin. Huber *et al.* (1991) provided the first direct evidence, by demonstrating the release of GlcNAc, subunits of chitin, by chitinase activity on the PM of *A. aegypti*. The long held belief that chitin is diagnostic of PMs (Peters, 1992) is rejected by some authors (Wigglesworth, 1930; Waterhouse, 1953a and 1953b; Jeuniaux, 1964; Peters, 1968; Rudall and Kenchington, 1973; Peters and Latka, 1986; Rudin and Hecker, 1989; Huber *et al.*, 1991) despite this being established by a number of methods (Tellam *et al.*, 1999) because of the potential for erroneous detection. False negative results can be caused by either the presence of chitin being masked by other PM components or its secondary loss from the PM after formation. In addition, false positives can

arise from the detection of heavily glycosylated proteins within the PM (Berner *et al.*, 1983). As a result, reports that chitin is absent from the PM of a number of insects, including adult *Anopheles stephensi*, should be interpreted with caution. Equally this may indicate that the ability of the midgut to synthesize chitin is an ancestral feature that may have been lost by some insects (Gooday, 1990).

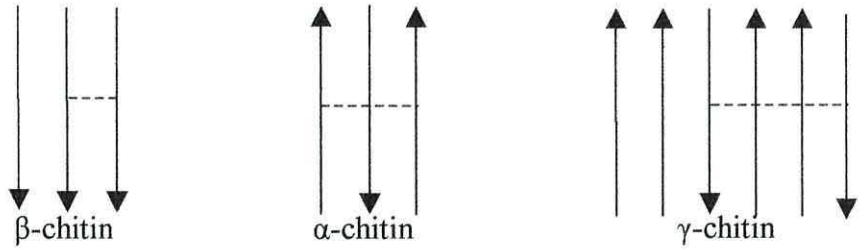
Chitin is the second most abundant biopolymer in nature and the most widely used organic structural component in invertebrates (Peters, 1992). The consensus view emerging is that chitin is fundamental to the integrity of most PMs, since it is the primary structural component of the PM, forming an integral part of the matrix (Neville, 1975). Structurally, chitin is the simplest glycosaminoglycan, being a β 1,4-homopolymer of N-acetyl-D-glucosamine. The β 1,4 linkage contributes to the strength of the molecule, producing a polysaccharide sufficiently compact and strong for this purpose (Peters, 1992). Hydrated chitin microfibrils give strength and elasticity to the PM in a similar way to proteoglycans (Neville, 1975).

Fig. 3. The chemical structure of chitin.
Adapted from Neville (1975).



Chitin contributes to the mechanical properties of the PM, as it is a viscoelastic polymer that is tough, flexible and resistant to degradation. Chitin polymers commonly form microfibrils that are linked by hydrogen bonds between amine and carbonyl (–N–H ··· O=C–) groups. These microfibrils commonly form bundles containing groups of at least ten microfibrils (Rudall and Kenchington, 1973). X-ray diffraction analysis shows that chitin is structurally anisotropic, with 3 polymorphic forms known as α, β and γ-chitin that differ in the orientation of the chains in successive bundles (Rudall, 1963) (Fig. 4).

Fig. 4. Molecular interpretation of chitin polymorphs.
Adapted from (Rudall, 1963). In β-chitin the chains are arranged in a parallel manner; in α-chitin in an anti-parallel orientation; there are groups of three chains in γ-chitin, alternating between two parallel strands and one anti-parallel strand. The dotted lines denote the expected position of di-sulphide bonds formed between individual chitin chains.



PMs normally consist of α- and γ-chitin (Rudall and Kenchington, 1973; Kenchington, 1976), whilst the β form is commonly used to form cocoon fabrics (Merzendorfer and Zimoch, 2003; Muzzarelli, 1977). The flexibility of the PM and cocoons may be explained by the high degree of hydration of α and β-chitin afforded by less tight packing of individual chains in these forms (Merzendorfer and Zimoch, 2003).

The synthesis of chitin is poorly understood (Arakane *et al.*, 2003; Arakane *et al.*, 2004), and yet a full understanding of chitin biosynthesis could lead to a better understanding of the factors affecting PM formation. The synthesis of chitin is described by the following reaction:

$$\text{UDP-GlcNAc} + (\text{GlcNAc})_n \longleftrightarrow (\text{GlcNAc})_{n+1} + \text{UDP}$$

Where UDP-GlcNAc denotes chitin synthase; $(\text{GlcNAc})_n$ the individual N-acetyl-D-glucosamine monomers and $(\text{GlcNAc})_{n+1}$ the chitin polymer. Chitin synthase (CHS) is known to be the key enzyme within this biosynthetic pathway, as it catalyses the incorporation of GlcNAc into the chitin polymer (Tellam *et al.*, 2000b). There are two isoforms of chitin synthase: CHS1 is expressed by the epidermis and other tissue of ectodermal origin, whereas CHS2 is produced specifically by the portion of the gut epithelium responsible for the production of a PM (Ibrahim *et al.*, 2000). Transcriptional and post-transcriptional regulation of midgut-specific CHS expression allow them to be upregulated in response to ingestion of a blood meal (Becker, 1978). Glutamine synthetase (GS) is responsible for the catalysis of glutamine, and is therefore essential during the initial pathway of chitin synthesis, since just 0.3 mM of the GS inhibitor (L-methionine S-sulfoximine) results in the complete inhibition of PM formation in *A. aegypti* (Smartt *et al.*, 1998). The enzyme glucosamine fructose-6-phosphate aminotransferase is also thought to be essential for the formation of the PM in *A. aegypti* (Kato *et al.*, 2002). PM secretion is sensitive to inhibitors of chitin synthesis at both the protein (polyoxin D) and RNA

(amanitin) level (Clarke *et al.*, 1977). However, their use in experiments to demonstrate the presence of chitin in the PM is not widespread, since they can only provide indirect evidence (Spence, 1991). (see section 7.).

Chitin is always complexed with protein in arthropods, and the structure of the PM is no different. The chitin microfibrils forming the substructural network are embedded in a superstructural proteineous matrix (Richards and Richards, 1977; Peters, 1992), highlighting that chitin is just one component in the complex structure of the PM, and that there is also a need to understand the properties of the other constituents before the physicochemical properties of the PM can be fully understood.

1.6.3. PM Proteins

Proteins invariably account for a large proportion of the PM dry weight, commonly up to 50% (Ono and Kato, 1968a). For example, protein is estimated to form 35-40% of *Bombyx mori* larval PM (Peters, 1992). The number of PM proteins within each PM varies greatly between insects. The PM of adult blackflies has been shown to contain just two major proteins (Ramos *et al.*, 1994), as opposed to sandflies and blowflies (East *et al.*, 1993; Elvin, 1996), that possess a PM consisting of 5-15 proteins. Similarly, six major proteins have been identified in the PM of *L. cuprina* larvae (Stamm *et al.*, 1978). Mosquitoes have a PM composed of a more complex array of proteins, with the identification of over 40 proteins in *Anopheles gambiae* and *Aedes aegypti* by 2-D gel electrophoresis (Moskalyk *et al.*, 1995; Moskalyk *et al.*, 1996). Moreover, 2-D gel analysis of the PM of adult *Glossina morsitans morsitans* revealed approximately 40 proteins (Lehane *et al.*, 1996; Tellam *et al.*, 1999).

The PM consists of mostly glycosylated proteins with different solubilities (Tellam, 1996). PM proteins are assigned to one of four classes, depending on their ease of extraction from the PM using increasingly severe solubilising agents (Elvin, 1996). Class I proteins are those PM proteins that are easily removed by physiological buffers such as Ringer's solution. They are commonly digestive proteases or gut contents rather than genuine PM proteins, and comprise only 1% of *L. cuprina* PM. Class II proteins are eluted using mild detergents, and represent just 2% of *L. cuprina* PM proteins. Such treatment is believed to disrupt the weak protein-protein, protein-chitin, or protein-oligosaccharide interactions, such as those that form between proteoglycans. Class III proteins are extracted by strong detergents, such as 6 M urea. These proteins are the peritrophins, which form the majority of the proteins that can be solubilised from the PM, making up 11% of *L. cuprina* PM. Class IV proteins are not extractable from the PM, since they cannot be solubilised, even by strong denaturants. Elvin (1996) demonstrated that they form 87% of *L. cuprina* proteins.

The amino acid analysis of PM proteins first took place over twenty years ago (Adang and Spence, 1983), revealing the major differences in the protein spectra of PMs even when protein content is identical between species (Stamm *et al.*, 1978). The presence of bacteria on the PM can have a profound effect on the readings, making it necessary to rear insects under sterile conditions to obtain PMs that are devoid of bacterial contamination (Peters, 1992). Although peritrophins and mucins are known components of the PM (Elvin, 1996), their structures and functions still remain largely unknown (Elvin, 1996). Mucins are much larger in comparison to peritrophins, are rich in proline, alanine and threonine residues and are highly glycosylated (Stamm *et al.*, 1978; Elvin, 1996; Tellam *et al.*, 1999).

1.6.3.1 Integral PM proteins: the peritrophins

The term "peritrophin" is used to describe proteins that are integral components of the PM, since they are bound so tightly that they can only be eluted from the PM using strong denaturants such as 6 M urea and 6 M guanidine-HCl (Tellam *et al.*, 1999). The peritrophins form a large protein family in insects, with members that are extremely variable in size and sequence. Ten peritrophins from five different insect species (Tellam *et al.*, 1999) from both PM 1 and 2 types have been identified so far (see Fig. 5). peritrophins are characterised by possessing one or more chitin binding (type-2) domain (CBD) known as the peritrophin-A (P-A) motif (Tellam *et al.*, 1999). The sequence of P-A domains is poorly conserved, but are typically 65-70 amino acids long and characterized by a 6 cysteine motif having a consensus sequence of $CX_{13-20}CX_{5-6}CX_{9-19}CX_{10-14}CX_{4-14}C$ (where C is cysteine and X is an amino acid other than cysteine) and several conserved aromatic residues (Elvin, 1996; Wang and Granados, 1997a). It is these conserved cysteine residues that mediate the chitin binding interactions by forming the intra-domain disulphide bonds responsible for their affinity for chitin and resistance to proteolytic digestion. The peritrophin-A domain of peritrophins allow the formation of multiple cross-links between peritrophins and chitin in the PM, a theory supported by the high affinity binding of P-95 to the PM (Tellam *et al.*, 2000a). However, P-A domains are not confined to peritrophins, as they may also be present in mucins (Gorman *et al.*, 2000), serine proteases (such as Tequila in *Drosophila*) (Tjoelker , 2000) and class-II chitinases (Elvin, 1996) since these molecules are also associated with chitin. Invertebrate CBDs were first identified in peritrophin-44 from *L. cuprina* (Shen and Jacobs-Lorena, 1999). PM proteins commonly have 2-5 CBDs, whilst chitinases have 1-3 CBDs at the C-terminus in addition to a catalytic domain at the N-terminus. The multiple domains within PM proteins are believed to have arisen from gene duplication during evolution, driven by the expansion in function of genes encoding multiple CBDs, and thus capable of binding more than one chitin fibril (Vuocolo *et al.*, 2001).

Apart from the P-A domains, peritrophins and peritrophin-like (PL) proteins bear little resemblance in sequence. However, they are all secreted proteins, and so have a signal peptide at the N-terminus in addition to a sequence rich in cysteines and prolines, that commonly causes them to be highly acidic, polar proteins (Elvin, 1996; Schorderet *et al.*, 1998). peritrophin-44 and P-48 are true peritrophins from the PM 2 of *L. cuprina* larvae that comprise more than 70% of the peritrophin component (Elvin, 1996). Both are strongly bound to and uniformly distributed throughout the larval PM, suggesting that they have a role maintaining the structure and function of the PM (Schorderet *et al.*, 1998). P-44 and P-48 are usually exposed on the PM surface, where they are easily accessible to digestive proteases, and yet they are highly resistant to proteolysis. Both P-44 and P-48 have numerous isoforms, derived from gene families that are co-expressed in the cardia (Becker, 1978). Such multi-gene families may be required to synthesize sufficient amounts of each protein to support rapid PM synthesis, which can be 5-10 $mm.hr^{-1}$(Gaines *et al.*, 2003). A P-48 homolog to the *L. cuprina* P-48 termed Cb-peritrophin-48 (Cb-P-48) has been identified in *Chrysomyia bezziana* larvae. Most peritrophins are acidic and have potential for N-linked glycosylation. P-44 and P-48 from *L. cuprina* contain 2 potential N-linked glycosylation sites and Cb-P-48 7 N-linked glycosylation sites. P-95 from the PM 2 of *L. cuprina* has 4 potential N-linked glycosylation sites.

1.6.3.2 Mucin-like peritrophins

Recently, the boundary between peritrophins and mucins has become less distinct than first thought, since the criteria used to denote them are no longer mutually exclusive (Tellam *et al.*, 1999). This reflects the evolutionary link between these two types of molecules, since peritrophins are believed to have evolved from mucins with the acquisition of CBDs (Tellam *et al.*, 1999). Mucins that contain one or more P-A domain(s) are termed "mucin-like peritrophins" (Van-Klinken *et al.*, 1995; Casu *et al.*, 1997). In fact peritrophin-55 and P-95 from *L. cuprina*, originally thought to be strictly peritrophins, are "mucin-like peritrophins" since their amino acid sequence is rich in serine, prolines and threonines, resulting in high levels of O-linked glycosylation (Tellam *et al.*, 1999). P-30 from *L. cuprina* is characterised by P-B domains, consisting of a specific sequence of 8 cysteines; with the consensus $CX_{12-13}CX_{20-21}CX_{10}CX_{12}CX_2CX_8CX_{7-12}C$ and one potential N-linked glycosylation site (Barry *et al.*, 1999). P-55 contains a single P-B domain with 1 potential N-linked glycosylation site and a single proline-threonine rich domain with substantial O-linked glycosylation.

Fig. 5. Amino acid sequence alignment (by ClustalW) of the CBDs from a number of peritrophins to illustrate the relationship between peritrophin-A (P-A), P-B and P-C domains, based on the unique spacing between cysteine residues of the CBD. Conserved cysteine residues are boxed and semi-conserved aromatic residues are indicated by an asterisk. *Lucilia cuprina* unless otherwise stated. Abbreviations: P, peritrophin; Cb, *Chrysomyia bezziana*; Dm, *Drosophila melanogaster*. **A.** The sequence of P-A domains, typically 65-70 amino acids long, characterized by a 6 cysteine motif having a consensus sequence of $CX_{13-20}CX_{5-6}CX_{9-19}CX_{10-14}CX_{4-14}C$ (where C is cysteine and X is an amino acid other than cysteine), there is a small amount of inter-domain amino acid similarity. **B.** P-B domains are typically 70-85 amino acids long, consisting of a specific sequence of 8 cysteines; with the consensus $CX_{12-13}CX_{20-21}CX_{10}CX_{12}CX_2CX_8CX_{7-12}C$, there is a small amount of amino acid similarity, mainly of aromatic residues. **C.** P-C domains, a register of 6 cysteines within a 68-70 long consensus sequence of $CX_{8-9}CX_{17-21}CX_{10-11}CX_{12-13}CX_{11}C$, frequent sequence similarity of aromatic residues.

A

```
Tequila.I Dm    PN C PEMQGPLP----HYFIHPTN C SRFYE C HMRDAWE----------YE C PAGL--HF
Tequila.II Dm   -- C PPTGATLP----NYWAHGTD C SRYYG C LEGCVKE----------FK C PDGL--YW
P44.I           ET C EYTPDG-------FIADPNS C QSYGY C KNNQLVG---------TGK C PDGY--LY
P44.II          NA C LHATDN------SFVADPTN C NGYCY C SNKTATC----------TT C PEFQ--LF
P44.III         SI C RLVPNA------VYVGNPNE C GEYIS C FNGIGTEG----------R C ASG---YF
P44.IV          FV C QVNGGNPTEEKPVFISDGQT C MGYYK C TSRNGPG---------IWGK C PKGL--HF
P44.V           DR C GNLNRE------FVGAIRTE C KNFLI C KNETSQGMAYNYAKYIGLP C TDKNYPFF
P48.I           KY C ELVKIG------TLMPSMIS C QDYYI C RLNNQPI---------PVK C GANT--VF
P48.II          NP C ENKDR-------TFAPSSKA C NEWHY C LNGNIVA---------NGS C QPGQ--IF
P48.III         NI C DIMQNG------QFFGDFEN C QNWQK C NNGRLQK----------GI C LGNL--VY
P48.IV          TL C TSSND-------GPLPDKLT C SVYYI C EQDTTSTPTT--YKWIKTS C PNGQ--YF
P48.V           NR C EYTTGSTTY---WVNAVSND C TKFST C RNGRKITNE-------DGS C NSGY--YF
P95.I           SY C QLVPTG------TKFPSLIS C QKYYI C RDNGQVI---------ETS C SGND--VF
P95.II          NP C DNNVV-------GFVSDPND C QRWIY C EKNEILS---------SGN C AYGH--YF
P95.III         NI C QLVPTG------TKFPSLIS C QKYYI C RDNGQVI---------ETS C SGND--VF
P95.IV          EI C NETELN------KIIPNSGV C SIYFE C TVSNNNN-----YTWKKNS C ENGE--YY
P95.V           DR C EFVN---AN---FVNAVDPT C MNYLV C QDGKEIVK---------GT C VSG---YF
                                             **                          *

Tequila-I Dm    NVAIDV C DFPVN---------AK C ES--(32-90)
Tequila-II Dm   NDQQKR C DSYSS---------SQ C GCPD(29-85)
P44-I           NNKLGI C DSPAN---------VK C IS--(89-146)
P44-II          DSKQIK C VYALEK--------PE C TA--(148-201)
P44-III         NKQLGG C QTTNP---------- C LA--(221-283)
P44-IV          NE--GK C VTPFT---------FP C TF--(284-356)
P44-V           NEVSGT C EKTSPKS----DTYKL C V---(26-83)
P48-I           DKDTQG C VPEAQ---------AN C IL--(87-140)
P48-II          DASKNS C IYG-----------A C NS--(154-210)
P48-III         DTKNGM C LQNDG---------TM C ER--(225-292)
P48-IV          DVFGDG C LDRAKRR-----VYTG C NR--(291-360)
P48-V           NEADQY C NMGDFTN--YAETNGA C QN--(30-87)
P95-I           SKDKQK C VSASS---------AN C NI--(91-144)
P95-II          NKDTKL C AAG-----------D C PY--(153-209)
P95-III         SKDKQK C VSASS---------AN C AG--(212-277)
P95-IV          DVNTKS C KNRQEAT-----PVEG C DR--(276-341)
P95-V           NEQEQI C TNNDTNLPDYRKNNGA C YL--(287-347)
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B

```
P30-I           LV C PTTPKYNQKINNL- C VAEKDLKVVWPDYTNVSNYYI C QGLGNP--------VLQN C FP
P30-II          SP C MLPV-YTHTFVPT- C EGN-DIYRLWPNYKNVEKYYW C QAPYMP--------VIKD C PK
P55             GV C DSNVDYNSTLITP- C LGN-DIIVLWPNYLNFNTYYK C VEFGKP--------QLMD C PP
DmPro9 Dm       AA C SAVPQYTEKIDPVN C PQ--SGFKLPNYENASVYYQ- C TAQLQSDGSIVVTGVLTS C GE
                          *                    *  *     **

P30-I           NTIFSFYQQK C TK C DKYVPAPH C -KDLKNA----K C ED (20-104)
P30-II          GTYFNYYAQS C TD C LAFVEAPD C NKLMKQEGKPNK C VK (117-204)
P55             NTYFTYYFQQ C TG C DNFIPAPT C -EYLKQTT-DVE C VP (20-106)
DmPro9 Dm       DTYFNYALQR C VA C ANYFPSAQ C -SSLPIN---VT C VP (20-111)
                  **
```

C

```
P15             -- C DPDGNNQPE C T-GKNVNVPARNFWDPTHYWL C KSAGAVAESVR C PDAEGFDSAKGA C
P15 Cb          LA C DPDGNNQPQ C S-SNNVNVPVRNFWDPTHYWL C KSASGVAESVP C PVAEGFDPAKGA C
P15a Dm         NA C DPNSDNQPD C SDASNVQTNIRNFWDPTRYWW C ESSTSTATAVL C PLSTGFDPTKKE C
P15b Dm         LD C NPDGNGEPD C VGRSGEIS--RDFWDPTHYWQ C -SSTGQAELVA C EQNTGFDPKTGS C
                                              **    **                  *

P15             VPFSQWKWTEP C PK (20-89)
P15 Cb          VPFDQWKWTEP C PK (18-89)
P15a Dm         VSWSEWSWTAY C -- (21-92)
P15b Dm         VDWSVWQWYPP C SE (20-91)
                  * *
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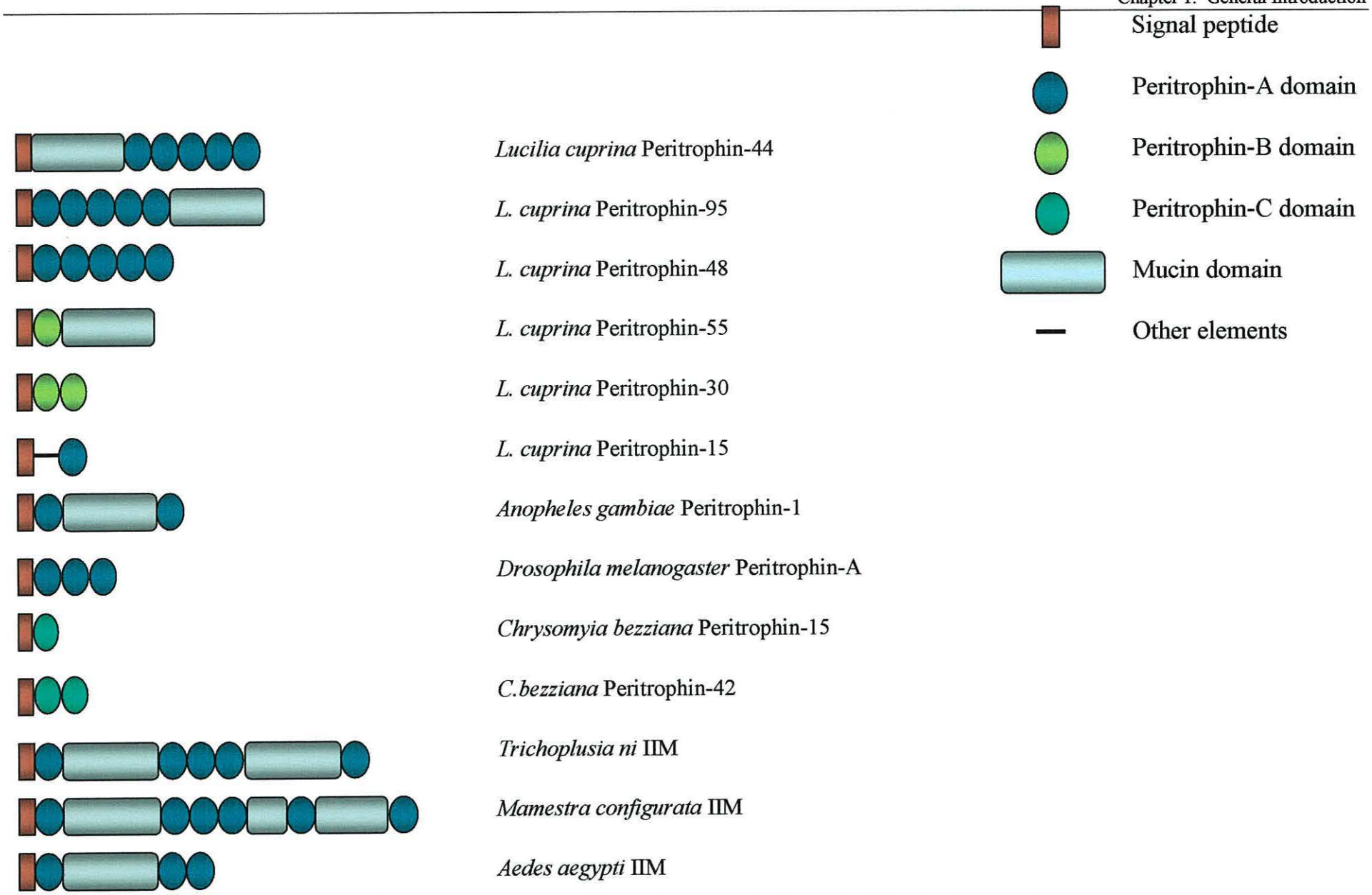


Fig. 6. Diagrammatic representation of the domain structures of invertebrate mucin-like and chitin-binding proteins. Adapted from Tellam *et al.* (1999) and Sarauer *et al.* (2003).

1.6.3.3 Mucin/ peritrophin-like proteins

Peritrophins termed "peritrophin-like" (PL) proteins distinguish those peritrophins that have roles outside of the PM from integral PM proteins. The first PL proteins to be identified were encoded by the genes 'Gene analogous to small peritrophins' (Gasp) and PeriA (Gaines *et al.*, 2003). They have been shown to have sequence similarity to known peritrophins, but are nonetheless expressed in the embryonic tracheae and adult ovaries of *Drosophila melanogaster* respectively. Recently, two sequences were identified in the Malpighian tubules and hindgut of adults and larvae of the cat flea, *Ctenocephalides felis* with homology to both mucins and peritrophins, and are thus termed "mucin/ peritrophin-like" (MPL) proteins (Gaines *et al.*, 2003). This is the first report of MPL proteins with hindgut and Malpighian tubule (HMT) tissue specificity. The MPL proteins in *C. felis* can not be construed as neither true peritrophins nor mucins, since adult cat fleas are known to lack a PM (Elvin, 1996; Shen and Jacobs-Lorena, 1998).

This further supports the hypothesis that peritrophins may also be present in other tissues that interface with the environment (such as those where nutrient and gaseous exchange occur) that are also potential entry points for pathogens and parasites. Since the midgut epithelium and trachea in insects must perform these two functions, it is not surprising that they utilise similar proteins for this task. Most peritrophins are believed to create a strong matrix by either binding tightly to chitin and other protein components of the PM via protein-chitin and protein-protein interactions (see Fig 2.), creating a three-dimensional network (Wijffels *et al.*, 2001). Some peritrophins have specialised functions within the matrix, for example peritrophin-15 proteins from the type-2 PM of *D. melanogaster*, *L. cuprina* and *Chrysomya bezziana* are believed to function solely as capping proteins for the ends of chitin fibrils (Gaines *et al.*, 2003). They are characterized by a peritrophin-C domain; $CX_{8-9}CX_{17-21}CX_{10-11}CX_{12-13}CX_{11}C$, resulting in unique spacing between the six cysteine residues (Tellam *et al.*, 1999) that allow a sufficiently tight association with the tip of a chitin microfibril for this purpose. Some peritrophins, including Ag-Aper1 have multiple proline-rich regions, believed to aid the cross-linking of chitin in the matrix by forming a hinge, allowing P-A domains more flexibility when binding chitin (Williamson, 1996; Tellam *et al.*, 1999). Williamson (1996) also hypothesised that these same regions may facilitate the formation of cross-links between peritrophins through protein-protein interactions (Wang *et al.*, 2004).

Recently, Wang (2004) proposed that the CBD proteins within the PM are present in a partially degraded form to explain the phenomenon by which some CBD proteins such as Peritrophin-1 and Peritrophin-2 from the PM of *T. ni* larvae are present as partial fragments rather than full-length molecules (Van-Klinken *et al.*, 1995). The exact mechanism may involve

their proteolysis either before or after such CBD proteins have been incorporated into the matrix. Indeed, such an effect of the protease rich insect gut on the CBD containing proteins during formation of the PM may function to modulate the properties of the PM, particularly its fine structure, and thus permeability.

1.6.4. Mucins

A suitable definition of mucins has been debated for many years since the identification of mucin domains remains a bioinformatic challenge due to little sequence conservation within these motifs that have characteristic biased amino acid composition (Lang *et al.*, 2004). Mucins are macromolecules composed of long, unbranched side chains (usually GAGs) that are covalently linked to a protein core via O-glycosidic bonds during post-transcriptional processing (Van-Klinken *et al.*, 1995). However, all mucins are very large proteoglycans (PGs) that protect the gastrointestinal tract and other mucosal surfaces of endodermal origin (Lehane, 1976a; Stamm *et al.*, 1978; Dimitriadis and Kastritsis, 1984; Peters, 1992); insect intestinal mucins are integral components of the PM, of analogous function and have been identified in the majority of PMs studied (Ruoslahti, 1988). Mucins are ideal components of extracellular matrices, such as the PM, as a result of their space filling properties (Kjellen and Lindahl, 1991). At the level of matrix organisation, mucins may aid the assembly and binding of matrix components by forming cross-links. It is believed that they act to regulate the thickness of the chitin fibrils within the matrix, which in turn affects the characteristics of the PM. In addition hydrated proteoglycans are known to provide strength against compressive forces. This together with the strength provided by chitin and peritrophins against tensile forces may account for the great mechanical strength of the PM.

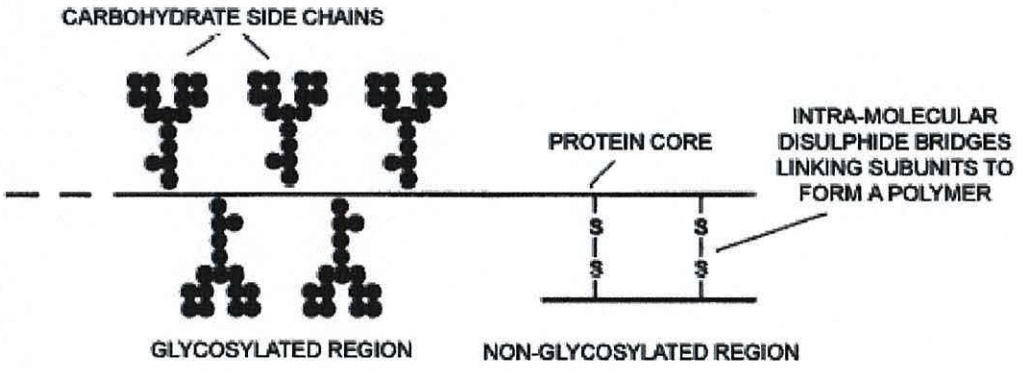
The protein core of vertebrate and invertebrate secretary mucins contains distinct mucin domains, rich in cysteine residues, termed cysteine rich domains (CRDs) in addition to tandem repeats rich in proline, threonine and serine residues (PTSBs) to which the GAG side chains are linked (Shen and Jacobs-Lorena, 1998). The CRDs of invertebrate mucins and peritrophins, known as CBDs, are required for PM structural integrity (Allen *et al.*, 1998). Whereas, those in vertebrate mucins, termed D-domains, aid mucin polymerisation (Stamm *et al.*, 1978). The extensive glycosylation accounts for their characteristic high molecular weight (200 kDa), since oligosaccharide side chains comprise 50-85% by weight of mucins.

Mucins are heterogeneous molecules, resulting from a structure that is open to extensive modulation due to the variable carbohydrate side chains, of which there are usually five (see Section 1.5.1). The glycosylation accounts for the enormous variability that exists in the glycoprotein pattern at different life stages in the same species (Kjellen and Lindahl, 1991). It is

this glycocosylation pattern which dictates the function of the molecule since these residues influence the conformation and thus biological activity of mucins. In fact, it has been reported that specific biological activity can be exerted by a single GAG side chain (Forstner and Forstner, 1994). The massive carbohydrate moieties of mucins confer hydration, viscoelasticity and protease resistance of mucins (Gum *et al.*, 1992; Perez-Vilar and Hill, 1999).

Concatenation (multimerisation) of mucin monomers into oligomers is mediated by the self-dimerisation of mucins via inter-molecular disulphide bonds, forming oligomeric mucin, with several mucins forming a mucus gel layer (Harding, 1984). The core protein functions primarily as a scaffold for the appropriate spacing of GAG side chains with disulphide bonds located in the non-glycosylated or 'naked' regions of the peptide (Gallagher and Corfield, 1978).

Fig. 7. Schematic representation of the main features of a secretory mucin. The dense oligosaccharide clusters within mucin domains are proteolytically resistant and result in the molecule forming an extended, bottle-brush appearance. From Allen (1993).



Diptera are unique in confining mucins to the electron dense layer (EDL) of the PM, whereas the majority of other PMs have uniformly distributed mucins (Lehane, 1997). The role of mucins in determining PM permeability (Peters and Wiese, 1986) is suggested by their abundance in the luminal surface of the PM (Dorner and Peters, 1988). Mucins can be localised within the PM by detecting glycoprotein side chains other than GlcNAc, which is also a unit of chitin. Dorner and Peters (1988) detected GlcNAc and GalNAc residues on both sides of the PM in *Anopheles stephensi*, *Aedes aegypti, Odagmia ornata* and *Culex pipiens* larvae, in contrast to Fructose, which localised to only the epithelium side of the PM in *A. aegypti* larvae (Billingsley, 1990). All five residues, except fructose have been identified in *Glossina* PM using lectin-gold conjugates (Glattli, 1988; Lehane *et al.*, 1996).

1.7. Physiological Functions of the PM

The strategic position of peritrophic matrices (PMs) place them at the center stage in many physiological processes. PMs are believed to be an "enigma" due to the lack of knowledge about their function within the insect (Richards and Richards, 1971). Vertebrates primarily utilise mucus to line and protect the epithelium of the gastro-intestinal tract whereas insects, lacking mucus, instead posses a midgut lined with a PM, which acts in an analogous way. The PM is an engineering achievement, since it is able to perform a number of protective functions against mechanical, chemical and biological insults whilst not even being a 10th of the thickness of vertebrate mucus (50-450 μm) (see Section 1.3). It was assumed for many years that the sole function of the PM was to protect the midgut epithelial cells from damage by coarse particles within the food bolus. Several functions have been attributed to the PM to date, although none have been conclusively proven. The widespread occurrence of the PM in arthropods indicates that it must have some essential roles, as it must make a significant contribution to the adaptive fitness of the organism to be so well preserved during evolution (Berner *et al.*, 1983). Conversely complete blood meal digestion takes place in the absence of a PM in the mosquito *Anopheles stephensi* (Billingsley and Rudin, 1992; Villalon *et al.*, 2003), which implies that the adult PM is a vestigial structure that does not confer any selective advantage or disadvantage in digestion. There is evidence in support of a role for the PM in digestion in some larvae (Terra, 1990; Terra and Ferreira, 1994), including *A. aegypti* (Wigglesworth, 1972), *M. domestica* (Terra *et al.*, 1988), *Rhynchosciara americana* (Terra *et al.*, 1979), *Erinnyis ello* (Lepidoptera) (Santos *et al.*, 1983) and *Tenebrio molitor* (Coleoptera) (Terra *et al.*, 1985) larvae. It may be concluded that much work remains in elucidating PM function, as emphasised by Richards and Richards (1977): 'some significance is eluding us'.

The PM is believed to be multifunctional, as numerous roles have been proposed since its discovery. These include the mechanical protection of the midgut epithelium; a semipermeable filter for digestive enzymes and blood proteins; a retention barrier to restrict proteinase inhibitors to the gut lumen; prevention of clogging of microvilli by lumen material and anti-pathogen protection of the midgut epithelium (Richards and Richards, 1977). Most functions depend upon the selective permeability properties of the PM, and multiple, simultaneous functions are likely for most PMs.

1.7.1. Protection from mechanical damage

The commonest function attributed to the PM is that it protects the midgut epithelium cells against damage by abrasive food particles. The gel-like nature of mucin assists the lubricating

role of the PM. This function of the PM was once believed to be irrelevant in fluid feeders (Billingsley and Rudin, 1992), but it has since been shown by Berner *et al.* (1983) that sharp edged haemoglobin crystals within the blood meal of haematophagous arthropods could potentially damage the delicate brush borders of midgut epithelial cells. In addition, Zhuzhikov (1964) proposed that the chitin microfibrils within the PM serve to reinforce it against the pressure caused by osmotic swelling of the food bolus due to the high molecular weight constituents in the latter. Such pressure, if transmitted to the midgut epithelium, could lead to damage or rupture. However, it is not an absolute requirement because it has been shown that a PM is not required for digestion of the blood meal in *An. stephensi* and *A. aegypti* (Villalon *et al.*, 2003). However, sublethal damage to the midgut epithelium caused by heme crystals may lead to a loss in competitiveness, reflected by a corresponding reduction in the overall fitness of the mosquito.

1.7.2. Permeability barrier

Traditionally thought to be an inert sieve (Derksen and Granados, 1988), the permeability of the PM depends on ultrafiltration relative to pore size (Richards and Richards, 1971; Spence, 1991 Peters, 1992). PM permeability is crucial to many of its functions. Many studies have concluded that it is of great importance to the PMs role in nutrition (Tellam, 1996), thought to be attributed to the compartmentalisation of enzymes and nutrients (Peters and Wiese, 1986; Zhuzhikov, 1970).

Numerous studies have been carried out to investigate PM permeability, both *in vitro* and *in vivo*. It has been successfully measured directly, *in vivo* by feeding tracer particles or molecules of known size to insects and monitoring their distribution between the endoperitrophic space (ENPS) and the ectoperitrophic space (ECPS) (Rudzinska *et al.*, 1982). Indirectly, it has been measured by inferring pore size from the size of the digestive enzymes, cells and ribosomes that reside in the ENPS (Adang and Spence, 1981; Miller and Lehane, 1990). *In vitro* studies commonly involve the mounting of the PM so that permeability can be tested under controlled conditions (Richards and Richards, 1977). Such tests initially achieved relatively inconsistent results, although a pore size of less than 30 nm is generally agreed (Rudzinska *et al.*, 1982). More recent studies of PM permeability in insects have determined pore sizes of 10 nm or less. IgG molecules have an effective diameter of 10 nm, which implies that the pores may restrict the flow of ingested immune molecules through the PM. Consequently, potential targets for the immune mechanisms of vectors would be restricted to the ENPS. The PM is a microfilter with narrow limits of permeability (Adang and Spence, 1983; Miller and Lehane, 1993b; Peters and Wiese, 1986). The degree of PM permeability differs among the insect orders (Peters and

Wiese, 1986). The PM of Diptera contains pores ranging from 1-9 nm in diameter (Derksen and Granados, 1988) and larval Lepidoptera from 17-18 nm.

The actual process of influx of particles through the PM is believed to be much more complex than that which occurs in a simple semipermeable membrane, as it depends on more than just simple ultrafiltration relative to pore size (Adang and Spence, 1981). A number of studies support this theory, including the study by Miller and Lehane (1993a), who demonstrated the permeability of the PM from adult *G. m. morsitans* to alkaline phosphatase increased in the presence of calcium ions during *in vitro* studies. Another interesting report, by Zhuzhikov (1964) demonstrates that some PMs may be selectively permeable. Indeed, an *in vitro* study of the PMs of *Musca domestica* and *C. erythocephala*, known to consist of 2 and 3 separate membranes respectively, identified their directional polarity to human salivary amylase, as the enzyme was able to pass from the ECPS to the ENPS, but not in the opposite direction.

The variability in PM permeability can be explained by the composition of the PM, as components such as glycoproteins and peritrophins are responsive to changes in environmental conditions. Viruses have been reported by Derksen and Granados (1988) to remove glycoproteins from the PM of *T. ni* (Spence and Kawata, 1993), the resulting perforations being implicated in viral penetration of the PM (Miller, 1991). *Glossina* PM contains glycosaminoglycans (Lehane, 1976a; Barbehenn and Martin, 1997), the properties of which vary with pH. This suggests that changes in midgut pH could alter permeability of the PM by influencing the distribution of anionic charges on the molecules (Willadsen *et al.*, 1993).

Similarly, Miller and Lehane (1993a) proposed that calcium ions neutralise anionic charges on glycoprotein complexes in the PM, which alters the conformation of the molecule and thus the porosity of the PM. Lehane (1976a) proposed that some insects utilise the properties of PM proteins in order to control PM permeability. This is supported by studies in which a number of peritrophins were implicated in determining PM permeability in indirect studies of the PM of *L. cuprina* larvae (Elvin, 1996; Tellam *et al.*, 2000a).

Recent evidence has been obtained showing that PM permeability is reduced on binding of specific lectins and antibodies raised to these components in the PM. P-44 and P-95 are abundant components of the PM that are uniformly distributed and strongly associated with the PM of *L. cuprina* (Willadsen *et al.*, 1993; Eisemann *et al.*, 1994a and 1994b), indicating that they have a central role in determining many properties of the PM. Previous investigations (Eisemann and Binnington, 1994) showed that the permeability of *L. cuprina* PM was reduced by treatment with gut extracts and lectins. The resulting retarded growth rate and increased mortality in blowfly larvae may be attributed to interference with the proper movement of digestive enzymes and food molecules across the PM caused by the non-specific inhibition of

PM porosity resulting from the formation of an impervious gel-like layer on the luminal side of the PM (Tellam and Eisemann, 1998). This topic is discussed further in Section 8.

A number of studies have determined the permeability of intact PM, and assayed for the effect of disruption, and the resulting enhanced permeability, by calcofluor, polyoxin-D or other inhibitors on PM permeability (Edwards and Jacobs-Lorena, 2000; Tellam and Eisemann, 2000; Wang and Granados, 2000). However, such toxins may have other undesirable physiological effects on the insects studied. The permeability properties of the PM are fundamental to its role in digestion: mainly spatial organisation and enzyme conservation (Barbehenn and Martin, 1992). The selectivity of PM permeability also offers protection in some insects from ingested toxins (Lehane, 1997). This is discussed further in Section 1.7.4: Protection from toxins.

1.7.3. Enhancing digestion efficiency

1.7.3.1 Countercurrent flow

The role of the PM in digestion has been investigated extensively. The PM effectively partitions the midgut lumen into an endo-, ecto- and intra-PM space (Edwards and Jacobs-Lorena, 2000), the physiological significance of which is the separation of the midgut lumen into an active peripheral zone and a central region (Zhuzhikov, 1964). As a result the PM acts as a dialyser (Langley, 1966), allowing digestion to be organised spatially by controlling fluid flow and the distribution of molecules (Zimmermann and Peters, 1987). Efficiency of gut function is increased (i) through the formation of a buffered zone next to the midgut epithelium, (ii) the partitioning, immobilisation and countercurrent flow of digestive enzymes, (iii) regulation of water and ion movements, and (iv) prevention of non-specific binding of undigested food. The PM favours the formation of an unstirred layer next to the midgut epithelium, within the ECPS, which aids absorption of solutes by epithelial cell membranes (Terra *et al.*, 1979). The countercurrent flow set up by the PM would confer a number of advantages, including the endo-ectoperitrophic recirculation of digestive enzymes (Dow, 1981). This increases their efficiency, by ensuring that the freshest enzymes encounter food depleted of nutrients (Terra, 1990) and reducing their loss with the frass (Pimenta *et al.*, 1997). Conversely, Villalon *et al.* (2003) believe that the PM actually limits the rate of digestion in adult *An. stephensi* and *A. aegypti*, since the absence or disruption of the PM leads to more rapid trafficking of enzymes between the midgut lumen and the epithelium. In addition, the PM may in this way inadvertently assist the survival of pathogens within the insect gut, as the PM of sandflies actually promotes *Leishmania* survival, by acting as a permeability barrier to hydrolytic enzymes, reducing the exposure of parasites to proteolytic damage (Derksen and Granados, 1988).

Derksen and Granados (1988) proposed that the PM creates a favourable environment for digestion by maintaining a pH gradient of 7.8-9.5 between the midgut epithelium and the lumen in Lepidopteran larvae. In addition the PM may act as an amphoteric ion exchanger, since proteoglycans within the PM are substantially hydrated and therefore capable of conferring an osmotic force that could play a role in regulating the movement of water and ions across the PM (Peters, 1976; Peters, 1992; Zimmermann and Peters, 1987). These attributes are of particular use in the dehydration and concentration of watery meals such as blood meals (Tellam *et al.*, 1999). Waterhouse (1957) also suggested that the PM prevents the non-specific binding of undigested food to the midgut epithelium, which can clog microvilli, a theory supported by Terra (1990). The success of insects is in part due to their ability to exploit highly divergent niches, made possible in part by such diverse gut functions.

1.7.3.2 Immobilised enzymes on the PM

The immobilisation of proteases on the PM has the potential to increase the efficiency of digestion (Shen and Jacobs-Lorena, 1998). However, there is little evidence in support of this hypothesis, since the trypsins sequenced to date (Eguchi *et al.*, 1982; Peters and Kalnins, 1985) lack the binding domains necessary for their interaction with chitin or protein components of the PM. Although, the adsorption and immobilisation of trypsins and aminopeptidases has been demonstrated in some insects (Peters and Kalnins, 1985) . The immobilisation of enzymes to the PM allows their conservation when compared to soluble enzymes in the ENPS that are lost more rapidly with the frass (Walker *et al.*, 1980). Aminopeptidases have been detected on the PMs and PEs of a number of insects including leucine-aminopeptidase in *Drosophila melanogaster* larvae (Eguchi *et al.*, 1982), larvae of the silkworm, *Bombyx mori* (Terra *et al.*, 1979), and *Rhynchosciara americana* (Peters and Kalnins, 1985). Aminopeptidase activity may also be localised to a particular region of the PM: aminopeptidases have been detected in just the posterior midgut of *D. melanogaster* larvae, and only the anterior midgut of *Locusta migratoria* (Eguchi *et al.*, 1982; Peters and Kalnins, 1985). Aminopeptidases are strongly attached to the PMs in which their adhesion was tested (Peters and Kalnins, 1985). Peters suggested that immobilised enzymes can be incorporated into the PM either directly during synthesis or subsequently once the PM is fully formed (Terra *et al.*, 1979). This mechanism of conserving enzymes by immobilisation may be essential in PM 1 producing insects, since the re-circulation of enzymes in the ECPS can only take place in insects with a PM 2, where the PM forms a continuous barrier to loss in the faeces (Barbehenn and Martin, 1992; Barbehenn, 2001).

1.7.4. Protection from toxins

The PM not only acts as a barrier to toxins, but is also believed to have a role in detoxification of certain toxins by acting as a scavenger. The larvae of *Orgyia* and many phytophagous insects are able to tolerate ingested tannic acid due to the PM acting as an ultrafilter, retaining high molecular weight phenols within the ENPS (Cross *et al.*, 1984; Grisham *et al.*, 1987).

1.7.4.1 PM protection against tannins

The striking resemblance of the PM to the gastric mucus of vertebrates is inferred by its physical location and chemical composition (Cross *et al.*, 1984). Several studies indicate that the mucin component of mucus in the human GI tract protects the underlying epithelium from damage by dietary pro-oxidants by acting as a free radical scavenger (Grisham *et al.*, 1987). The carbohydrate moieties of glycoproteins and proteoglycans of the PM, specifically sugars related to glucose, such as GlcNAc, Gal and Fuc may similarly suppress oxidative damage to the insect midgut epithelium (Peters, 1976; Peters and Wiese, 1986). The permeability of the PM is the most important factor determining the sensitivity of herbivorous insects to ingested tannins (Barbehenn and Martin, 1994), as this barrier can potentially protect them from oxidation of such pro-oxidants in the gut lumen and the resulting oxidative stress. The peritrophic envelopes (PE) of tannin-tolerant larvae of the white tussock moth, *Orgyia leucostigma* (Lymantriidae) are impermeable to tannins, so that 90-100% of ingested tannic acid is retained in the ENPS and egested with the frass (Barbehenn and Martin, 1992). However, containment of tannin within the ENPS of *Malacosoma disstria* (tannin sensitive) larvae is insufficient to protect the gut, since only 20% of ingested tannin is recovered in the frass despite the PE being seemingly impermeable (Adang and Spence, 1983; Santos and Terra, 1986). The pores within the PEs of larval Lepidoptera are not sufficiently small to obstruct the diffusion of tannic acid, since tannin molecules are much smaller (3.5-4.7 nm in diameter) than pores in the PE of Lepidoptera (7 nm) (Barbehenn and Martin, 1994). This does not account for the failure of dietary tannin to diffuse across the PEs (Barbehenn, 2001). Alternative explanations include the presence of substances in the matrix that strongly adsorb polyphenols (Barbehenn and Martin, 1998), or the polyanion exclusion hypothesis, in which negatively charged side chains (anionic sites) on proteoglycans in the PE act as an electrostatic barrier capable of repelling negatively charged tannin molecules (polyphenolate anions) (Miller and Lehane, 1993b), preventing their diffusion through the PE (Bernays, 1978). Some Acrididae (grasshoppers) deal with the toxicity of dietary tannins through a combination of hydrolysis of tannic acid into inactive gallic acid coupled with adsorption of tannin by the PM. *Schistocerca gregaria* is extremely tolerant of ingested tannic acid as a result of the hydrolysis of 70% tannin (Bernays and Chamberlain, 1980), with 34-84%

of the remainder being sequestered by the PM (Bernays, 1981). Proteoglycans within the caecal PMs are believed to act as the sink for tannins in this case (Summers and Felton, 1996). The tolerance of *Helicoverpa zea* larvae to ingested tannin has been attributed to the scavenging function of the PE successfully interfering with the generation of pro-oxidants in the midgut (Felton and Summers, 1995; Barbehenn *et al.*, 1996; Barbehenn *et al.*, 2003). The protection of the midgut epithelium from oxidative damage by dietary pro-oxidants is an additional function for PEs that are believed to act as anti-oxidants (Rayms-Keller *et al.*, 2000; Beaty *et al.*, 2002).

1.7.4.2. The role of the PM in resistance to heavy metals

Mucins are also believed to have a role in determining the resistance of aquatic arthropods to heavy metals and other toxic insults. Rayms-Keller *et al.* (2000) successfully isolated cDNA from *A. aegypti* expressed specifically in larval midgut in response to exposure to the heavy metals iron and cadmium. The expression of *A. aegytpi* intestinal mucin 1 (*AEIMUC*1) is induced by sublethal levels of the heavy metals copper and cadmium (Rayms-Keller *et al.*, 1998). However, higher concentrations of Cu or Cd are lethal by affecting PM integrity and formation (Beaty *et al.*, 2002), indicating that the response is metal and dose dependent. Concentrations of heavy metals found in contaminated aquatic ecosystems are sufficient to affect *AEIMUC*1 expression to the point of compromising PM integrity have the potential to reduce the resistance of aquatic arthropods to other biological and chemical insults (Morlais and Severson, 2001). The fact that *AEIMUC*1 expression is also induced by virus infection supports the hypothesis that the PM protects the insect midgut epithelium against a range of environmental insults by the qualitative and quantitative modulation of mucin and other constituents (Granados *et al.*, 2001).

1.7.4.3. The detoxification of the by-products of blood digestion

The heme produced as a result of digestion of the blood meal can reach substantial levels in the midgut of some mosquitoes, and presumably other haematophagous insects. Pascoa (2002) observed the adsorption of heme to the PM of *A. aegypti* in the form of electron-dense granules, which reached its saturatable limit 48 h post blood-meal. This indicates that the PM has a role in heme detoxification by sequestering it from the rest of the midgut. In addition, the PM may have a role in the elimination of toxic ammonium ions generated by the digestion of the blood-meal, for example glutamine synthetase (GS) from *A. aegypti* can utilise ammonia as a subtrate for the synthesis of chitin, which may be incorporated into the PM, efficiently eliminating the toxin by incorporating it into the PM, a process that does not incur water loss (Kato *et al.*, 2002).

1.7.4.4. The PMs role in protection from DDT

Abedi and Brown (1961) identified a strain of *A. aeypti* larvae resistant to DDT and linked this to a 9-fold increase in the rate of PM formation and 6-fold greater rate of DDT excretion compared to susceptible strains. This led them to the conclusion that the hypersecretion of PM is a defensive mechanism to remove DDT.

1.7.5. The PM as a barrier to pathogen invasion

The anti-parasite protection role of the PM was first proposed by Mercer and Day (1952), a function that is implied by the location of the PM, as the gut is the first line of contact with a number of physical, chemical and biological hazards (Miller and Lehane, 1993b; Shahabuddin and Kaslow, 1993; Tellam, 1996). In particular, the PM is the first point of contact during parasitic infection due to the tough exoskeleton and cuticle that lines the foregut, hindgut, trachea and many other vulnerable internal tissues, preventing entry via these routes (Ramos *et al.*, 1994; Zieler *et al.*, 1999; Zieler *et al.*, 2000).

In nature, *D. melanogaster* breed on rotting fruit from a broad range of plant species, although figs (genus *Ficus*) appear to be the most important niche of this species (Lachaise and Tsacas, 1983). Both the rotting fruit and the microorganisms growing on it provide food for drosophilids, although it is the latter, especially yeast, that act as the major nutritional source (Wagner, 1944; Wagner, 1949; Camargo and Phaff, 1957). This is because the fly medium must contain a source of vitamins and minerals vital to development, including biotin, folic acid and riboflavin, in addition to water, protein and carbohydrate (Sang, 1978; Begon, 1982). In this way *Drosophila* could potentially be exposed to pathogens in the field, leading to *per os* infection.

Pathogens and parasites transmitted by haematophagous insects are invariably taken up with the blood meal. They must reach the midgut epithelium in order to undergo further development. However, the PM completely surrounds the blood meal and separates the ingested parasites from the midgut. Therefore penetration of the PM is an obligatory stage in the lifecycle of many arthropod borne diseases, including malaria, river blindness and leishmaniasis (Richards and Richards, 1977). However, the intervening ECPS constitutes a relatively safe zone, where the parasite can develop without the risk of expulsion with the faeces by the force of a subsequent meal (Bignell, 1981); hence, it is an ideal site for the parasite to develop. The PM does not form an absolute barrier, since this would not permit the development of the pathogen within the insect, which in turn would not function as a disease vector. This hypothesis is supported by studies on the penetration of the PM of blood-sucking insects by bacterial and protozoan parasites (Shahabuddin *et al.*, 1995b). In addition, ultrastructural studies carried out

by Sieber (1991) provide direct evidence that the PM of mosquitoes plays a significant protective role against ingested parasites. However, its role is not clear-cut, since the absence of a PM in *A. aegypti* and *An. gambiae*, in which the PM had been disrupted by fungal chitinase or Polyoxin D, did not result in increased susceptibility to *P. gallinaceum*, indicating the PM may not be of major importance in determining the resistance of mosquitoes (Tellam, 1996).

Since PM pores are too small to permit particles the size of parasites, bacteria and viruses passage through them (Fuhrman *et al.*, 1992) parasites and pathogens use one of three strategies to gain entry to the midgut epithelium of insects:

1. Parasites invade the midgut epithelium during the window of opportunity that occurs prior to PM formation.
2. The pathogen persists in the blood meal and invades the gut once the PM degrades.
3. The parasite penetrates a fully mature PM after undergoing further development within the gut lumen.

1.7.5.1 The window of opportunity strategy

In the first case, in which the parasite traverses the midgut before the PM forms, the PM does not constitute a significant barrier. Most viruses and microfilariae use this window of opportunity strategy. Perrone and Spielmann (1986) demonstrated that microfilariae of *Brugia malayi* penetrate the mosquito gut soon after ingestion, before PM formation, despite having the potential to degrade the PM, since they encode a chitinase (Laurence, 1966). Similarly, the majority of *Onchocerca volvulus* microfilariae traverse the gut within 30 minutes of ingestion by blackflies (Duke and Lewis, 1964; Reid and Lehane, 1984), in which PM secretion is initiated within 10 minutes of taking a blood meal (Perrone and Spielmann, 1986). According to Ramasamy (1997a,b) the ookinetes of *Plasmodium vivax* penetrate the midgut of *An. tessellatus* prior to PM formation, whereas the majority of *Plasmodium* ookinetes develop later, so that infection requires the disruption of PM integrity in incompatible vector-parasite interactions (Ponnudurai *et al.*, 1988). Thus, successful penetration of the midgut epithelium is governed by the temporal relationship between parasite development and PM formation in type I PM insects (Billingsley and Rudin, 1992), which in turn determines parasite-vector specificity (Walters *et al.*, 1987).

1.7.5.2 The sit and wait strategy

Parasites such as *Leishmania* persist in the gut lumen until the PM breaks down, the promastigotes developing further by transforming into amastigotes, which invade the midgut

(Rayms-Keller *et al.*, 2000). The disintegration of the sandfly PM a few days after ingestion of the blood meal allows the promastigotes to invade and attach to the midgut epithelium. Conversely, Schlein *et al.* (1991) suggest that *Leishmania* uses the enzyme chitinase to penetrate intact PM in the same way as *Plasmodium* parasites, indicating that they do not use this sit and wait strategy.

1.7.5.3 Invasion of the PM

Plasmodium parasites commonly use the third strategy of invasion of the PM. Following ingestion, gametocytes develop into motile ookinetes, a process that can take up to 24 hours. Thus, the PM has fully matured once invasion of the midgut epithelium occurs, forming a formidable barrier. However, some parasites and pathogens are able to impair PM integrity (Shahabuddin and Kaslow, 1993; Shahabuddin *et al.*, 1993) by altering mucin expression, thereby reducing the resistance of the insect to pathogens. (Huber *et al.*, 1991) and (Sieber *et al.*, 1991) suggest that *Plasmodium gallinaceum* ookinetes secrete chitinase in order to digest and penetrate the PM of *A. aegypti*, a theory supported by evidence that inhibition of chitinase by allosamidin completely blocks transmission of malarial parasites (Ramasamy *et al.*, 1997; Vimal *et al.*, 2000). The vector-parasite specificity of *Plasmodium* infectivity in mosquitoes is determined by a number of factors, including the ligand-lectin binding of ookinetes to midgut cell walls (Perrone and Spielmann, 1988; Ponnudurai *et al.*, 1988), and the rate limiting potential of the PM, since different mosquito species form PMs at different rates (Ponnudurai *et al.*, 1988; Rudin and Hecker, 1989). The presence of mostly N-acetylglucosamine (GlcNAc) residues on *Ae. aegypti* PM, mainly N-acetylgalactosamine (GalNAc) sugars on *An. stephensi* PM and the absence of lectins on PMs of other species may explain the different susceptibility to the same *Plasmodium* species (Sieber *et al.*, 1991). Similarly, the protozoan parasite *Babesia microti* elegantly uses the contents of a specialised organelle, termed the arrowhead structure, to traverse the mature PM of the tick *Ixodes dammini* (Harmsen, 1973).

Trypanosomes, the causative agents of sleeping sickness in humans and nagana in livestock, are transmitted by tsetse flies. The tsetse fly produces a PM 2 prior to feeding, suggesting that the PM should limit invasion by ingested parasites. Indeed some dipteran PMs are completely impermeable to trypanosomes (Peters, 1992). This parasite was once thought to enter the ENPS exclusively via the open end of the PM in the hindgut (Ellis and Evans, 1977; Peters, 1992; Miller and Lehane, 1993a). However, there is evidence in support of trypanosomes directly penetrating the PM (Ellis and Evans, 1977). The passage of trypanosomes from the endo- to the ECPS is an essential process during *T. brucei* development (Harper and Granados, 1999). The PM forms an effective barrier once mature, since the natural frequency of *T. brucei*

infections in tsetse flies is less than 0.04% (Harmsen, 1973), so that significant parasite survival is only observed in immature flies, lacking a fully mature PM.

1.7.6. Mucins and host defense against pathogens

Invertebrate Intestinal Mucin (IIM) from *Trichoplusia ni* and Hemomucin from *Drosophila* are believed to protect the insect midgut in an analogous way to vertebrate mucins, since they are biochemically similar (Theopold *et al.*, 1996). Hemomucin, a *Drosophila* hemolectin, was originally isolated from hemocytes, but is also present in the PM and the chorion of mature oocytes where it is involved in defence responses (Menrath *et al.*, 1995). There have also been a number of mucin-type glycoproteins identified in other invertebrates. The protective coating of the female reproductive duct in *Schistosoma mansoni* is composed of mucin-like proteins. Tachycitin, an antimicrobial peptide (AMP) from the horseshoe crab *Tachypleus tridentatus* possesses a mucin-like structure with a CBD pocket fashioned from three disulphide bonds (Tse and Chadee, 1991; Strous and Dekker, 1992; Kawabata *et al.*, 1996; Suetake *et al.*, 2000). The wide tissue distribution of these mucins is atypical for PM proteins, but nonetheless reflects the potential range of novel functions possessed by mucins.

The vertebrate mucus barrier is maintained by the regulation of mucin production and degradation, since only intact mucin molecules form an effective barrier against enteric pathogens (Holzer, 2000), with repair of the underlying mucosa being the ultimate defence (Byrd *et al.*, 2000). Many enteric pathogens and their toxins have potent secretagogue (stimulate mucin release) effects. The hypersecretion results in either the expulsion of the pathogen or an inhibition of mucin synthesis (Perez-Vilar and Hill, 1999). The genes encoding human MUC2 and MUC5AC, the major mucins secreted by the gastrointestinal tract in humans, are upregulated in response to bacterial exoproducts, such as lipopolysaccharides (Van-Klinken *et al.*, 1995; Theopold *et al.*, 1996).

Mucins are well known for their ability to bind pathogens, playing a critical role in mediating parasite-midgut interactions, thereby inhibiting infection (Peters, 1992). Specifically, the oligosaccharide side chains of mucins exposed on the PM surface mimic those of cell surface receptors (Forstner and Forstner, 1994). By binding pathogens they are competitively inhibiting receptors on underlying epithelial cells, which protects the midgut epithelium from pathogens (Tse and Chadee, 1991). Once the mobility of the pathogen has been impeded by mucins within the PM, they are susceptible to the natural renewal of the PM. Similarly to vertebrate mucus, the PM is in a dynamic rather than static state, constantly being replenished by the midgut epithelium and lost with the excreta (Shen *et al.*, 1999; Tellam *et al.*, 1999). AgMUC1 and ICHIT from *Anopheles gambiae* show similarity to *Trypanosoma cruzi* mucin-like glycoproteins,

suggesting they may act to immobilise *Plasmodium* parasites during invasion, and so reduce parasite transmission (Peters, 1992). A more direct antipathogenic function has been attributed to MUC1 of *Aedes aegypti* (*AEIMUC*1), since it is upregulated in response to Dengue infection (Raymus-Keller, A., unpublished data). Interestingly, MG2, a human salivary mucin, has potent antibacterial activity, and has been shown to have significant similarity to *AEIMUC*1. Ligands (= specific sugar residues) are commonly exposed on the luminal surface of the PM (Rudin and Hecker, 1989), where they are believed to be central to the recognition of host tissues by parasites, thereby impeding the passage of the parasite or aiding PM penetration and midgut invasion (Lehane and Msangi, 1991). For example, GlcNAc, a ligand bound to the PM of *G. m. morsitans* (Lehane *et al.*, 1996) is known to aid the binding of trypanosomes via a specific lectin (Ibrahim *et al.*, 1984). Thus, species differences in the expression of such residues may help explain vector-parasite specificity (Theopold *et al.*, 1999). The ligand GalNAc, recognised by *Plasmodium berghei* ookinetes is present in the midgut of *An. stephensi* (susceptible), but is absent from *A.aegypti* (refractory) mosquitoes (Rudin and Hecker, 1989). The marked difference in the proportion of glycoproteins between adult and larval PMs of *Calliphora vicina* (Shen *et al.*, 1999) indicates that the prevalence alters during development, suggesting that these differences may reflect varied functions of the PM.

Surprisingly, PM proteins may also aid parasite invasion of host insects, since the invertebrate intestinal mucin gene, AgMUC1, from the PM of *An. gambiae* has a similar structure to mucins present on the coat of the *Plasmodium* parasite (Gutierrez *et al.*, 1999). Similarly, an antigen identified in the ECML of *Triatoma infestans*, the vector of Chagas disease, is also found on the outer membrane of *Trypanosoma cruzi* (Neville, 1975; Kenchington, 1976; Tristram, 1977; Peters, 1992). These molecules may be able to bind to each other, effectively aiding the parasite to dock onto and then penetrate the PM.

1.7.7. Specialised functions

In addition to its role within the midgut, the PM has evolved to form numerous divergent, specialised structures and functions including cocoon construction (Turner and Ferrante, 1979; Peters, 1992) and faecal pellet coverings (Kathirithamby, 2000). A highly specialised role for the PM was recently identified by Moll (2001), who found that the meconium and the meconial peritrophic membranes of mosquito pupae act to sterilize the gut during metamorphosis and adult emergence.

1.8. Disruption of PM structure

The synthesis of the adult *C. erythrocephala* PM has been shown by Becker (1978) to be temporally regulated by the ecdysteroids ecdysone and juvenile hormone, probably through the regulation of chitin biosynthesis (Becker, 1980; Zimmermann and Peters, 1987). Moreover, potential ecdysone response elements have been identified in the regulatory region of the *Drosophila* (Tellam *et al.*, 2000b) and *A. aegypti* (Ibrahim *et al.*, 2000) chitin synthase gene. This may help account for the differences observed in PM structure in the different life stages of insects possessing the same type of PM. A range of agents, such as the chitin synthesis inhibitor polyoxin-D (Abedi and Brown, 1961), DDT (Rupp and Spence, 1985; Russell and Dunn, 1996) and *Bacillus thuringiensis (Bt)* endotoxin, are known to alter the glycoprotein composition of the PM of *Manduca sexta* larvae, causing it to become more fragile (Kramer and Muthukrishnan, 1997). Alternatively, the PM of some insects is degraded naturally in the hindgut by spines or teeth that mechanically fray an intact PM into pieces. The adult fleshfly, *Parasarcophaga argyrostoma* secretes two PMs continuously, which are degraded by small teeth in the hindgut, a process that may be completed by rectal pads and chemical degradation (Nagel and Peters, 1991). The following describes the degradation of specific components of the PM in more detail.

1.8.1. Degradation of the chitin component of the PM

Chitinolytic enzymes are secreted by the epidermis, midgut, salivary glands and fat body in insects, where they are used primarily to digest the chitin of their exoskeletons and cuticle lining the fore- and hind-gut during the molting process (Lu *et al.*, 2002; Arakane *et al.*, 2003). Chitinase has been isolated from the gut of several insect species, including the tobacco hornworm *Manduca sexta* (Zheng *et al.*, 2003), the spruce budworm *Choristoneura fumiferana* (Babiker *et al.*, 2001; Kimura, 1981) and the silkworm *Bombyx mori* (Brandt *et al.*, 1978). Chitinases were first shown to degrade the PM of insects in the late 1970s. Chitinases produced by pathogens have a key role in determining the virulence of insect-borne parasites that infect insects via the *per os* (oral) route, by perforating the PM, and thus allowing them access to the underlying epithelium (Schlein *et al.*, 1991; Shahabuddin and Kaslow, 1994). This is a particularly effective strategy in facilitating the infection of mosquitoes and other insects with a PM 1, since chitinases have been shown to dramatically alter the structural integrity of PM 1s (Tellam *et al.*, 1999). Similarly, chitinase has an effect on the structural integrity of PM 2. For example, Becker (1980) showed that chitinase dramatically reduced PM 2 production in *C. erythrocephala* larvae. In contrast, it has been suggested by Tellam and Eisemann (2000) that

chitin is only a minor component of PM 2. This hypothesis is supported by the finding that the PM of *L. cuprina* larvae was also not affected by treatment with a sublethal dose of polyoxin D, a potent inhibitor of chitin synthesis (Rudin and Hecker, 1989). Alternatively, the PM 2 of *L. cuprina* larvae may be just as susceptible to chitin degradation as that of *C. erythrocephala* larvae. A property which is masked by the protection of the β-1-4 glycosidic linkages of chitin within the PM from the action of exo- and endo-chitinases by the close association of P-15 and other peritrophins (Eisemann *et al.*, 2001; Tellam and Eisemann, 2000).

Plasmodium parasites have been shown to disrupt the PM in ultrastructral studies. Sieber *et al.* (1991) and Huber *et al.* (1991) demonstrated that chitinase activity occurs in malarial ookinetes within the mosquito midgut. The PM was also shown by the authors to be digested by *Serratia marcescens* chitinases. Vinetz *et al.* (2000) further investigated *Plasmodium* ookinete chitinolytic activity, and identified a gene encoding chitinase in *P. gallinaceum*, the PgCHT1 gene. The PM of *A. aegypti* and *An. stephensi* differ in their composition as *A. aegypti* PM contains chitin (Berner *et al.*, 1983), whereas the PM of *An. stephensi* reportedly does not (Sampson and Gooday, 1998), suggesting penetration of these species involves different biochemical mechanisms. The role of chitinase in the pathogenesis of *B. thuringiensis* (Schlein *et al.*, 1992; Schlein *et al.*, 1991) and the trypanosome *Leishmania* (Shahabuddin *et al.*, 1993) have also been examined. Both investigations lead to the conclusion that the parasite endogenous chitinases are acting to weaken the insects PM, allowing easy access to the midgut epithelium.

The effectiveness of chitinase is emphasised by an experiment in which the addition of exogenous chitinase to the blood meal of the mosquito *Anopheles freeborni*, prevented the formation of a PM (Filho *et al.*, 2002). Transgenic tomato cultivars expressing chitinolytic enzymes have enhanced resistance to damage by *T. ni* larvae, due to disruption of the chitin component of the PM leading to increased PM permeability (Kramer and Muthukrishnan, 1997). Details of other experiments in which chitinase has been utilised as a means of enhancing virulence are given below (Table 4).

Table 4. Examples where the virulence of insect pathogens is enhanced by supplementation with chitinolytic enzymes.

Insect	Pathogen	Origin of chitinase	Reference
Spodoptera littoralis	*Bacillus thuringiensis*	*Serratia marcescens*	(Regev *et al.*, 1996) (Schlein *et al.*, 1992)
Trichoplusia ni	*Bacillus thuringiensis*	*Streptomyces albidoflavus*	(Sampson and Gooday, 1998)
Culicoides nubeculosus	*Bacillus thuringiensis*	*Serratia marcescens*	(Sampson and Gooday, 1998)

Table 5. Pathogens possessing their own chitinase activity.
Adapted from (Shahabuddin and Kaslow, 1993).

Insect	Pathogen	Reference
Glossina spp.	*Trypanosoma brucei brucei*	(Schlein *et al.*, 1991; Schlein *et al.*, 1992)
Ixodus dammini	*Babesia microti*	(Rudzinska *et al.*, 1982)
Autographa californica	*Nucleopolyhedros virus*	(Lepore *et al.*, 1996)
Anopheles spp.	*Plasmodium falciparum*	(Fuhrman *et al.*, 1987)
Aedes aegypti	*Plasmodium gallinaceum*	(Huber *et al.*, 1991)
A. aegypti, Anopheles spp.	*Brugia malayi*	(Fuhrman *et al.*, 1992)
Phlebotomus spp.	*Leishmania donovani*	(Schlein *et al.*, 1991)
Phlebotomus spp.	*L. infantum*	(Schlein *et al.*, 1991)
Lutzomyia spp.	*L. brasiliensis*	(Schlein *et al.*, 1991)
Epilachna vigintioctopunctata	*Alcaligenes paradoxus*	(Otsu *et al.*, 2003a)
E. vigintioctopunctata	*Enterobacter cloacae*	(Otsu *et al.*, 2003b)
Aedes spp. Anopheles spp.	*Brugia malayi*	(Fuhrman *et al.*, 1987)
Culicoides pungens	*Onchocera gibsoni*	(Gooday, 1990)
Bombyx mori	*Aeromonas*	(Ono and Kato, 1968b)
Galleria mellanella	*Serratia marcescens*	(Lysenko, 1976)

Chitinases consist of peritrophin-A domains, characterised by a register of 6-cysteines, that are implicated in chitin binding, in addition to a catalytic domain (Venegas *et al.*, 1996). The peritrophin-A domains within *B. mori* and *B. malayi* chitinases have been shown to bind chitin (Arakane *et al.*, 2003). The requirement of *Plasmodium* chitinase for the parasites infectivity to mosquitoes was demonstrated by the reduction in infectivity caused by the knockout of the *Plasmodium berghei* chitinase gene *PbCHT1* in null mutants (Dessens *et al.*, 2001). Similarly, a knockout experiment confirmed that chitinase is essential for *P. falciparum* invasion of the PM (Shahabuddin, 1995b).

Chemical disruption of the PM has also been widely investigated. A wide range of agents have been tested in adult and larval (Edwards and Jacobs-Lorena, 2000) mosquitoes (Diptera), including exogenous chitinase, Calcofluor, Cycloheximide, Polyoxin D and DDT. Formation of the adult PM was most inhibited by Polyoxin D, whereas DDT proved to be the most effective at disrupting the larval PM, and the effect of bacterial chitinase was variable (Wang and Granados, 2000). Polyoxin D is a potent competitive inhibitor of insect chitin synthesis that has been shown to dramatically reduce the formation of the PM 2 from *C. erythrocephala* larvae (Becker, 1980). Calcofluor effectively inhibits chitin synthesis (Shen and Jacobs-Lorena, 1997; Filho *et al.*, 2002; Merzendorfer and Zimoch, 2003) since it blocks *T. ni* PM formation and increases

larval susceptibility to baculovirus infection, the effect being temporary and reversible, as it is linked to IIM degradation.

As with the insect cuticle, the PM is regulated by a triple enzyme system, employing chitin synthases and chitinolytic enzymes to control chitin metabolism, thereby affecting its synthesis and degradation respectively (Shen and Jacobs-Lorena, 1997). The midgut chitinase of *Anopheles* mosquitoes is activated by trypsin 10 hours post blood meal, which may be used to degrade the PM after digestion (Shen and Jacobs-Lorena, 1997). The precise expression of insect midgut chitinases may also be involved in the regulation of PM thickness and permeability through modulation of its structure (Kramer and Muthukrishnan, 1997). Filho *et al.* (2002) demonstrated that chitinase completely blocked PM formation in *A. aegypti*, whereas allosamidin, a potent chitinase inhibitor, resulted in the formation of a thickened PM. Chitin degradation is a two-step process in which chitinase hydrolyses chitin into oligomers of GlcNAc that subsequently become the substrate for N-acetylglucosaminidase, which degrades the oligomers into monomers (Zimmermann and Peters, 1987).

1.8.2. Disruption of Peritrophins

Removal of key peritrophins from the PM has the potential to disrupt the structural integrity of the PM weakening chitin-protein and protein-protein interactions (Shen and Jacobs-Lorena, 1999). However, peritrophins are highly resistant to the action of proteinases due to their tight association with chitin reducing their exposure to hydrolysis and the presence of numerous intradomain disulphide bonds that mask the cleavage sites present in CBDs.

1.8.3. Mucin degradation by enteric pathogens

A number of pathogens have developed novel strategies in order to overcome the mucin blockade by producing virulence factors that disrupt the balance between mucin production and degradation, such as proteinases and glycosidases. In vertebrates the pathogens *Vibrio cholerae* (Silva *et al.*, 2003), *Entamoeba histolytica* (Tse and Chadee, 1992), *Yersinia enterocolitica* (Mantle and Husar, 1994), and *Shigella* (Henderson *et al.*, 1999) produce mucin-degrading enzymes (mucinases). The PM 2 of larval Lepidoptera is not a significant protective barrier to viruses that produce the mucin-digesting enzyme, enhancin (Tanada, 1959). This virus infection-enhancing protein is a 33 kDa cysteine protease, capable of degrading high molecular weight proteins such as mucins, that was first discovered in the armyworm *Pseudaletia unipunctata* (Corsaro *et al.*, 1993). Wang and Granados (1997b) discovered that the baculoviruses *Autographa californica nuclear polyhedrosis virus* (AcMNPV), and *Trichoplusia ni granulosis virus* (TnGV) also code for this enzyme. *Trichoplusia ni* granulosis virus (TnGV) produce an

enhancin that specifically degrades Insect Intestinal Mucin (IIM) (Bolognesi *et al.*, 2001; Pechan *et al.*, 2002), a major structural protein of the *T. ni* PM (Derksen and Granados, 1988), which disrupts PM integrity (Wang *et al.*, 1994), allowing the virions access to the midgut epithelium (Derksen and Granados, 1988). Denaturing gel electrophoresis (SDS-PAGE) was used to identify the protein targeted by enhancin, which demonstrated that the protein profile changed during infection with TnGV, with the disappearance of high molecular weight proteins (Granados *et al.*, 2001). *T. ni* PM treated with enhancin are also significantly more permeable to AcMVPV virions due to the destruction of PM structural integrity (Wang *et al.*, 1994). The increased mortality due to AcMNPV infection in lepidopteran larvae treated with enhancin is also correlated with altered protein profiles (Derksen and Granados, 1988) and a more fragile PM in virus treated larvae (Wang and Granados, 1998). Wang and Granados (1998) identified a mucin similar to the insect intestinal mucin (IIM) from *T. ni* in *P. unipuntata* PM that was targeted by TnGV enhancin, resulting in enhanced infection and mortality (Corsaro *et al.*, 1993). Enhancin has since been shown to improve the efficiency of the biopesticide Bt from two- to seven-fold in most insects studied depending on the dose of enhancin and Bt, with a massive 250-fold enhancement in *T. ni* larvae (Hayakawa *et al.*, 2004). Thus, some parasites and pathogens are able to compromise PM integrity or alter mucin expression, resulting in reduced resistance of the vector. Enhancin has a potential use as a biopesticide through the engineering of transgenic plants to synthesize enhancin gene products. This ensures that the pest receives a constant dose, with prolonged disruption of the PM facilitating the entry of opportunistic pathogens and their toxins to the susceptible midgut epithelium. The expression of TnGV enhancin by transgenic tobacco resulted in retarded growth of *Pseudaletia separata* and *Spodoptera exigua* larvae (Pechan *et al.*, 2002). Insect resistant maize lines produce enhancin in response to insect wounding, resulting in severe damage to the PM in the form of perforations and abrasions (Rudin and Hecker, 1989).

Recently, Osta *et al.* (2004) identified a C-type lectin with mannose specificity (CTLMA) from the midgut of *Anophelene* mosquitoes that protects ookinetes against melanization, a mosquito innate immune response. Thus this immune gene acts as a protective antagonist that promotes vector susceptibility to infection by blocking pathogen recognition. Similarly, host mucins may provide adhesion ligands that facilitate invasion, since it is thought that specific sugar residues are central to the recognition of host tissues by parasites, thereby aiding PM penetration and midgut invasion (Theopold *et al.*, 1999). In addition mucins may act as a convenient source of metabolic energy for pathogens that have evolved to exploit them. (Ponnudurai *et al.*, 1988; Rudin and Hecker, 1989). Differences between species in the expression of such residues may explain vector-parasite specificity in mosquitoes (Peters *et al.*,

1983). The presence of lectins and glycoproteins with mannose specific residues on the lumen side of the PM was first demonstrated in the larvae of *Calliphora erythrocephala* by Peters (1983). Many species of commensal and pathogenic bacteria have the ability to bind to mucins. *Proteus*, a common gut bacterium adheres to the PM using pilli via mannose-specific lectins (Sajjan and Forstner, 1990). Similarly, *E. coli* and mammalian mucus have mutual adherence properties that may serve to aid colonisation of beneficial bacteria. Moreover, some mucins within mucus or the glycocalyx provide initial binding sites for pathogens such as the bacterium *E. coli* serotype 0157:H7 (Ensgraber and Loos, 1992; Ghosh *et al.*, 1996; Giannasca *et al.*, 1996; Vimal *et al.*, 2000), *Salmonella typhimurium* and the protozoan parasite *Cryptosporidium parvum* (Cevallos *et al.*, 2000). Infective *Cryptosporidium parvum* bind to the Gal and GalNAc residues on the human mucins MUC2 and MUC5 via Gal or GalNAc lectins (Ravdin *et al.*, 1993). A Gal-specific lectin from *Entamoeba histolytica* binds to human mucins and epithelial cells of the GI tract (Moncada *et al.*, 2003a and 2003b).

Unlike vertebrates, the surface of the insect midgut epithelium does not possess lectins with mannose affinity, and so lacks the ability to serve as a suitable binding substrate. Instead, the PM is believed to have gained mannose specific lectins by becoming an extension of the surface of midgut cells, which subsequently lost this function during evolution (Peters *et al.*, 1983). The presence of mannose specific lectins on the lumen side of the PM in larvae of the blowfly, *C. erythrocephala*, which act as adhesion ligands for the bacteria *Proteus vulgaris* and *P. morganii*, is in favour of this mode of evolution (Lehane and Msangi, 1991). The agents of Trypanosomiasis, *Trypanosoma cruzi* and *T. brucei*, express mucin-like molecules that act as membrane anchors. Likewise, GlcNAc, a ligand bound to the PM of *G. m. morsitans* (Lehane *et al.*, 1996), is capable of binding to the corresponding lectin of trypanosomes (Richards and Richards, 1977).

1.9. PM as a target for vaccination

Plant lectins have also been found to bind to the chitin component of the PM (Czapla and Lang, 1990). The mortality of the European corn borer *Ostrinia nubilalis* and the Southern corn rootworm *Diabrotica undecimpunctata* can be attributed to the ingestion of plant lectins with GlcNAc and Gal specificity (Lehane, 1996; Casu *et al.*, 1997). The current trend in vector control research is toward the production of novel control strategies (East *et al.*, 1985). The control of insect-borne disease by vaccination of haematophagous insects is being investigated. The low cost, high degree of specificity for the target parasite and lack of undesirable side-effects are potential advantages of vaccination over pesticides (Billingsley, 1993). Novel or

'concealed' antigens of the gut, especially those exposed on the surface of the PM, offer a valuable method of artificially inducing immunity (East *et al.*, 1993; Tellam *et al.*, 1994; Tellam, 1996). PMs are prime targets for vaccination due to their location, function and composition. The PMs of parasitic arthropods are directly exposed to ingested host fluids containing immune molecules. This coupled with their chemical composition and physiological function suggests that PMs are potentially the target of immune attack by host antibodies from hosts vaccinated with whole or extracts of PMs (Sukarsih *et al.*, 2000). However, it may be difficult to produce adequate quantities of the PM extract to have the desired effect since the gut is also the site of digestive proteolysis, implying immune molecules may be degraded before they are able to interact with target molecules. In addition, the rate of intake of immune molecules must be sufficient to have an impact on structure that is being continuously produced. Studies show that anti-PM antibodies are potentially a successful approach to vector control. An 82% reduction in larval weight from *in vitro* assays of vaccination against the Old World screwworm, *Chrysomyia bezziana* is very encouraging, especially since control of this pest using traditional methods is difficult (Willadsen, 1999; Sukarsih *et al.*, 2000).

Alternatively, several non-immune targets can be used as targets to block malaria transmission, including chitinases, trypsins and proteases. *Plasmodium* ookinetes secrete chitinases in order to penetrate the PM of the mosquito midgut (Ramasamy, 1992), indicating that anti-chitinase vaccines may be effective (Shahabuddin, 1998). Trypsin is required by mosquitoes during digestion of the blood meal, and is utilised by the *Plasmodium* parasite to activate the chitinase used to invade the PM (Shahabuddin *et al.*, 1995a). Anti-trypsin antibodies are effective inhibitors of trypsin activity (Tellam *et al.*, 1994). However, anti-protease antibodies have been unsuccessful in controlling *L. cuprina* larvae (Rand *et al.*, 1989).

1.9.1. Vaccination against ticks

Rand (1989) successfully vaccinated cattle with anti-PM antibodies from the gut of the tick *Boophilus microplus* (Willadsen *et al.*, 1989). Treatment with the Bm86 protective antigen resulted in profound immunological damage due to lysis of midgut cells (McKenna *et al.*, 1998). However, co-vaccination of Bm86 with BmA7 has been shown to enhance immunity compared to that produced by Bm86 alone (East and Eisemann, 1993; East *et al.*, 1993).

1.9.2. Vaccination against blowflies

Similarly, sheep vaccinated with PM extracts from the sheep blowfly *L. cuprina* (East and Eisemann, 1993; Tellam and Eisemann, 1998) were immunised against infection. They are protected by the growth of larvae being significantly retarded, the mean weight of larvae from

immunised sheep being 50% of that from control sheep ($P<0.05$), with a corresponding increase in mortality. The strongest anti-larval effect was exerted by the PM extract containing proteins and glycoproteins (Willadsen *et al.*, 1993), specifically P-44 and P-95 (Eisemann, 1990). P-44 alone has been shown to reduce larval growth by 60% (Casu *et al.*, 1997). The P-95 vaccine antigen causes some antibody-mediated inhibition of *L. cuprina* larvae (Wijffels *et al.*, 1999).

1.9.3. Vaccination against mosquitoes

In contrast, research into vaccination against mosquitoes has been relatively unproductive (Ramasamy, 1992). Rabbit anti-sera raised against midgut extracts from *Anopheles tessellatus* did reduce their fecundity (Ramasamy *et al.*, 1996). However, putative midgut-specific antigens identified resulted in less effective vaccines, since they only inhibited the formation of the PM in the posterior midgut, which is not essential for protection from *Plasmodium* infection or survival of the vector (East and Eisemann, 1993). If the antibodies are in a concentrated form they are capable of causing substantial mortality (Wang *et al.*, 2001). A number of antigens have been identified in *A. aegypti* that could be potential targets for infection blocking vaccines (Kaslow, 1993). Thus, anti-mosquito antibodies have the potential to disrupt mosquito physiology, but further work is required before vector control using vaccines becomes a realistic option (Willadsen *et al.*, 1993).

1.9.4. How do PM derived vaccines work?

The mode of action of such candidate vaccines is still not fully understood, although a number of theories exist. One such hypothesis is that binding of anti-PM antibodies to the PM forms an impenetrable barrier that reduces its permeability to products of digestion and enzymes, subsequently leading to starvation due to a reduction in the ability of the insect to utilise ingested food. This theory is supported by the identification of a thick, amorphous layer on the lumen side of the PM in anti-P-44 and P-95 antibody treated larvae of *L. cuprina* (Tellam and Eisemann, 1998). Specifically, the binding of the antibody is believed to be preventing the normal movement of digested food particles, which are consequently precipitated on the PM. The mechanism by which such antibodies retard the growth of the larvae resembles that exerted by crude anti-PM extracts, since the dense layer observed by transmission electron microscopy on the lumen side of the PM in treated larvae indicates that it is an antibody-induced layer which reduces the permeability of the PM as a result of binding to their respective protective antigens (Tellam *et al.*, 1992).

PM formation itself could potentially be disrupted by the binding of antibodies, especially in PM 1s, since the formation zone covers the whole of the midgut, rendering it

vulnerable to immune attack. However, interference during PM 2 formation by antibodies would be restricted to those regions where antibodies would have access to formation zones. This precludes the majority of PM 2s from immune attack, as the cardia isolates the formation zone from the contents of the gut lumen.

Lastly, antibodies may be targeting functional molecules bound to the PM. The Bm86 antigen in *B. microplus* has been localised to the surface of midgut epithelial cells using immunofluorescence and immunogold labelling (Willadsen *et al.*, 1989). The surface glycoprotein to which it binds contains a number of repeats, including 6-cysteine motifs that are characteristic of receptors. The uptake of this antibody by feeding has been shown to cause severe damage to the parasite. It is available commercially as a vaccine under the name tickGARD as it induces effective protection of cattle against the parasite (Willadsen *et al.*, 1989) showing that this approach can be successful.

1.10. Aims and Outline of this Thesis

The PM has received considerable attention during past decades, and yet several elementary questions remain to be solved. The hypothesis that the PM has a protective function remains to be determined fully. Peritrophins have the potential to fulfil an important role in reinforcing the structure of the PM and hence protection against pathogens and their toxins. In this doctoral research project I aim to reveal the peritrophins critical to determining the structure and function of the PM, using a multi-faceted approach:

1. To identify novel putative peritrophins from the type 2 PM of *Drosophila melanogaster* through the use of Suppression-PCR Subtractive Hybridisation and Reverse Transcriptase-PCR (RT-PCR). This method will be applied to isolate differentially expressed genes from the cardia of both adults and larvae.
2. To indirectly determine the distribution of peritrophins within the PM using *in situ* hybridisation and confocal imaging to identify the specific secretion zone(s) within the cardia in which the transcripts of the genes identified above.
3. To investigate the function of the PM in *Drosophila* by identifying suitable mutant strain(s) in which a deficiency in single or multiple genes encoding peritrophins lead to a PM-compromised phenotype in adults and/ larvae or using RNA interference (RNAi) as a tool to specifically knockdown expression of a number of peritrophins.
4. To study the susceptibility of PM-compromised (mutant) and PM-normal (wild type) *Drosophila* to *per os* infection with midgut infective bacteria by conducting survival analyses.
5. To analyze the expression of genes encoding peritrophins in response to key physiological states in *Drosophila*, by means of Real Time RT-PCR.
6. To conduct a comparative study in *Glossina*, using the *Glossina* EST database to identify homologs of *Drosophila* peritrophins.

Chapter 2. Identification of novel peritrophins from the peritrophic matrix of *Drosophila melanogaster* adults and larvae

2.1. Abstract

In order to gain significant insights into the components of the type 2 PM of *Drosophila* a systematic search for genes specifically expressed within the cardia was conducted using suppression subtractive hybridisation (SSH), an expression profiling technique, to identify differentially expressed genes. The method has the potential to isolate genes encoding unique PM proteins since it does not discriminate against rare transcripts with enrichment of those genes that are differentially expressed. Several sequences that may be putative peritrophins, and thus implicated in PM structure have been identified. Fifteen adult and thirty-one larval genes were homologous to known peritrophins, of which nine and twelve were unique respectively, and another four were homologous to genes without known function. This work provides a starting point for the development of a molecular profile of the type 2 PM in *Drosophila*.

2.2. Introduction

It is a priority during the post-genomic era to ascribe functions and expression profiles to genes and gene products identified by the genome project of the highly valuable model organism, *Drosophila*. Whilst the physiological properties of the PM are well known, there is little understanding of its molecular basis. One way to better appreciate the molecular framework that forms the PM is to conduct gene profiling by isolating and analyzing genes specifically expressed in the separate tissues that secrete the PM. I have therefore utilised a differential gene expression analysis technique to systematically search for genes that are involved in PM formation in *Drosophila*. There are a number of techniques available to compare differences in gene expression, including mRNA differential display (Liang and Pardee, 1992; Morlais and Severson, 2001), representational difference analysis (RDA) (Lisitsyn *et al.*, 1993), serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995; Yamamoto *et al.*, 2001) and standard macroarrays. The majority of these techniques have been superceded by the development of more sensitive methods, such as cDNA microarrays (Schena *et al.*, 1995) and subtractive hybridisation (SH). Despite being a relatively technical and labour-intensive technique, SH has a number of advantages over previous techniques, since it can be used to isolate previously uncharacterised cDNAs and does not discriminate against rare transcripts.

Subtractive Hybridisation (SH) and its sister method Suppression Subtractive Hybridisation (SSH) are techniques designed to accurately identify differentially expressed target genes that are either uniquely expressed or highly induced when compared between two populations of mRNA. SSH is particularly useful when limited amounts of source tissue are

available, since it is a PCR-based subtraction technique that allows the conversion of mRNA into a form that can be amplified prior to subtraction. cDNA can be amplified by PCR, once the DNA has been fragmented so as to prevent bias during PCR, as smaller fragments are preferentially amplified. The kinetics of SSH allow the identification of differentially expressed genes through the hybridisation of denatured double-stranded cDNA (tracer) to an excess (at least 10 fold, critical for enrichment) of denatured double-stranded cDNA (driver). This strategy enriches the differentially expressed transcripts represented by the non-reassociated fraction of tracer since only those sequences common to both the driver and tracer populations form duplexes. After hybridisation, driver-tracer hybrids and unhybridised driver are removed during the subtraction step. Thus, the tracer is enriched for sequences specifically expressed by the tracer tissue and depleted of sequences common to tester and driver. Consequently, multiple rounds of subtraction can be carried out to enhance the sensitivity of the probe sufficiently to detect even rare (0.005-0.01%) sequences that are unique to the tracer (target) within the morass of cDNA (Diatchenko *et al.*, 1996). In addition to subtraction, the desired sequences are actively isolated from the pool of cDNA by positive selection provided by the exponential amplification of tracer-tracer hybrids that possess adaptors at both ends of the duplex DNA. In this way the cDNA population can be enriched, by increasing the representation of less abundant transcripts between 50- to100-fold, which frequently correspond to the most interesting genes or gene products. The isolation of differentially represented clones can also be enhanced by probing duplicate blots with a subtracted probe versus an unsubtracted probe, or two subtracted probes (tracer-driver and driver-driver) since only one-way comparisons can be made using SSH (See Fig. 9 for clarification).

Our current understanding of the role of peritrophins in the structure and function of the PM is still very limited. The studies involved in this chapter provide valuable information about the molecular characteristics of the PM in *Drosophila*, through the identification and characterisation of numerous novel integral PM proteins. A number of PM chitin binding proteins, in particular peritrophins and mucins have been described in invertebrates, including a family of peritrophins in *L. cuprina* whilst only a few have been identified in *A. aegypti*, *An. gambiae*, *T. ni*, *M. configurata* and *C. bezziana* (see Fig. 6). In contrast only Gasp and Peritrophin-A have been studied in the PM of *Drosophila*. In addition a number of peritrophins are listed as being putative structural constituents of the PM (Gene Ontology GO: 0016490). However, to date none of these chitin-binding proteins, identified by annotation of the *Drosophila* genome database have been characterised further.

Chitin binding domains (CBDs) are extracellular domains that consist of six conserved cysteines that probably form three disulphide bridges. Chitin binding has been demonstrated for a protein containing only two of these domains. An analysis of amino acid sequences from the databases available indicate that copies of CBDs are present in most peritrophic matrix proteins; Tequila, a serine protease and Hemolectin, a sugar binding protein from *Drosophila*; Tachycitin, an antimicrobial peptide isolated from Horseshoe crab hemocytes; animal chitinases; some baculoviruses and a number of proteins of unknown function.

Chitin binding proteins in invertebrates have only recently been characterised, despite the great importance of chitin in invertebrates. The first CBD to be characterised, Peritrophin-44 from the type 2 PM of *L. cuprina*, is known to contain five tandem CBDs, each comprising of a typical peritrophin-A domain (Elvin, 1996). A number of studies have verified the chitin binding properties of peritrophins through in-vitro studies, including P-44 (Elvin, 1996) and *An. gambiae* Aper1 (Shen and Jacobs-Lorena, 1998). Only the structure of the chitin-binding domain of tachycitin is known. It has been found to consist of a distorted beta-sandwich formed from a group of two and three beta-sheets connected via a bending loop, to form a chitin binding site at Cys-40 to Gly-60 (Suetake *et al.*, 2000).

The CBD is currently recognised by some databases as either the Chitin binding motif 14 (CBM_14) or Chitin binding Type 2 domain (ChtBD 2). Whereas, the consensus of these two conserved domains are not distinct except for the absence of the sixth cysteine in the ChtBD 2 motif, despite its presence in target sequences.

2.3. Materials and Methods

2.3.1. Fly Strains

The fly strain used throughout the study is the strain used by the Berkeley and European Drosophila Genome Projects (BDGP and EDGP) to construct the P1, BAC, cDNA and Celera plasmid libraries utilised during the genome sequencing of *Drosophila melanogaster* (Rubin *et al.*, 2000). Genotype: y[1]; cn[1]; bw[1]; sp[1], an M cytotype (P cytotype denotes those strains containing P-element insertions) and isogenized line.

2.3.2. Oligonucleotides

The following oligonucleotides were used during the SSH procedure, in the order given (see Fig. 8 for details of their relative positions):

SMART II oligonucleotide: 5'-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3'

cDNA synthesis primer: 5'-AAGCAGTGGTAACAACGCAGAGTAC$T_{30}N_1N$-3'

Adaptors:

Adaptor 1, 5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT
3'-GGCCCGTCCA

Adaptor 2R, 5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT
3'-CGGCTCCA

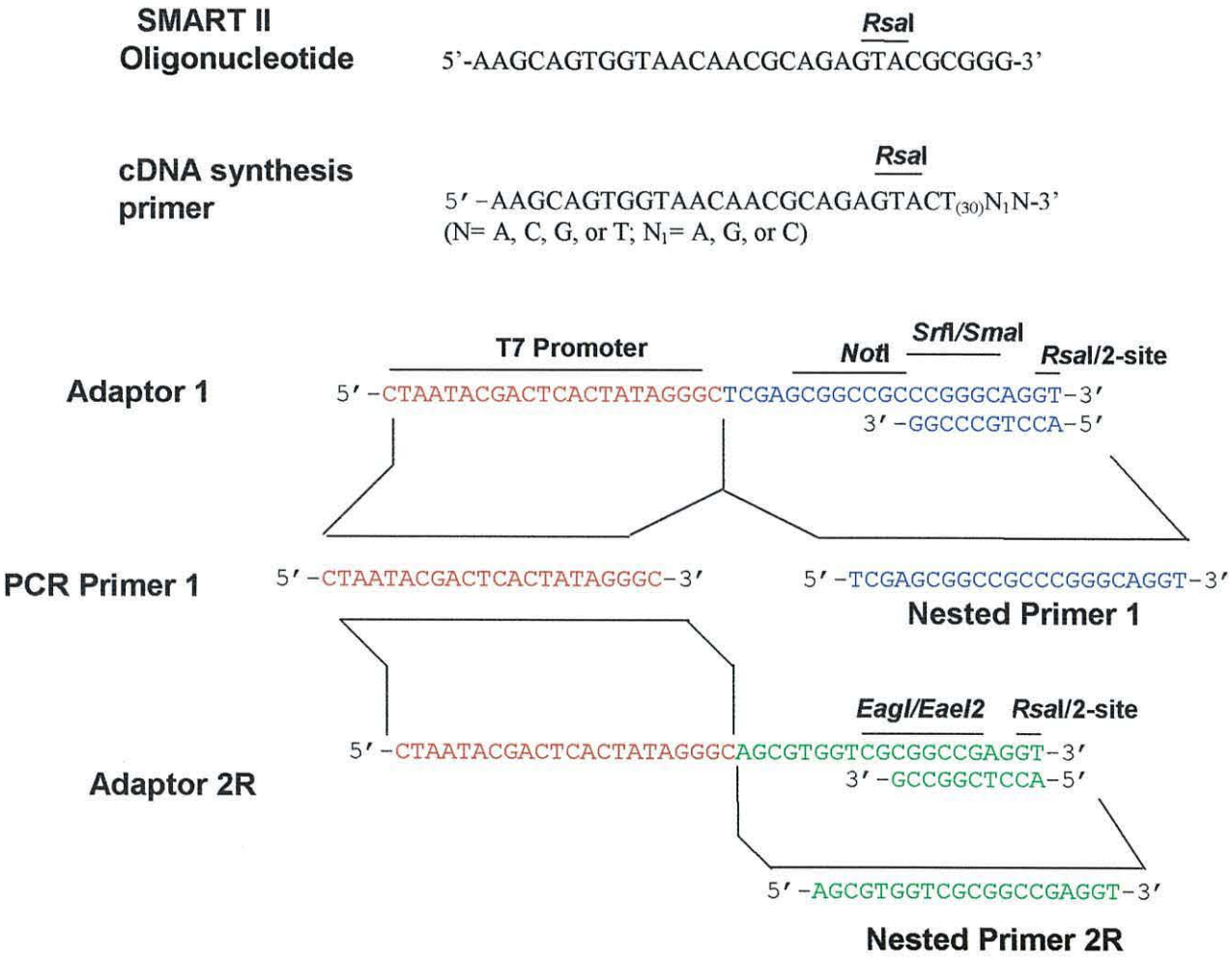
PCR primers:

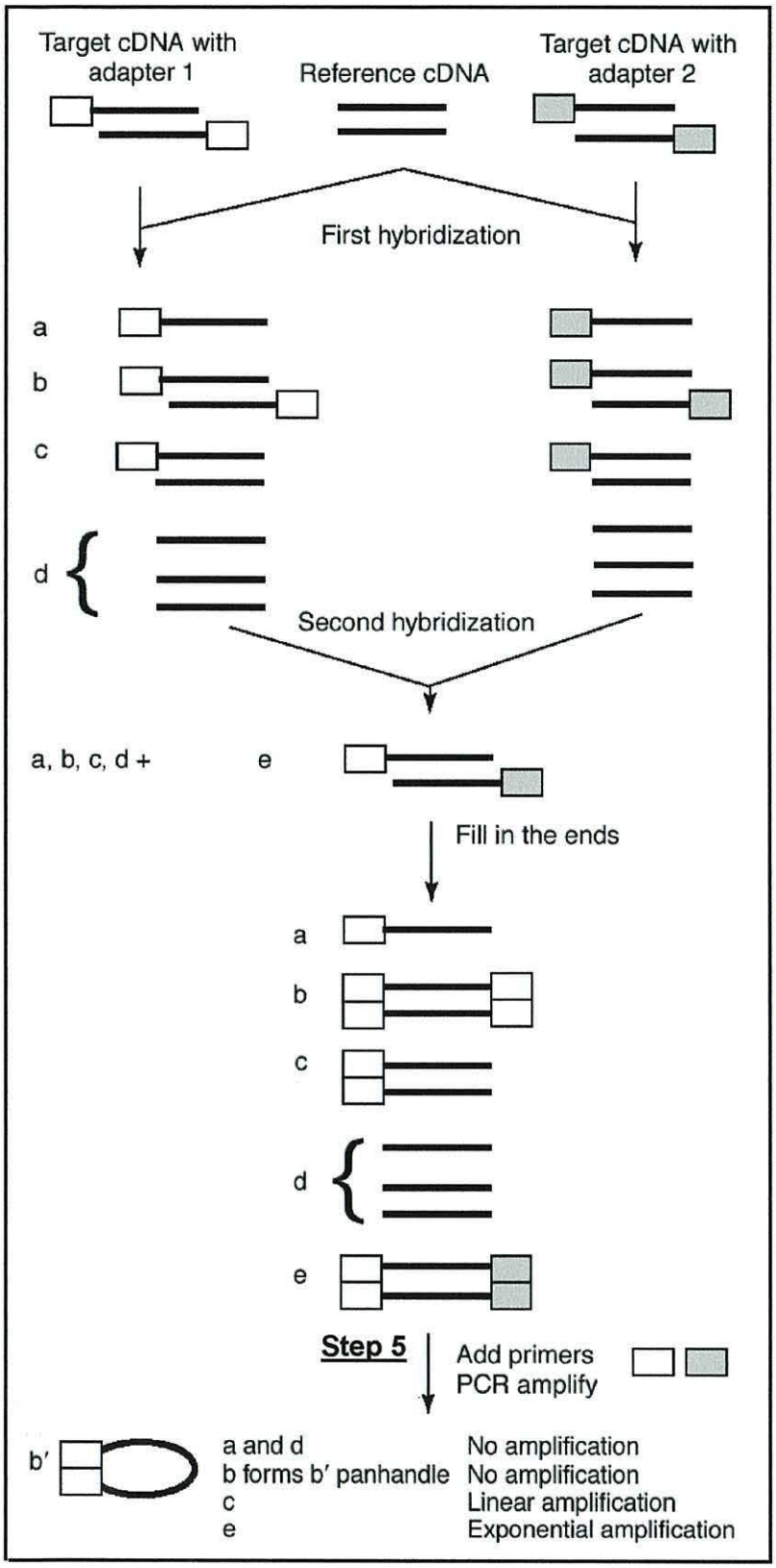
Primer 1, 5'-CTAATACGACTCACTATAGGGC-3'

Nested Primer 1, 5'-TCGAGCGGCCGCCCGGGCAGGT-3'

Nested Primer 2R, 5'-AGCGTGGTCGCGGCCGAGGT-3'

Fig. 8. Schematic diagram depicting the relationship between the oligos and adaptors used in the SSH protocol (from PCR-Select Differential Screening Kit User Manual CLONTECH). The SMART II oligonucleotide and cDNA synthesis primer are both used during first-strand cDNA synthesis, the former codes for a stop codon, hence one third of cDNAs are not translated. PCR primer 1 is used during the first PCR and the nested primers during the second PCR.





1. cDNA synthesized from mRNA extracted from two populations to be compared.

2. Tester and driver cDNAs are restricted with *Rsa*I, a 4-base-cutter that yields blunt ended fragments.

3. **Subtraction**
The tester cDNA is subdivided into two pools to be ligated to a different cDNA adapter.

4. **Hybridization**
Two hybridizations are performed.

a) The first serves to to normalize the ss tester cDNA fraction by equalizing the concentration of low and high abundance sequences, enriching for differentially expressed sequences. An excess of driver cDNA is added to each sample of tester cDNA. The samples are heat-denatured and allowed to reanneal.

b) The second further enriches the sample for differentially expressed sequences since only the remaining normalized and subtracted ss tester cDNAs are able to form new hybrids (e). The two first hybridization samples are mixed without denaturing and fresh denatured driver cDNA added.

5. **Suppression**
The entire sample undergoes PCR amplification. Only (e) type molecules that have both adaptors are exponentially amplified, further enriching for differentially expressed sequences. This is the suppression PCR effect.

Fig. 9. Diagram of the critical steps during the suppression subtractive hybridisation (SSH) procedure. Adapted from Dessens (2000). Solid lines depict the *Rsa*1 restricted tester and driver cDNA. Boxes represent adaptors, also corresponding to the PCR primers: clear boxes represent adaptor 1 and PCR primer 1 sequence; solid boxes adaptor 2R and PCR primer 2R sequence.

2.3.3. RNA Extraction and mRNA Isolation

Total RNA was isolated from approximately 5 mg of frozen cardia and carcass samples, ground under liquid nitrogen using a mortar and pestle, and extracted using the RNeasy Mini Kit (Qiagen), before being purified by the subsequent removal of contaminating genomic DNA with RNase-free DNase 1 (Ambion), following the manufacturer's protocol. The total RNA was quantified by UV absorbance at 260 nm and a sample run out on a formaldehyde/agarose/EtBr gel. Approximately 0.5 μg and 2 μg of mRNA was purified from cardia and carcass tissue respectively using Dynabead mRNA Direct Kit (Dynal).

2.3.4. cDNA Subtraction

This technique was carried out using the PCR-Select^{TM} cDNA Subtraction Kit (CLONTECH, Palo Alto, USA) according to the manufacturer's instructions.

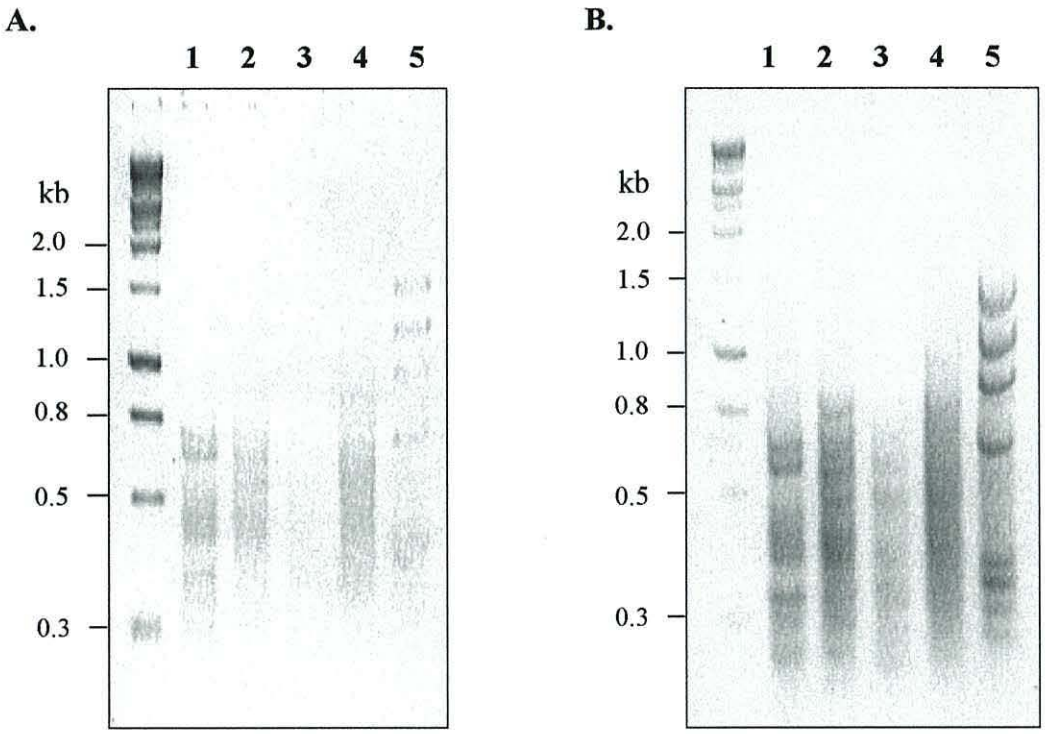
2.3.4.1. Driver synthesis

First strand cDNA is synthesised from mRNA using 1 μl AMV RNase H$^-$ reverse transcriptase Superscript II (20 U μl^{-1}) (Invitrogen) and 1 ng cDNA synthesis primer. Second strand cDNA synthesis was performed by according to the manufacturer (SMART cDNA synthesis kit). Blunt ending of the DNA ends using T4 DNA polymerase was carried out according to the manufacturer's protocol. The resulting cDNA pellet was resuspended in 50 μl deionised ultra-pure water and restricted using *Rsa*I in a 50 μl reaction volume containing 10 units of enzyme (Promega) for 1.5 h at 37°C. The cDNA was purified by phenol/chloroform extraction and ethanol precipitation, and resuspended in 5.5 μl of deionised ultra-pure water to a final concentration of ~300 ng μl^{-1}. Diluted driver cDNA (2 μl) was ligated to 2 μl of adaptor 1 and adaptor 2R in separate 10 μl reactions using 1 μl T4 DNA ligase (3U μl^{-1}) in the buffer supplied by the manufacturer and incubated at 16 °C overnight, resulting in the subtracted cDNAs driver-adaptor 1 (2-1) and driver-adaptor 2R (2-2). The ligation reaction was terminated by the addition of 1 μl of 0.2 M EDTA/glycogen to the reaction and heating to 72 °C for 5 mins to inactivate the ligase.

2.3.4.2. Tester synthesis

*Rsa*I restricted ds tester cDNA was synthesised as described for the driver, before being diluted (1 μl) in 5 μl of water. The diluted tester cDNA was ligated to both adaptors in the same way as for driver cDNA, to produce the subtracted cDNA corresponding to tester-adaptor 1 (1-1) and tester-adaptor 2R (1-2).

Fig. 10. Analysis of ds cDNA synthesized for adult cardia vs carcass library construction.
A. Primary PCR of differentially expressed cDNAs.
B. Nested PCR of ds cDNAs.
Lanes 1-5 denote different reactions: 1. Forward subtracted tester cDNA (S1); 2. Unsubtracted tester control (1-c); 3. Reverse subtracted tester cDNA (S3); 4. Unsubtracted tester control (2-c); 5. PCR control subtracted cDNA.



2.3.4.3. Unsubtracted control preparation

Portions of (2 µl) tester-adaptor 1 (1-1) and tester-adaptor 2R (1-2) are mixed prior to ligation to generate the unsubtracted tester control (1-c). Similarly driver-adaptor 1 (2-1) and driver-adaptor 2R (2-2) are combined to form unsubtracted driver control (2-c). The ligation reactions are inactivated in the same way as for adaptor ligation to subtracted driver.

2.3.4.4. Subtractive hybridisation

During the first hybridisation step, 1.5 µl driver ds cDNA corresponding to carcass for cardia tester, and cardia for carcass tester, is combined with 1.5 µl of tester-adaptor 1 and tester-adaptor 2 cDNA and 1 µl hybridisation buffer [(50 mM Hepes, pH 8.3) 0.5 M NaCl (0.02 mM EDTA, pH 8) (10% w/v) PEG 8000] in separate tubes. The cDNAs are incubated at 98 °C for 1.5 min, to denature, and then annealed at 68 °C for 8-10 h. In the second hybridisation, paired hybridisation samples (1-1 with 1-2, and 2-1 with 2-2) are combined in the presence of freshly denatured driver (1 µl) and hybridisation buffer (1 µl). The samples are then incubated at 68 °C for an additional 8-10 h. The hybridisation product was then diluted in 200 µl of dilution buffer (20 mM Hepes at pH 8.3, 50 mM NaCl, 0.2 M EDTA) and incubated at 68 °C for 7 min.

Two PCR amplification steps are carried out for each subtracted and unsubtracted sample using the Advantage 2 PCR Kit according to the manufacturers instructions (Clontech Labs Inc., Palo Alto, USA). The primary PCR served to preferentially amplify hybrid cDNAs with different adaptors on each end. The reaction contained 1 µl diluted subtracted or unsubtracted cDNA, 2.5 µl 10x Advantage 2 PCR buffer, 0.5 µl dNTPs (10 mM), 1 µl PCR primer 1 (10 µM) and 0.5 µl 50x Advantage 2 polymerase mix in a total of 25 µl. The following thermal cycling conditions were used: 75 °C for 7 min; 27 cycles of: 94 °C for 30 sec; 66 °C for 30 sec; 72 °C for 1.5 min; 68 °C for 7 min. An 8 µl sample of each reaction was analysed on a 2% agarose/ EtBr gel in 1x TBE buffer [10x TBE: 0.89 M Tris base, 0.89 M boric acid, and 20 mM EDTA (pH 8.0)] to ensure that a distinct smear of PCR products is visible, 3 additional cycles can be used if this is not the case (see Fig. 10A). The primary PCR product was diluted 10-fold in deionised water, and a 1 µl sample was used as a template for the secondary PCR. During the second PCR, nested PCR primers are used to further enrich for differentially expressed cDNAs, thereby reducing background. The reaction is performed in a total of 25 µl, with the same components as the primary PCR, except for the replacement of PCR primer 1 with the nested PCR primers 1 and 2R. Thermal cycling was performed for 12 cycles: 94 °C for 30 sec; 68 °C for 30 sec; 72 °C for 1.5 min; final extension of 72 °C for 5 min. An 8 µl sample of each reaction was analysed by 2% agarose/EtBr gel electrophoresis in 1x TBE buffer to ensure that PCR product is visible, with the addition of 3 more cycles if required (Fig. 10B).

2.3.4.5. Subtracted cDNA library construction

Secondary PCR products were inserted into the pCR II cloning vector using a T/A cloning kit (Invitrogen). The pCR II inserts were screened using M13 F and R primers, under similar conditions as the secondary PCR, for 23 cycles. Amplified inserts were then prepared for cDNA dot blots by combining 5 µl of each PCR product with 5 µl fresh 0.6 M NaOH. A 96 Pin Multi-Blot Replicator (V and P Scientific, Cat # VP409) was used to transfer 1 µl of each mixture, arrayed in duplicate onto a nylon membrane (Hydrobond-N, Amersham), creating four identical blots. The membranes were neutralised by soaking in 0.5 M Tris-HCL (pH 7.5) and deionised water, before being cross-linked at 120000 µJ/M^2 in a UV cross-linker (UVP, model CL-1000 UV).

2.3.4.6. Random primer labelling of cDNA probes

Subtracted (20-90 ng) and unsubtracted (50-100 ng) cDNA probes were diluted 3 µl in 9 µl with deionised water, and incubated at 95 °C for 8 min, followed by snap-freezing. The probes were ^{32}P-labelled by random priming in a reaction composed of 3 µl reaction buffer [(-dCTP) 333 mM

Tris-HCl (pH 8.0), 33.3 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 170 mM dATP, 170 mM dGTP, 170 mM dTTP] 1 µl random primer mix, 5 µl [α-^{32}P] dCTP (50 U Ci, 3000 Ci mM^{-1}, aqueous solution) and 1 µl Klenow enzyme (exo^-). The reaction was incubated at 37 °C for 30 min, before being terminated with 5 µl stop solution (0.1 M EDTA, 0.5 mg ml^{-1} tRNA). The probes were then purified using S-300 HR microspin columns (Amersham Biosciences), before verifying that the radioactivity is > 10^7 cpm (counts per minute) per 100 ng probe.

2.3.4.7. Hybridisation screening of cDNA library

The blotted membranes were pre-hybridised for 1 h at 72 °C in hybridisation buffer: 50 ml 20x SSC (175.3 g NaCl and 88.2 g $Na_3C_6H_5O_7.2H_2O$, pH 7.0 L^{-1}), 20 ml 50x Denhardts solution, 10 ml10% SDS (20 % 200g L^{-1}), 2 ml sheared heat-denatured salmon sperm DNA (Invitrogen, Cat # 15632011, 10 mg ml^{-1}) and deionised water to a final volume of 200 ml. ^{32}P-labelled cDNA probes are combined with 50 µl 20x SSC and 50 µl blocking solution, before being denatured at 95 °C for 5 min and snap-frozen. This mixture was then combined with 200 ml of hybridisation buffer and the membranes were hybridised at 72 °C overnight, with continuous agitation. The membranes were subsequently washed four times under low-stringency conditions with 0.5% (w/v) SDS in 2x SSC buffer, and twice at high-stringency conditions with 0.5% (w/v) SDS in 0.2x SSC buffer at 68 °C for 20 min each. The membranes were then individually sealed in polythene prior to being exposed to a Storage Phosphor Screen (Molecular Dynamics) overnight. The resulting images were analysed on a Personal Molecular Imager FX (Biorad) phosphorimager with Quantity 1 (Amersham) software.

2.3.5. Bioinformatics

2.3.5.1. Database searches

A combination of databases was used to identify the putative function of the positive sequences through the detection of conserved domains. These include the Blast-X, -N and -P programs from NCBI (http://www.ncbi.nlm.nih.gov/) (Altschul *et al.*, 1997), PIX system, HGMP-RC (http://www.hgmp.mrc.ac.uk/Registered/Webapp/pix/) (Williams and Faller, 1999), Pfam (http://www.sanger.ac.uk/Software/Pfam/) (Bateman *et al.*, 2002), SMART (http://smart.embl-heidelberg.de), ExPASy (Swiss-Prot) (http://ca.expasy.org/) (Letunic *et al.*, 2002; Schultz *et al.*, 1998), Flybase (http://flybase.bio.indiana.edu/) and BDGP (http://www.fruitfly.org/).

2.3.5.2. Signal Sequence

Analysis of the presence (so as to classify secreted and non-secreted proteins) and position of signal peptide cleavage sites in the amino acid sequences was performed with software from the The SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen *et al.*, 2004; Nielsen *et al.*, 1997). The presence of N-linked glycosylation sites and mucin type GalNAc O-glycosylation sites were predicted using the NetNGlyc and NetOGlyc 3.1 Servers.

2.3.5.3. Alignment of CBDs

The amino acid sequences corresponding to the individual CBDs within each CB protein were aligned using the integrated database known as the Conserved Domain Database (NCBI) viewed using Cn3D 4.1 (http://www.ncbi.nlm.nih.gov) (Marchler-Bauer *et al.*, 2003) and Megalign (DNAStar). The alignment is not shown in this work, as there was insufficient time to conduct phylogenetic analyses, thus only the two consensus sequences are shown.

2.3.6. Reverse transcription PCR (RT-PCR)

The guts from adult *D. melanogaster* were excised and carefully dissected to separate cardia and midgut. In addition tissues were harvested from the adult carcass and whole embryos and larvae. The gut from third instar larvae were removed and dissected to separate the cardia, midgut and hindgut. In addition the salivary gland, Malpighian tubules and trachea tissue were also collected. cDNA was prepared using total RNA derived from each of these tissues by first strand cDNA synthesis using Oligo (dT) primer and RNase H RT enzyme (SuperScript First-strand Synthesis System for RT-PCR, Invitrogen). The cDNA is used as a template for PCR analysis using the gene specific primers detailed in Table 6, and the conditions: 95 °C for 1 min; 25-30 cycles of: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min; final extension 72 °C for 10 min. The reactions contained 2.5 µl 10x PCR buffer, 2.5 µl $MgCl_2$, 2.5 µl dNTPs, 1 µl sense primer, 1 µl reverse complement primer and 0.1 µl Taq DNA polymerase (Invitrogen) with a total reaction volume of 25 µl.

Table 6. Gene Specific sense (S) and reverse complement (RC) primers used in RT-PCR analysis.

Name	Sequence (5'-3')	Product length (bp)
CG6004 (S)	CTGGGGACACTGTCGAGGTATA	458
CG6004 (RC)	GCGGCCGAGGTACCACAAGTT	
CG17826 (S)	CGCAGAGTGCAAAGATG	650
CG17826 (RC)	TCTTGTACACAAGTCTCCAG	
CG14125 (S)	CGGAGCCAACTGAAAAT	232
CG14125 (RC)	GGCCGACGTACCTTCATT	
CG14645 (S)	CCCCCCGAAACCCAGTT	303
CG14645 (RC)	CGGACAACCGGGTTTCA	
CG3906 (S)	GTGCACCTCGATCACATT	276
CG3906 (RC)	TTAACTTGAATCACGGCCAGC	
CG11672 (S)	CCTGTCATCTTGCGGGTGCATA	375
CG11672 (RC)	GGCCGAAGGTACAGCAGATT	
CG12726 (S)	GCCCACCGTGCGAACCAAAA	294
CG12726 (RC)	CCAGCGCGGCATTCATAGTA	
CG7252 (S)	GATCAGTGTACCTGCGATTG	402
CG7252 (RC)	TACAGTCCACATTCCACCAG	
CG5084 (S)	TTCCGCATGGTCCTTGGTGAG	543
CG5084 (RC)	GCCGCCTGGATGGACTGAAC	
CG15918 (S)	AGCGAGGCCAAGTCCAAGTC	640
CG15918 (RC)	ACGGGCTGCTTCAAAGGTG	
DmPro7 (S)	GATGGCGATGTCAATGTCTGCTC	404
DmPro7 (RC)	GCCTTGCTGGGTATGAAAACGAT	
CG17814 (S)	ACTCAATTGCACCATGAAGTC	580
CG17814 (RC)	TCAACAGTAAGGAGTCCAAGAC	
DmPro8 (S)	GCAAGCAAAGGAGTCGCAAAG	1086
DmPro8 (RC)	CTTGTTGTGGTCTCTGGTACACAG	
CG31893 (S)	TGACCCTCTGCGTTGCCATC	495
CG31893 (RC)	TTAAGTGGATGCTTCGGAAC	
CG7567 (S)	TGAAGCTGCCCACCAAC	518
CG7567 (RC)	GTGCCCTCCACGAAATC	
DmPro9 (S)	GACGCATTCCTTCTCCAC	784
DmPro9 (RC)	GTTGCTGTCACTGGGTGAG	

2.4. Results

2.4.1. Identification of cDNAs that Encode PM Proteins

A cardia-specific partial cDNA library, consisting of 96 clones was constructed and screened (Fig. 11) using SSH to isolate genes encoding putative PM peritrophins. From the differentially expressed genes identified using the SSH libraries, we were able to identify putative homologs of known peritrophins from the partial genomic sequence of the clones using the *Drosophila melanogaster* genome database. Of the 22 differentially expressed cDNA clones isolated from the adult cardia library by SSH, 5 clones (23%) were identical to previously known putative consituents of the PM in *Drosophila*, 1 clone (5%) was homologous to other peritrophins and 9 clones (41%) were novel putative peritrophins. A total of 40 out of 96 clones from the larval cardia SSH library were differentially expressed, and so characterised further by sequencing, of which 15 clones (36%) were found to be identical to known putative constituents of the PM in *Drosophila*, 4 clones (9%) contained chitin binding domains and 12 clones (29%) represented novel peritrophins (Table 7).

Table 7. Summary of the classification of cDNA clones isolated from SSH partial cDNA library.

Class of cDNA	Adult cDNAs sequenced	Larvae cDNAs sequenced
Identical to known peritrophins	5 (23%)	15 (36%)
Related to known peritrophins	1 (5%)	4 (9%)
Novel peritrophins	9 (41%)	12 (29%)
Other *Drosophila* proteins	3 (13%)	7 (17%)
Other	4 (18%)	4 (9%)
Total	22	42

There were cases of single gene products being represented by multiple clones from the SSH libraries, which accounts for the disparity between the total number of differentially expressed sequences and genes of interest within Table 7 and 8 respectively. Twelve out of seventeen unique gene products code for putative constituents of the *Drosophila* PM (Table 8). However, a number of known putative structural constituents of the PM in *Drosophila* (Flybase) were not identified in this SSH study (Table 7). Those genes encoding putative peritrophins are further characterised in Table 9, with details of their location within the genome depicted in Table 10.

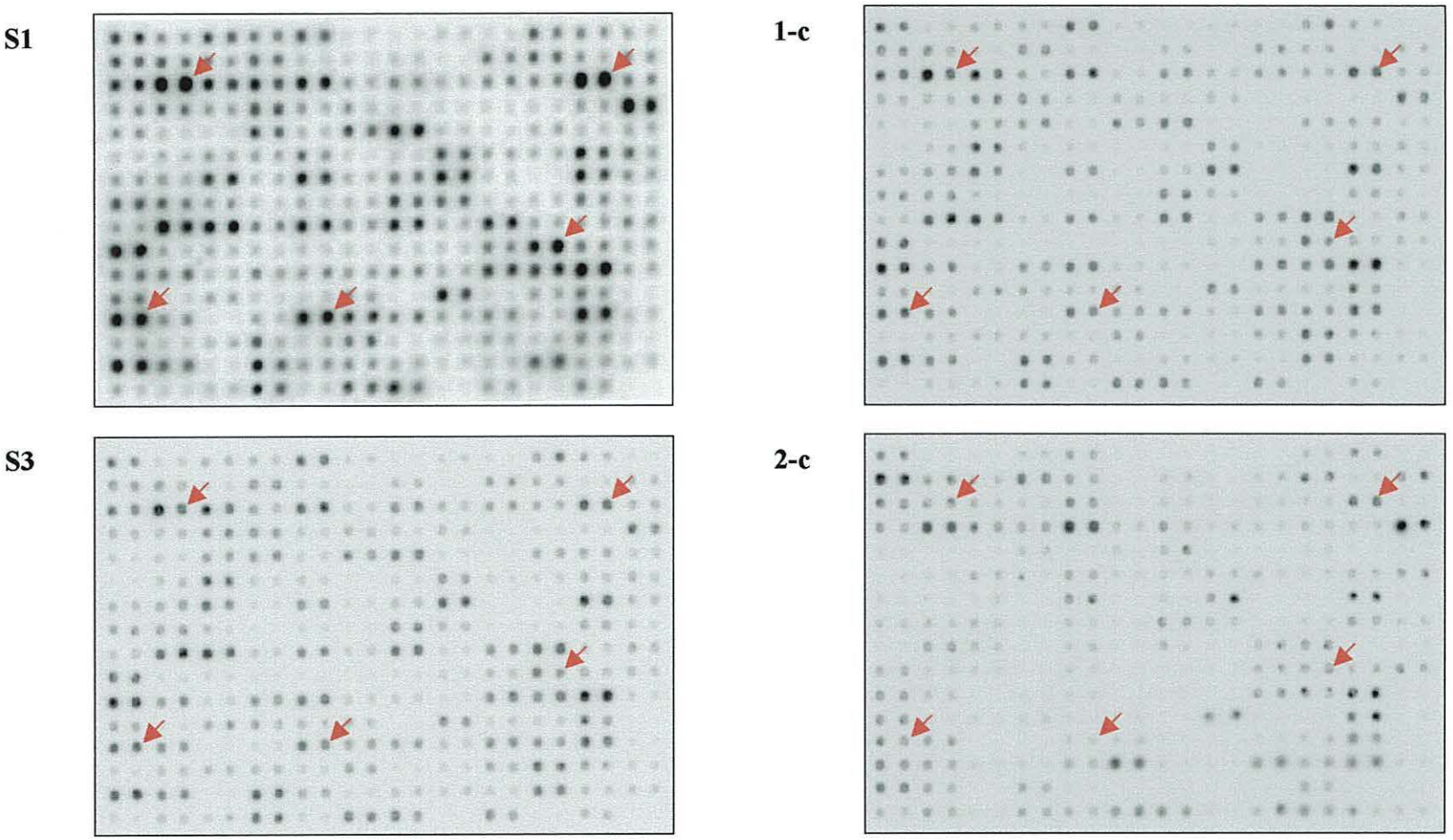


Fig. 11.
Dot blots showing results from SSH to identify differentially expressed genes in the cardia of *D. melanogaster* larvae.
Note: S1: subtracted tester cDNA probe; S3: subtracted driver cDNA probe, 1-c: unsubtracted tester control cDNA probe, 2-c: unsubtracted driver control cDNA probe; and arrowheads: indicate examples of clones that are positive for SSH screen (upregulated, differentially expressed).

SSH Library	Gene Identifier [$]	GenBank Accession number [‡]	Flybase number [†]	Query length (bp)	*Drosophila* Homolog [¶]	Putative function *	Similarity (%)[л]	Identical (%) [Ψ]	Score [δ]	E-value [ε]	Signal P [β]
Adult	*DmPro1*	Gb AAF50015.1	FBgn0036203	535	CG6004-PA	Peritrophin	97	97	313	9e-85	None
	DmPro2	Gb AAF49984.1	FBgn0036227	272	CG17826-PA	Peritrophin (N)	89	86	93	9e-19	26/27 (SSA-VC)
	DmPro3	Gb AAF49979.1	FBgn0036232	435	CG14125-PA	Peritrophin (N)	97	96	110	3e-24	20/21 (ALS-AR)
	-	Gb AAF52145.1	FBgn0040687	214	CG14645-PA	Peritrophin (N)	85	82	64	1e-09	20/21 (ALA-YD)
	-	Gb AAF47017.1	Fbgn0034871	333	CG3906-PA	Unknown	88	88	149	8e-36	22/23 (TIA-QP)
	-	Gb AAF54211.1	FBgn0037563	475	CG11672-PA	Unknown	99	98	184	6e-46	19/20 (ADC-QE)
Larva	*DmPro2*	Gb AAF49984.1	FBgn0036227	718	CG17826-PA	Peritrophin (N)	97	96	407	4e-113	26/27 (SSA-VC)
	DmPro4	Gb AAF49985.1	FBgn0036226	893	CG7252-PA	Peritrophin	100	96	139	6e-32	24/25 (VVA-QL)
	DmPro5	Gb AAF57767.1	FBgn0034288	801	CG5084-PA	Mucin (N)	84	84	170	3e-41	19/20 (VSA-VP)
	DmPro6	Gb AAF57892.1	FBgn0034197	580	CG15918-PA	Mucin (N)	100	99	219	3e-56	21/22 (SEA-KS)
	DmPro7	Gb AAN11837.1	FBgn0036362	482	CG10725-PB	Peritrophin	99	98	260	5e-69	19/20 (CSA-AD)
	DmP-15a	Gb AAF52668.1	FBgn0040959	460	CG17814-PA	P-15a	98	97	200	5e-51	22/23 (GNA-CD)
	DmPro8	Gb AAF52351.2	FBgn0040950	369	CG13990-PA	Peritrophin (N)	90	85	45	4e-07	19/20 (INA-AS)
	DmP-15b	Gb AAF52667.1	FBgn0040958	495	CG31893-PA	P-15b	98	98	201	5e-51	18/19 (IYA-DL)
	-	Gb AAF56903.1	FBgn0038645	410	CG7567-PA	Unkown	95	93	174	3e-43	23/24 (SLS-LV)
	DmPro9	Gb AAF55589.1	FBgn0036225	784	CG7714-PA	Peritrophin (N)	100	99	110	3e-23	20/21 (AHA-AC)
	DmPro10	Gb AAF49986.2	FBgn0036225	557	CG5883-PA	Peritrophin	100	98	161	1e-41	30/31 (LNA-TD)

Table 8. Characterization of positive clones from SSH screen, chosen for RT-PCR analysis.
[$] Those genes assigned *DmPro* numbers were chosen for further study. [‡] Accession numbers corresponding to the GenBank and [†] Flybase databases are given. [¶] The closest *D. melanogaster* homolog; –PA (protein A), single predicted protein and -PB (protein B) two predicted proteins. * Putative functions were determined by homologs identified by BlastX against the NCBI GenBank database; Mucin (N), novel putative mucin; Peritrophin, previously identified by Flybase as a putative consituent of the peritrophic matrix (Flybase GO: 0016490); Peritrophin (N), novel putative peritrophin; and unknown, sequence for which the function is not indicated by homology. [л] Percentage number of amino acid similarities and [Ψ] amino acid identities between query and homologous sequences. [δ] Number of residues identical to the amino acid sequence of the protein homolog core. [ε] E-value of the homology. A combination of higher score and lower E-value is an indication of high homology. [β] Numbers indicate the position of the signal cleavage site in relation to the start codon, with the exact position within the amino acid sequence given in brackets.

Table 9. The position of predicted motifs within the amino acid sequence of the selected *Drosophila* genes.
[¶] The position of CBDs were predicted by the Conserved Domain Database (CDD) (NCBI).
[†] The position of N-linked glycosylation sites and [‡] mucin type, GalNAc O-glycosylation sites were predicted using the NetNGlyc and NetOGlyc 3.1 Server.

Gene Product	Predicted position of CBDs (aa) [¶]	Potential *O*-linked glycosylation (aa) [†]	Potential *N*-linked glycosylation (aa) [‡]
DmPro1	1244-1297, 1318-1370	3, 23, 254, 264, 356, 390, 424, 458, 525, 559, 992	293-308, 334-340, 462-484, 663-699, 829-875, 896-909, 929-976, 996-1025, 1154-1204
DmPro2	29-77, 82-128, 456-505, 562-611, 621-672, 697-751	117, 279	None
DmPro3	207-255	79, 95, 115, 129	28-197
DmPro4	30-83, 175-227, 251-311, 343-395, 420-472	318, 337	117-157
DmPro7	35-86, 91-146	124, 145	None
DmPro8	415-466	None	117-369
DmPro9	37-100	None	None
DmPro10	95-147, 154-206	29	None

Table 10. Detailed localisation of the sequence encoding putative peritrophins and other chitin-binding proteins within the *Drosophila* genome. A diagrammatic representation of the exact location of the genes of interest in this study is provided by FlyBase. All genes of interest are present as a single copy within the genome.
[§] The precise genomic locus of a gene within the genome.
[β] The Genbank accession number assigned to portions (contigs) of the whole *Drosophila* genome.

Gene Identifier	GenBank Accession number	Drosophila Homolog	Cytogenic position [§]	Scaffold [β]
DmPro1	Gb AAF50015.1	CG6004-PA	(3L) 68D3	AE003544
DmPro2	Gb AAF49984.1	CG17826-PA	(3L) 68E3	AE003543
DmPro3	Gb AAF49979.1	CG14125PA	(3L) 68E3	AE003543
DmPro4	Gb AAF49985.1	CG7252-PA	(3L) 68E5	AE003543
DmPro5	Gb AAF57767.1	CG5084-PA	(2R) 55B1	AE003801
DmPro6	Gb AAF57892.1	CG15918-PA	(2R) 53F13	AE003804
DmPro7	Gb AAN11837.1	CG10725-PB	(3L) 70B1	AE003538
P-15a	Gb AAF52668.1	CG17814-PA	(2L) 29C1	AE003621
P-15b	Gb AAF52667.1	CG31893-PA	(2L) 29C1	AE003621
DmPro8	Gb AAF52351.2	CG13990-PA	(2L) 26B11	AE003612
DmPro9	Gb AAF55589.1	CG7714-PA	(3R) 91C4	AE003723
DmPro10	Gb AAF49986.2	CG5883-PA	(3L) 68E3	AE003543

Comparison of the amino acid sequences of the genes of interest (predicted by translation of the clone) with those present within the *Drosophila* genome database indicate that the differentially expressed sequences vary from 84-100 % in similarity and 82-99 % in identity to the homologs (Table 8). All sequences gave high scores and low E values, indicative of high sequence homology to the homologous sequence within the database. Those sequences for which a known putative peritrophin (Flybase) was indicated as the homolog were termed P known, whereas those novel putative peritrophins that contain CBDs and other motifs common to peritrophins (Table 9), but were not previously identified as putative peritrophins were reported as P novel (Table 8).

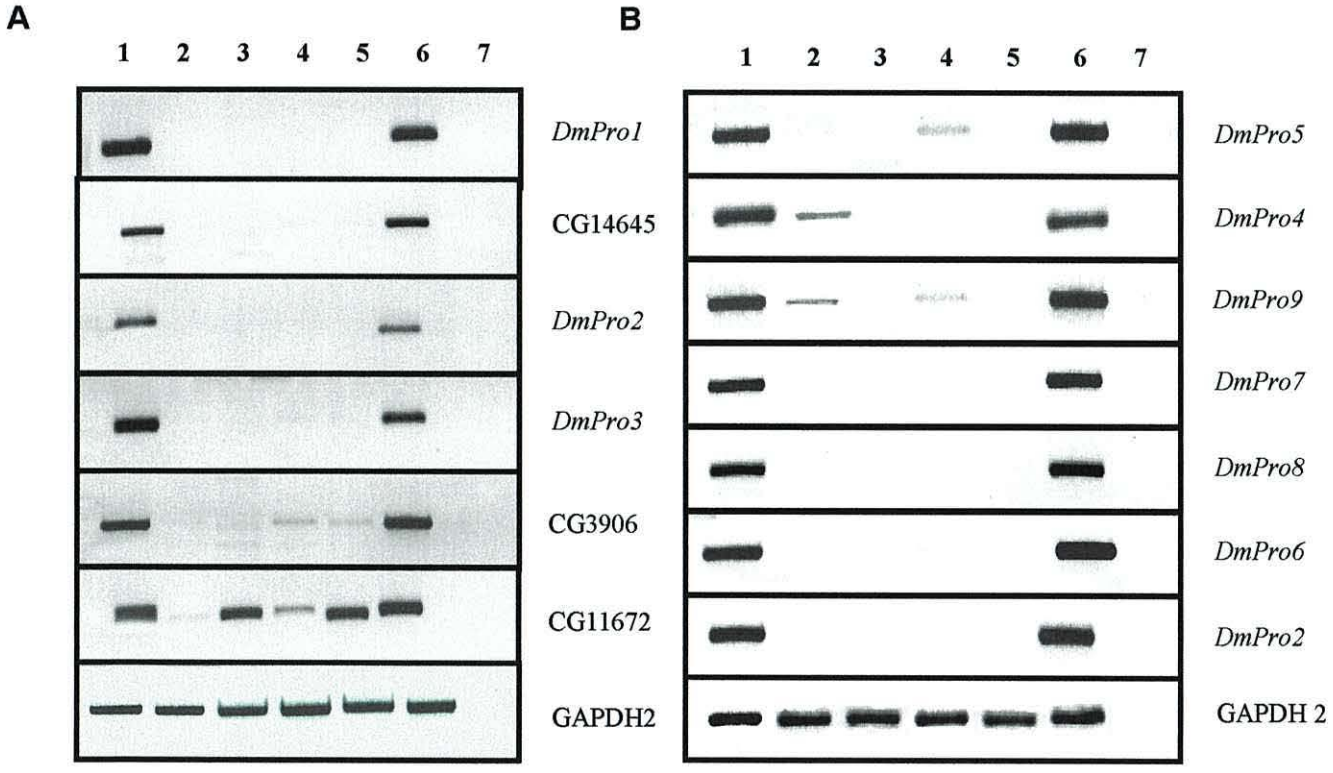
All genes identified as positive clones from the SSH screen have putative signal sequences at their amino termini (except *DmPro1*), indicating that they are secreted proteins. The putative signal peptides span between 20-22 amino acids (aa) from the start codon in most of the sequences (Table 8). This is in keeping with putative signal peptides of known peritrophins, including P-44 (23 aa), P-48 (20 aa) and P-95 (19 aa) from *Lucilia cuprina* and P-48 (20 aa) from *Chrysomya bezziana*.

The rational behind the selection of those sequences to be further characterised, termed *DmPro1* to *10* from here on [named according to the group of selected sequences being specifically or highly expressed within the *D. melanogaster* cardia (proventriculus)], was based on the underlying principle that the aim of this thesis was to specifically investigate putative peritrophins in Diptera, i.e. chitin binding proteins that play an integral role in determining the structure and function of the PM. Thus, for the purposes of this study those sequences with true cardia specific, or cardia and midgut specific expression were selected for further examination, the implications of which include that a number of sequences of interest that do not meet the above criteria were only partially characterised.

2.4.2. Gene expression analysis of cDNAs of peritrophins

To confirm the SSH data obtained (Table 8), and verify that the sequences of interest are indeed putative peritrophins, a series of reverse-transcriptase polymerase chain reactions were perfomed (Fig. 12) in order to determine their temporal and spatial expression patterns. RT-PCR was used to accurately locate the site of synthesis and to determine the developmental expression pattern of the mRNA coding for all adult and larval SSH positive clones.

Fig. 12. RT-PCR analysis of expression within specific tissues from fed adults and 3rd instar larvae. Each sample contained the same quantity of first strand cDNA (1ng).
A. Tissue specific expression pattern of mRNA in adults. Lane 1, first strand cDNA from cardia; lane 2, midgut; lane 3, carcass; lane 4, larvae; lane 5, embryos; lane 6, positive control (total cDNA pool derived from whole adults) and lane 7, no template control for each primer pair.
B. Tissue specific expression pattern of mRNA in larvae. Lane 1, cardia; lane 2, midgut; lane 3, Malpighian tubules; lane 4, tracheae of larvae; lane 5, adult; lane 6, positive control (total cDNA from total RNA of whole larvae); and lane 7, no template negative control for each primer pair. Lower row, GAPDH 2 RT-PCR and loading controls for each of the samples are also shown.



The tissue and developmental expression patterns of the genes corresponding to positive clones from the adult and larval PM SSH screen (Table 8) were determined from adult and larval tissues. The tissues included adult cardia, midgut (excluding the cardia) and carcass; larvae and embryoes for the RT-PCR profiles of putative adult PM proteins (Fig. 12A). Similarly, larval cardia, midgut (excluding cardia), Malpighian tubules, trachae and adults were included in the RT-PCR detection of transcripts encoding positive larval SSH sequences (Fig. 12B).

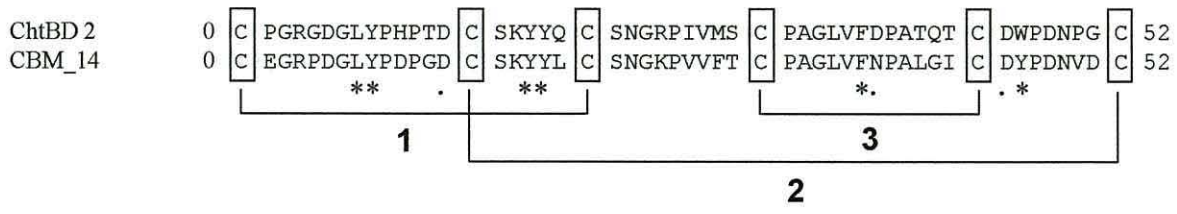
The results in Fig.12 show the tissue- and stage-specific expression profiles for the genes of interest. Reassuringly, most were not expressed in the other tissues tested, the expression of *DmPro1*, *DmPro2*, *DmPro3* and CG14645 being limited to the adult cardia tissue (Fig. 12A). Similarly, only the cardia expressed *DmPro2*, *DmPro6*, and *DmPro8* mRNA, whilst *DmPro4* and *DmPro9* mRNA were also expressed to a lesser extent in the midgut of larvae. Similarly, *DmPro5* and *DmPro9* were expressed in the tracheae of larvae (Fig. 12B). Whilst CG3906 transcripts are also produced in embryos and to a lesser extent larvae. Furthermore, CG11672 is expressed in all adult tissues embryos and larvae, suggesting that this gene is ubiqitously expressed (Fig. 12A). The result of the tissue-specific expression profiles directly reflect the specificity of expression expected for the constituents of the type 2 PM of *Drosophila*, by either being expressed exclusively in the cardia or the cardia and to a lesser extent in the midgut. Meaning that the PM is predominantly produced in the larval cardia, P-44 is expressed in all 3 larval instars, compared to little in eggs, pupae and adults.

RT-PCR analysis suggests that the genes of interest are predominantly expressed by the cardia, where they are believed to be constitively expressed, supporting the view that *DmPro1-10* are putative peritrophins.

2.4.3. Sequence comparison of the CBDs of peritrophins

The alignment of individual CBDs within the amino acid sequence of peritrophins allows the identification of the type of peritrophin or mucin molecule they represent (indicated by the presence of a peritrophin A-C domain). The CBDs from all ten CBD containing putative peritrophins in Table 9 align perfectly with the CBM_14 or ChtBD 2 consensus of the peritrophin-A domain (Fig. 13), indicative of true peritrophins such as P-44, P-48 and P-95 from *L. cuprina* (Fig. 5).

Fig. 13.
Alignment of the consensus sequences of the CBM_14 and ChtBD 2 CBDs from the putative peritrophins in this study. The six cysteines indicative of a CBD motif are boxed, and the conserved charged and aromatic residues are depicted by a single dot and an asterisk respectively. The disulfide bonds are illustrated by the lines 1-3, predicted from those present in the tachycitin molecule (Kawabata *et al.*, 1996).



2.4. Discussion

From the summary of the efficiency of the SSH technique in Table 7 and the list of putative functions attributed to each positive clone in Table 8 it is possible to appreciate that the majority of clones are either identical to known putative PM proteins or are novel peritrophins . This indicates that SSH is an effective tool for the identification and functional analysis of cardia specific genes, and so pave the way for a more thorough understanding of the PM in *Drosophila*. isolation and analysis of genes that are spatially or temporally expressed, i.e. those with specific roles. Of particular interest are the genes identified by SSH that are also listed as PM chitin binding proteins in flybase (see Table 8) including *DmPro1*, *DmPro10*, *DmPro4* and *DmPro7*. Unexpectedly *DmPro8*, *DmPro3* and *DmPro2* were not previously identified as structural constituents of the *Drosophila* PM and yet are proteins that do contain CBDs coded for by genes with cardia specific expression profiles. Surprisingly, CG7714 (*DmPro9*), previously identified by Tellam (2003) as a homolog of the *L. curpina* peritrophin-55 protein was not specifically expressed in the cardia, since it was also expressed to a lesser extent in the midgut and trachae in larvae (see Fig. 12B). In addition, expression of *DmPro5* was also detected in larval trachae. It is possible that *DmPro5* and *DmPro9* may have evolved to perform diverse functions other than being a structural constituent of the PM, made possible by the less tight regulation in expression in comparison to most PM proteins. This is supported by the abnormal expression of the gene encoding Gasp, another putative peritrophin in *Drosophila* that is also expressed in the embryonic tracheae. It is possible that peritrophins may be present in other tissues other than the peritrophic matrix, particularly where nutrient or gas exchange occurs, since this may be an additional site for invasion by pathogens and parasites, suggesting that similarly to Gasp, *DmPro5* and *DmPro9* may have a role in the protection of the embryonic trachae in addition to the larval PM. Moreover, the parallels between the PM of the midgut and the cuticle of trachaea, in terms of molecular structure and function may support this theory. Both the PM and cuticle have similar properties, implying that they may utilize similar proteins to provide a strong matrix which serves to act as a barrier to limit entry of parasites and pathogens, whilst still being an effective interface with the external environment (Barry *et al.*, 1999).

The putative PM proteins coded for by the genes *DmPro5*, CG17824 (identified by the *Drosophila* genome database through sequence similarity, Flybase) and *DmPro2* (SSH clones DmAd26 and DmL265) code for proteins that may contribute more to PM structure by having a greater potential range of interactions with other components of the PM when compared to those that code for only 2-5 CBDs. Even with partial proteolytic degradation of the mature peptides of these more prominent PM proteins (coding for 11, 8 and 6 respectively, see Table 9 for details)

there are still sufficient CBDs present to form multiple links with other components of the PM (Wang *et al.*, 2004). The CBDs within *DmPro2* and *DmPro3* align perfectly with the CBM_14 domain, which brings into question why there are not included in the Flybase list of peritrophins that possess this domain.

A number of gene products that have unknown functions (Table 8) may have not been classified as putative peritrophins due to the presence of only a partial CBD within the deduced amino acid sequence (data not shown). For example CG14645 is not classified as a chitin binding protein for this reason, however it may be an immune responsive cardia specific gene, since it has significant sequence homology to Pro1 from *Glossina morsitans morsitans* (Hao and Aksoy, 2002). Indeed the partial CBD may indicate that this particular protein once had a functional CBD that have since become aberrant.

Although relatively uncommon, post-transcriptional regulation of gene expression in eukaryotes is normally restricted to those sequences involved in particular situations where a rapid response is required (Latchman, 1995). The genes of interest in this study that code for peritrophins are present in the *D. melanogaster* genome as single copy loci. The single cell thick cardia epithelia is capable of rapid rates of secretion of the PM without the aid of post-transcriptional regulation as the PM is produced continuously. Whereas post-transcriptional control of trypsin and protease expression is explained by the rapid upregulation of digestive enzyme production in some haematophagous insects upon taking a blood meal (Devenport *et al.*, 2004; Muller *et al.*, 1993). It is not surprising that genes coding for peritrophins are clustered in the *Drosophila* genome, this is particularly evident from the ten homologs within the same 68E3-68F locus as *DmPro2* (Table 10), since this is often the case for families of similar proteins.

Insect peritrophins commonly possess between 2 and 5 tandem CBDs per molecule (Table 9). Indeed, the majority of *Drosophila* peritrophins identified in this study contain 2, 3 or 5 CBDs, in addition to highly N- or O-glycosylated regions, and thus have the potential to function as adhesive molecules, in an analogous way to hemomucin, a protein identified in *Drosophila* that contains mucin domains, localized to many barrier structures, including the PM (Theopold *et al.*, 1996). The substantial N- and O-linked glycosylation of DmPro1 suggests that it may contain a mucin domain, although it would be classed as a true peritrophin due the CBDs being type P-A, rather than P-B. The occurrence of N-linked glycosylation at single sites within the amino acid sequence of *DmPro1*, *DmPro3*, *DmPro4* and *DmPro8* is consistent with the domain structure identified in P-55 (Tellam *et al.*, 2003). Such domains would allow multiple interactions with chitin and are characteristic of peritrophins and mucins within the PM. Thus, such PM proteins would be expected to have vital roles in maintaining the structural integrity of the PM. *DmPro3* and *DmPro8* are examples of peritrophins identified by the adult and larval

SSH screen respectively, that have a single CBD. In addition, the putative PM constituents CG11142 and CG17147 (Flybase) contain just one CBD, in accordance with their significant sequence homology to Peritrophin-1 from *An. gambiae* and P-15 in *D. melanogaster*. Such peritrophins may have a similar role as P-15a (CG17814) and P-15b (CG31893) in *D. melanogaster*, since a single CBD is thought to be insufficient to have any use in forming interactions with chitin within the PM structure, implying that these molecules may still have an affinity for chitin, but have an alternative role in PM structure. As integral components of the PM they may act as much shorter, capping proteins that protect the ends of the chitin microfibrils from degradation by exochitinases present in the midgut (Wijffels *et al.*, 2001). Indeed this proposed role is consistent with the pattern of synthesis of P-15 and *DmPro3* in cardia from adult *Drosophila*. P-15 gene expression has been found to be predominantly expressed in the posterior region of the epithelia of the cardia (Wijffels *et al.*, 2001). Furthermore, *DmPro3* is localised to the first and third zones of the formation zone of the cardia in addition to the anterior portion of the midgut, immediately posterior to the cardia (see Chapter 3 for more details). Suggesting that such peritrophins are added to an immature PM late during PM synthesis, supporting their proposed role in the structure of the PM. Alternatively, the interaction of two peritrophins, each containing a single CBD may be sufficient to allow the interaction with chitin within the PM.

The consensus sequences of the two recognised chitin binding domains, CBM_14 (pfam01607.11.CBM_14) and ChtBD2 (pfam00494.10.ChtBD2) were aligned in Fig. 13 to emphasize the presence of the same conserved CBD motif, consisting of six cysteines in the two types of CBDs identified within the putative peritrophins under study. Despite the apparent presence of a sixth cysteine in the correct location of the majority of CBDs identified as ChtBD2, this is not recognised by the databases that continue to read only the first five cysteines which leads to them distinguishing CBDs as the two separate types. The three disulphide bonds illustrated in Fig. 13 that form between the six conserved cysteines were predicted according to to those determined for the tertiary structure of tachycitin (Kawabata *et al.*, 1996). The amino acid sequence identity between the CBDs of molecules with as distinct functions as peritrophins and chitinases can be as much as 40%. However, outside of CBD motifs there is no significant amino acid sequence similarity among peritrophins, suggesting that peritrophins arose either as a result of divergent evolution from a common ancestor or due to the insertion of a CBD from another location in the genome (Shen and Jacobs-Lorena, 1999). The latter hypothesis conforms with the available data, in terms of the large range in number of CBDs that peritrophins possess. The three disulfide bonds are likely to be the main force which stabilizes the tertiary structure of peritrophins, as shown by the tertiary structure of tachycitin. Likewise, the conserved charged and aromatic residues of CDBs are believed to be involved in protein-saccharide bonds (Wright

et al., 1991). In particular the close stacking of the aromatic rings from the amino acid sequence of the protein and sugar rings of the saccharide are thought to aid the binding of chitin to peritrophins. Furthermore, the specific spacing between aromatic residues within CBDs may determine the binding specificity of peritrophins to chitin, rather than any other saccharide such as cellulose, as shown by the specificity of *AgAper1* binding in *in vitro* studies (Shen and Jacobs-Lorena, 1998).

Chapter 3. Generation of a null peritrophic matrix phenotype by targeting specific peritrophins

3.1. Abstract

A number of approaches were used to attempt to develop a PM-compromised fly strain in which the integrity of the PM is severely affected by specifically targeting key chitin binding protein (peritrophin) components. However, unexpectedly even the deficiency *Df(3L)vin3*, in which eight peritrophins were knocked out failed to produce a severe phenotype, indicating that there is sufficient functional redundancy within peritrophins to stabilize PM structure in such mutant strains. However, the exact role each peritrophin plays within the structure in terms of the interactions between components of the PM remains unclear. peritrophins may prove to be as vital for maintaining the structural integrity of the PM as the chitin component

3.2. Introduction

Our understanding of the structure and function of the PM remains at a relatively nascent stage, despite the perceived importance of PMs in insects. A complete picture of the *in vivo* function of individual peritrophins has the potential to enhance our understanding of the physiological functions that have been attributed to the PM since its identification over 200 years ago. The aim of this study is to identify those genes encoding PM chitin binding proteins (peritrophins) that when silenced cause maximal disruption to the integrity of the PM. This is the first study in which reverse genetics have been used to disrupt PM integrity and function. Previous studies in which PMs have been artificially disrupted have utilised chemical or biological approaches (Zimmermann and Peters, 1987; Shahabuddin *et al.*, 1993; Wang and Granados, 2000) that are less than ideal when determining the physiological function of the PM due to the confounding factors of non-PM specific physiological effects of the toxins used. We will use the simpler method of direct dsRNA injection, before attempting to establish a germline-transformed strain of flies in which the gene(s) are permanently disrupted. The transgenic approach is ideal, as it would permit the generation of large numbers of comparable individuals for experimentation.

The completion of the *Drosophila* genome project (Adams *et al.*, 2000) provides an invaluable resource that can aid the further utilisation of this model organism to define biological functions encoded by its genes. In order to fully exploit this resource it is critical that functional studies utilize techniques that allow rapid and efficient progress from the initial characterisation of gene(s) to elucidation of their biological function. Traditionally, genes have been characterised by the screening of phenotypes in loss-of-function of mutants. Here, mutations are induced randomly within the genome, by chemical, physical or insertional mutagenesis. Even the most recent Berkeley *Drosophila* Genome Project (BDGP) screen has resulted in only 25%

coverage of the entire euchromatic portion of the *Drosophila* genome (Spradling *et al.*, 1999). Recently, the coverage provided by the P-element technique has been modestly improved upon (an 5-7% increase) by Parks *et al.* (2004), with a second deletion collection created by FLP recombinase of FRT-insertions providing genome coverage of 56%. However, the mutation approach is often not sufficiently precise to result in only the gene of interest being affected, since most existing deletions are large and generate random breakpoints. All these mutational approaches are time-consuming and laborious and not ideal for the rapid pursuit of gene function required. In contrast, RNA interference (RNAi) dramatically simplifies reverse genetics in *Drosophila* by systematically generating mutant phenotypes in order to assign gene function.

The advent of RNAi has made it possible to rapidly create specifically tailored loss-of-function phenotypes for the first time (Fire *et al.*, 1998; Kennerdell and Carthew, 2000; Carthew, 2001). RNAi is a gene silencing mechanism that is triggered by introducing double-stranded RNA (dsRNA) into cells, leading to the sequence specific post-transcriptional gene silencing (PTGS) of its cognate gene. This curious phenomenon was discovered by Fire *et al.* (1998) who found that sense RNA contaminated with antisense RNA (i.e. dsRNA) was substantially more effective at blocking gene function than only antisense RNA. Small interfering RNAs (siRNAs) are the agents responsible for triggering the RNAi mechanism (Zamore *et al.*, 2000) as these 21-23 nt long RNAs become associated with the dsRNA-induced nucleases that carry out sequence specific mRNA degradation (Hammond *et al.*, 2000) (see Fig. 14 for details of the RNAi mechanism). Thus, RNAi can be used to experimentally suppress specific endogenous RNAs, thereby providing a potent and specific means to functionally silence gene(s). This approach has provided a powerful new tool for functional genomic studies in a variety of organisms, spanning the breadth of eukaryotic phyla, including *Drosophila* (Kennerdell and Carthew, 1998), *Caenorhabditis elegans* (Fire *et al.*, 1998), trypanosomes (Ngo *et al.*, 1998), zebrafish (Wargelius *et al.*, 1999) and mice (Hunter, 1999). The injection of dsRNA into *Drosophila* embryos efficiently silences gene activity, but its effect is transient as the disruption of late acting genes is less consistently seen than embryonically expressed genes and dsRNA is not stably inherited (Misquitta and Paterson, 1999). As a result the direct injection of dsRNA has previously been limited to studies of embryonic gene function in *Drosophila*. A number of novel strategies have since been developed in order to extend the application of RNAi to investigate later stages of *Drosophila* development. Current methods include the intra-abdominal injection of dsRNA into adult flies (Dzitoyeva *et al.*, 2001; Manev *et al.*, 2003) and the stable *in vivo* expression of dsRNA by heritable transgenes (Fortier and Belote, 2000; Lee and Carthew, 2003; Reichhart *et al.*, 2002). The former method was also recently used to disrupt the defensin gene in adult *An. gambiae* (Blandin *et al.*, 2002). Moreover, in insects where

transgenesis is difficult, e.g. Tsetse flies, RNAi by injection will almost certainly become the method of choice, in the short term at least. In adult *Drosophila* RNAi is still routinely mediated by the expression of dsRNA by transgenic flies (Lee and Carthew, 2003), in which the transgene is either in an inverted-repeat (IR) (Lam and Thummel, 2000) or symmetrically transcribed configuration (Giordano *et al.*, 2002). While elegant, this approach is very time consuming and often difficult with particular genes.

3.3. Materials and Methods

The use of traditional genetic analysis techniques to identify those peritrophins that are crucial for PM structure is hampered by the lack of suitable existing mutant fly lines that could be used to target individual peritrophins . Therefore, following the lead of studies that have firmly established the use of RNAi in *Drosophila* (Fortier and Belote, 2000; Lam and Thummel, 2000) the disruption of the *Drosophila* PM was first attempted using RNAi as a means of knocking down gene expression.

3.3.1. Origin and Maintenance of Drosophila strains

As described previously in Chapter 2, the wild type and mutant strains used during this study were maintained on standard Cambridge fly (yeast/ cornmeal/ dextrose/ agar) medium supplemented with p-hydroxybenzoic acid methylester (Sigma) as an antifungal agent, and cultured at 25 °C with a light: dark period of 1:1. The wild type strain used is the isogenic M-type strain used for the *Drosophila* genome sequencing project (y[1]; cn[1]; bw[1]; sp[1]) (Adams *et al.*, 2000; Rubin *et al.*, 2000). The balancers FM7j (FM7j, y[93j] w B; 2[iso]; 3[iso]); TM2/ TM6C (w[1118][iso]/ y[+]Y; 2[iso]; TM2/ TM6C, Sb) and SM6a (w[1118][iso]/ y[+]Y; Sco/ SM6a; 3[iso]) were obtained from John Roote, Department of Genetics, University of Cambridge. The strains with deficiencies that uncover genes encoding PM chitin binding proteins were supplied by the Bloomington Stock Center, the details of which are given in Table 11 and Fig. 15 below. In addition, the other class of aberration is P-element insertions. There were a number of strains with P-element insertion sites at the correct location to cause a deletion or disruption to the genes encoding *DmPro1*, CG17814 (P-15a) and CG31893 (P-15b). The p insertion P{ry11}CycA^{ry40} causes a breakpoint at 68D4 which may in turn lead to a frameshift mutation in the gene *DmPro1*. Moreover, the p insertion at 29C1 of P{y$^{+mDint2}$ w$^{BR.E.BR}$=SUPor-P}fy^{KG00724}; ry^{506} may affect the genes coding CG17814 and CG31893. The phentoypes of the mutants were assayed by T.E.M. of triplicate PMs from adults and larvae of each strain.

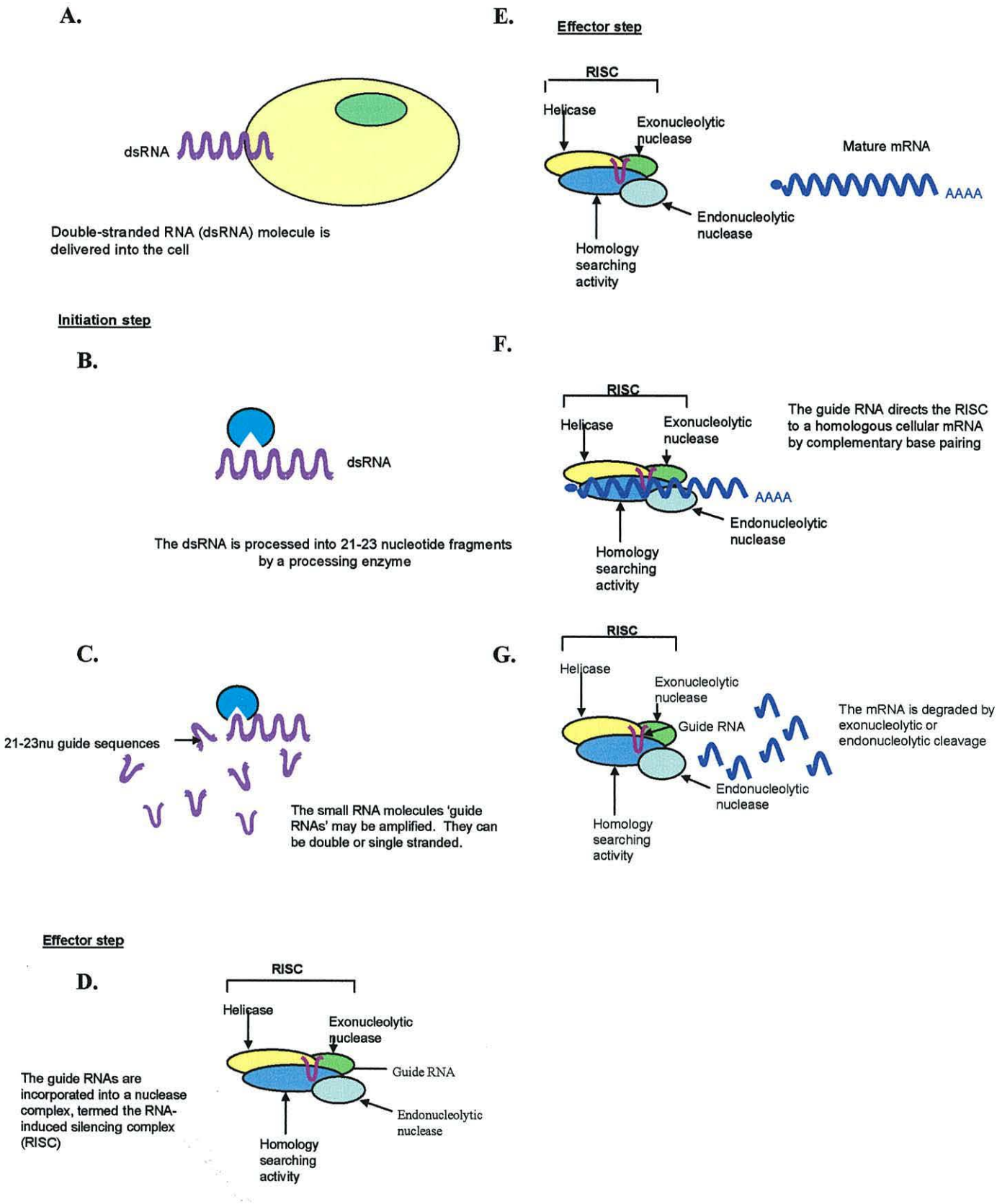


Fig. 14. Diagramatic representation of the RNAi mechanism.

3.3.2. Double-Stranded RNA Preparation

dsRNAs were produced using the generic high-copy plasmid pBluescript SK+ (Stratagene) to form constructs to be used as plasmid templates for transcription reactions. cDNA was PCR amplified using specific primers (see Table 12) and cloned between the T7 and T3 promoters of the plasmid (see Fig. 16), resulting in formation of the dsRNA constructs described in Table 7. The plasmid templates were prepared for use in transcription reactions by firstly linearizing the plasmid DNA by restriction at a site downstream of the insert in order to generate a run-off transcript of defined length which consists exclusively of the plasmid promoter and cDNA corresponding to the gene of interest. Next, a small proportion of the linearized DNA (1 µl of a 20 µl reaction) is examined on a 1% agarose gel to confirm that cleavage is complete. The restriction reaction is terminated using $1/20^{th}$ volume 0.5M EDTA and $1/10^{th}$ volume 3M Na acetate plus 2 volumes of 100% EtOH. The mixture is vortexed and precipitated at -20 °C for a minimum of 15 min, before centrifugation at 14 000 rpm. The pellet is then aspirated by removing the majority of the supernatant and briefly re-centrifuging before taking off the remaining fluid, the pellet is then dried briefly and resuspended in UPW at a concentration of 0.2-1 µg μl^{-1}. The template DNA may be treated with a mixture of proteinase K (100-200 µg μl^{-1}) and 0.5% SDS for 30 min at 50 °C if RNase contamination of the template is suspected, prior to transcription. Phenol/ chloroform extract (using an equal volume of each) the reaction and recover the purified template DNA by ethanol precipitation. Sense and antisense RNAs were synthesized using the T7 and T3 MEGAScript™ transcription kit (Ambion) as described by the manufacturer. Briefly, the transcription reaction was assembled at R.T., mixed thoroughly by pipetting and incubated at 37 °C for 2-4 h. A small aliquot (1 µl) of the transcription product can be analysed on a 2% agarose gel to verify that the optimum yield of ssRNA is obtained. The DNA template is removed from the transcription product with the addition of 1 µl RNase-free Dnase 1 (2U μl^{-1}) (Ambion), mixed well and incubated at 37 °C for 15-30 min. The transcription reaction is terminated by the addition of 115 µl nuclease-free water and 15 µl NH_4OAc stop solution to the 20 µl transcription product, and the RNA recovered by phenol/chloroform extraction (using an equal volume of each) and the addition of 1 volume of isopropanol, the purified RNA is precipitated at -80 °C for 15-30 min, before centrifugation at 14 000 rpm. The supernatant is removed and the RNA pellet re-centrifuged briefly before taking off the remaining fluid, so that only brief drying is required, ensuring that the RNA pellet can be easily resuspended in a small volume of nuclease-free water (typically 20 µl) on ice, at a concentration of 0.2-1 µg μl^{-1}. The ssRNAs were annealed in nuclease free water by mixing an equimolar concentration (100 ng μl^{-1}) of both S and AS strands, heating to 85 °C for 5 min, cooled down

Fig. 15. Cytogenic extent of mutation within the 68E3-4 interval. Coloured bars represent the overlapping ranges of the deficient stocks, with their corresponding Flybase identifiers. The individual genes affected are depicted as arrows, the direction indicates the orientation of the coding sequence, genes encoding putative peritrophins are solid arrows, empty arrows represent other genes within the locus.

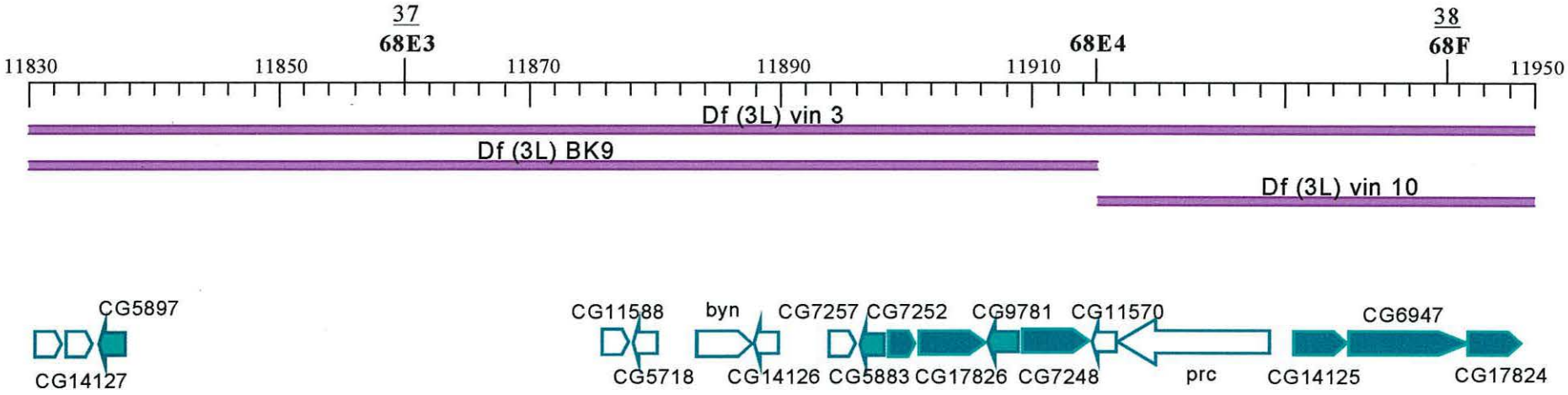


Table 11. The details available on the deficient stocks from Flybase (accurate on 25/11/02).

Deficiency	Breakpoints	Genotype	Disrupts gene(s)
Df(3L)vin3	[68C5-6] [68E3-4] [64C-F;71C] In	ru[1] h[1] gl[1] e[1] ca[1]/ TM3, ry[*] Sb[1] Ser[1]	*DmPro10* CG5897 CG6947 CG7248 *DmPro4* CG9781 *DmPro3* CG17824 *DmPro2*
Df(3L)vin10	[68E4-F8] [68F3-61A1]	-	CG6947 *DmPro3* CG17824
Df(3L)BK9	[68E2-3] [69A1]	ru[1] red[1] cv-c[1] Sb[1] sr[1] e[1]/ TM3, Sb[1]	CG7248

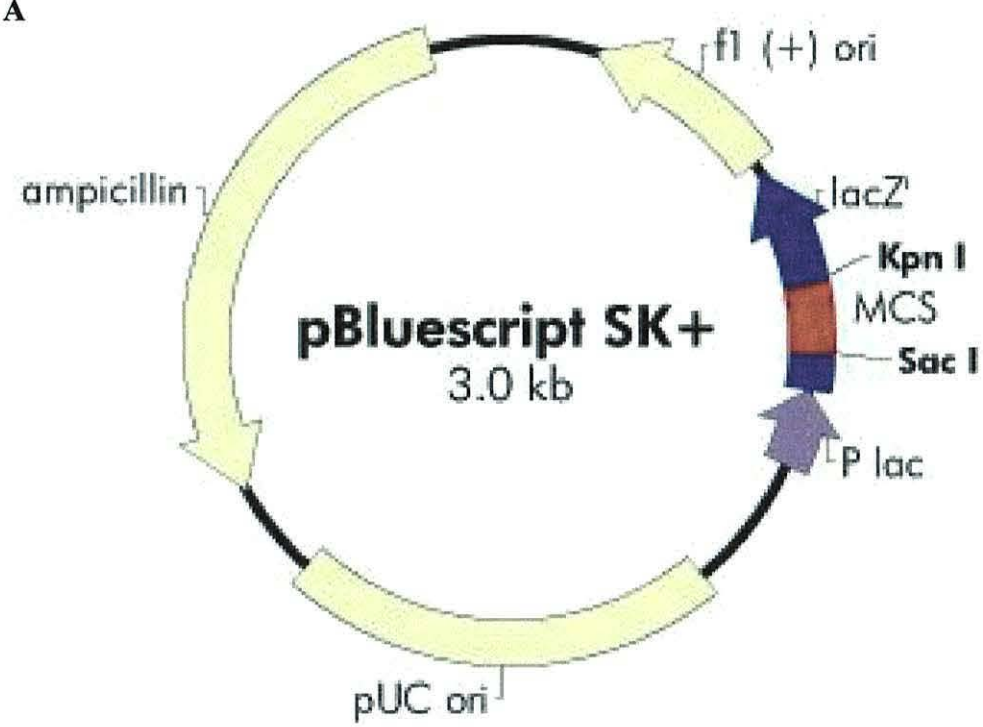
slowly for 18 h and stored as dsRNAs at -80 °C until use. The precise quantification of purified ssRNA (for successful annealing reaction) was determined using a combination of UV light absorbance (A_{260}) readings and the Picogreen RNA quantification protocol (Molecular Probes) on the Wallac Victor 2 1420 multilabel counter (Perkin-Elmer, Life Sciences, Turku, Finland) with the aid of Wallac Workout Software Version 1.5.

3.3.3. Double-Stranded RNA Injection into Drosophila

A microinjector (PM1000, MDI) and quartz needles (1 mm od x 0.7 mm id x 100 mm, Intracel) were used to introduce up to 200 nl of dsRNAs diluted in injection buffer (0.1mM sodium phosphate, pH 6.8; 5 mM KCl) to a concentration of ~ 0.8 $\mu g\ \mu l^{-1}$ into immobilised 2nd instar larvae and the thorax of CO_2 anesthetized adult flies, which were subsequently allowed to recover for 3 days.

Fig. 16.
A A restriction map of the pBlueScript SK+ vector.
B A schematic representation of the pBlueScript SK+, the multicloning site consists of the unique restriction sites, including: *Xho*I, *Sal*I, *Cla*I, *Hind*III, *Eco*RV, *Eco*RI, *Pst*I, *Sma*I , *Bam*HI, *Spe*I, XbaI, *Not*I and *Sac*II for the cloning of the gene of interest. The T3 and T7 promoter binding sites are indicated by arrows, as are the M13 primer binding sites for screening of clones.

A



B

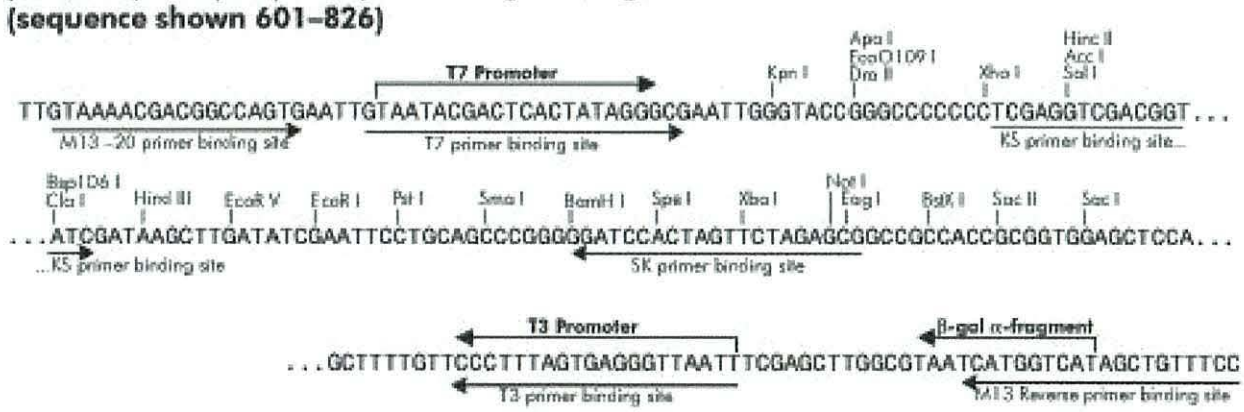
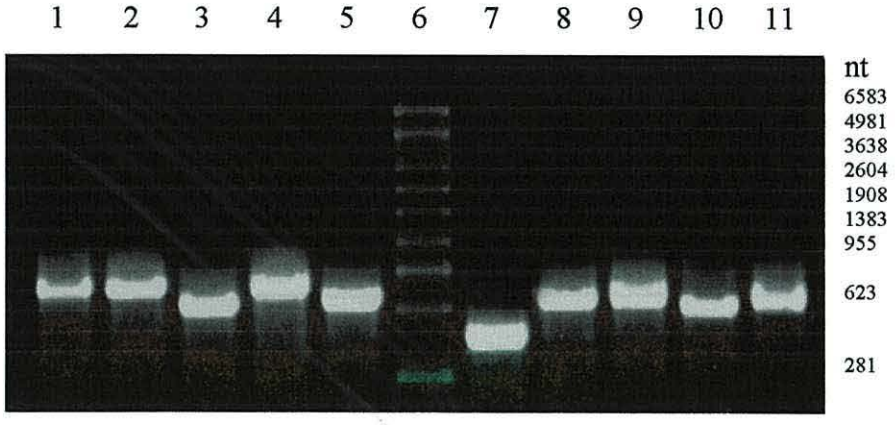


Table 12. Details of the constructs from which run off transcripts were synthesised. Note: pds_, dsRNA plasmid constructs; sense (S) and reverse complement (RC) primers; restriction sites added onto gene specific portion of the primer (detailed below); ClaI, CCATCGAT and XbaI, GCTCTAGA; T7 and T3, T7 and T3 promoters.

Construct	Primer Name	Primer Sequence (5'-3')	Transcript length (nt)
pds *DmPro1*	*DmPro1* S ClaI	CGTATACGACCGATAATC	T7 895
	DmPro1 RC XbaI	TGCATTTCTGGCTGCGAAG	T3 899
pds*DmPro6*	*DmPro6* S ClaI	AGCGAGGCCAAGTCCAAGTC	T7 698
	DmPro6 RC XbaI	ACGGGCTGCTTCAAAGGTG	T3 702
pds*DmPro3*	*DmPro3* S ClaI	GAGTCTTCTCGATTCTG	T7 872
	DmPro3 RC XbaI	TCCCGTGGGATTCAATG	T3 876
pds*DmPro2*	*DmPro2* S ClaI	TACGCAGAGTGCAAAG	T7 665
	DmPro2 RC XbaI	TCCAGTATGGCATTGAAG	T3 667
pds45	CG14645 S ClaI	CTCGCTCTAACCTCCCTACTAG	T7 432
	CG14645 RC XbaI	CTGCGTAAGCCACCCGTAAC	T3 437
pds*DmPro4*	*DmPro4* S ClaI	GGTTCCTCGGCGGTTCAATG	T7 508
	DmPro4 RC XbaI	CCATCGGCGCACTCCATCTC	T3 512
pds*DmPro5*	*DmPro5* S ClaI	TATTCCGCATGGTCCTTGGTG	T7 605
	DmPro5 RC XbaI	GTTATCTGGGTGGGCTTCTCCTG	T3 609
pds*DmPro8*	*DmPro8* S ClaI	CATCGGCAAGCTCTCCTGTAG	T7 913
	DmPro8 RC XbaI	GTCTCGCTGGTCGCACTTTC	T3 917
pdsGFP	GFP S ClaI	TTGTTGAATTAGATGGCGATG	T7 643
	GFP RC XbaI	TTTGGAAAGGGCAGATTGTG	T3 647
pdsY8	Y8 S EcoRI	GGAGACTCATACTACTA	T7 970
	Y8 RC XhoI	GCCTAGCTCGTTGGCTG	T3 974

Fig. 17.
Annealed dsRNA corresponding to the gene products: lane 1, *DmPro1*; lane 2, *DmPro3*; lane 3, cg15918; lane 4, *DmPro4*; lane 5, *DmPro2*; lane 6, RNA markers, 0.28-6.58 kb (Promega); lane 7, CG14645; lane 8, *DmPro5*; lane 9, *DmPro8*; lane 10, GFP coding gene; lane 11, Yellow Fever Virus coding gene (YF 8).



3.3.4. P-element vector IR construct design

3.3.4.1. pCaSpeR vector

Fig. 18. Plasmid map of pCaSpeR-hs/act (Thummel *et al.*, 1988).

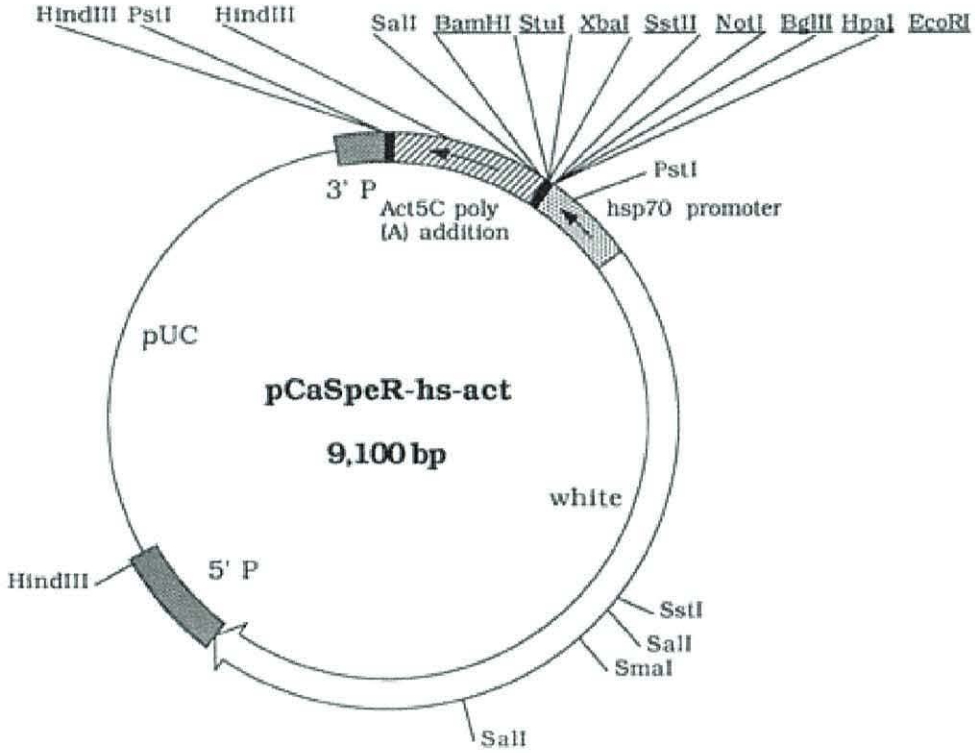


Table 13. Details of the PCR primers used to synthesize inserts A and B to create IRs in pCaSpeR constructs from which dsRNA transcripts are synthesised. Note: pC_, denotes specific pCaSpeR constructs; sense (S) and reverse complement (RC) primers; 5' clamp (brackets) and restriction sites adjoined to the gene specific portion of the primer (detailed below); BamH1, (CG)GGATCC; Xba1, (GC)TCTAGA; EcoR1, (CG)GAATTC; Not1, (GAAT)GCGGCCGC and Hpa1 (GC)GTTAAC. The RC primer for insert B includes sufficient non-palindromic sequence to create a spacer.

Construct	Primer Name	Primer Sequence (5'-3')	Product length (bp)
p*CDmPro1*	*DmPro1* S BamH1 A	TCAGAATCTCCTCCTGAAG	
	DmPro1 RC Xba1 A	TGCATTTCTGGCTGCGAAG	738
	DmPro1 S EcoR1 B	CGTATACGACCGATAATC	
	DmPro1 RC Not1 B	TGCATTTCTGGCTGCGAAG	818 (9 bp hinge)
p*CDmPro2*	*DmPro2* S BamH1 A	ATACGCAGAGTGCAAAG	
	DmPro2 RC Xba1 A	TCCAGTATGGCATTGAAG	604
	CG17286 S EcoR1 B	TGATCCGCGGTTTTTAC	
	DmPro2 RC Not1 B	TCCAGTATGGCATTGAAG	522 (9 bp hinge)
p*CDmPro3*	*DmPro3* S BamH1 A	CAAGGGTTACGCATTACC	
	DmPro3 RC Xba1 A	TCCCGTGGGATTCAATG	699
	DmPro3 S EcoR1 B	GAGTCTTCTCGATTCTG	
	DmPro3 RC Not1 B	TCCCGTGGGATTCAATG	813 (15 bp hinge)

3.3.4.2. pWIZ vector

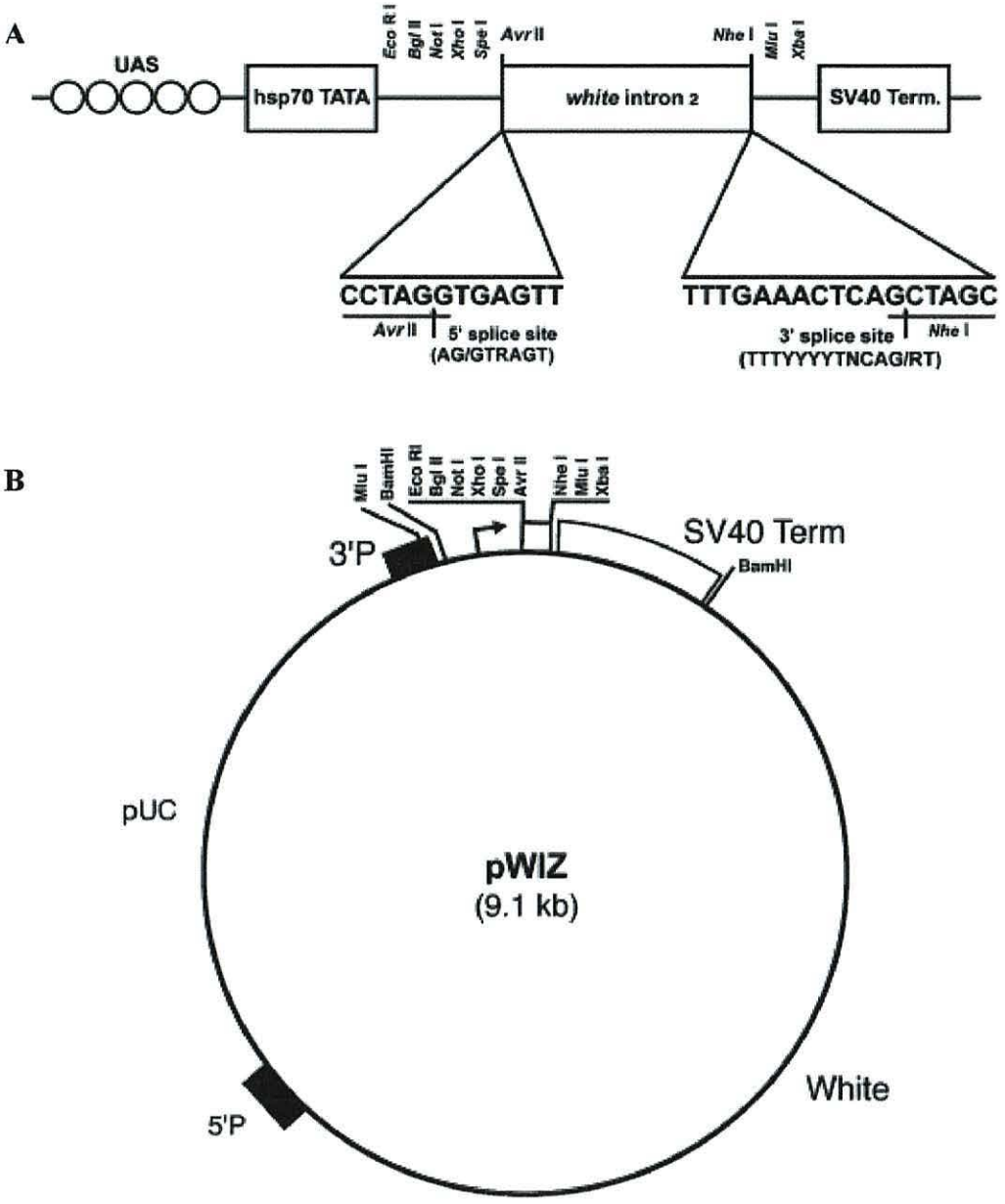


Fig. 19.
A A schematic representation of the pWIZ vector, developed by modifying the pUAST transformation vector through the insertion of the 74bp long intron 2 from the *white* gene. The multicloning site consists of the unique restriction sites *Eco*RI, *Bgl*II, *Not*I, *Xho*I, *Spe*I and *Avr*II on the 5' side and *Nhe*I and *Xba*I on the 3' side for the cloning of the gene of interest. The 5' and 3' splice sites are indicated by arrows.
B A restriction map of the pWIZ vector.
From Lee and Carthew (2003).

Table 14.
Details of the PCR primers used to synthesize gene specific inserts (A and B) to create IRs in pWIZ constructs from which dsRNA transcripts are synthesised. Note: pW_, denotes specific pWIZ constructs; sense (S) and reverse complement (RC) primers; restriction sites adjoined to the gene specific portion of the primer (detailed below); SpeI, (CTAG)ACTAGT; AvRI, (CTAG)CCTAGG; Xba1, (CTAG)TCTAGA; NheI, (CTAG)GCTAGC.

Construct	Primer Name	Primer Sequence (5'-3')	Product length (bp)
pW*DmPro1*	*DmPro1* S SpeI A	TCAGAATCTCCTCCTGAAG	757
	DmPro1 RC AvrII A	TGCATTTCTGGCTGCGAAG	
	DmPro1 S XbaI B	CGTATACGACCGATAATC	837
	DmPro1 RC NheI B	TGCATTTCTGGCTGCGAAG	
pW*DmPro2*	*DmPro2* S SpeI A	ATACGCAGAGTGCAAAG	604
	DmPro2 RC AvrII A	TCCAGTATGGCATTGAAG	
	DmPro2 S XbaI B	TGATCCGCGGTTTTTAC	522
	DmPro2 RC NheI B	TCCAGTATGGCATTGAAG	
pW*DmPro3*	*DmPro3* S SpeI A	CAAGGGTTACGCATTAC	699
	DmPro3 RC AvrII A	TCCCGTGGGATTCAATG	
	DmPro3 S NheI B	GAGTCTTCTCGATTCTG	814
	DmPro3 RC NheI B	TCCCGTGGGATTCAATG	

The protocol used to construct a hp-dsRNA expressing vector using pCaSpeR-hs/act and pWIZ is very similar. The target gene is amplified by PCR with a pair of oligonucleotides that include the desired restriction sites on their 5' ends to produce a cDNA fragment of ~600 bp long. The sequence of the DNA fragment to be inserted must be suitable for cloning, i.e. lack the restriction sites to be used for cloning, the consensus splice sites of pCaSpeR or pWIZ and any considerable secondary structure. The PCR product was inserted twice by sequential restriction and ligation reactions into the *Bam*HI and *Xba*I (insert A) and *EcoRI* and *Not*I (insert B) sites of pCaSpeR, and the *Avr*II and *Nhe*I sites of pWIZ. The 5' ends of the restricted vector were dephosphorylated by treatment with calf intestinal phosphatase (CIP) prior to the ligation reaction. This resulted in constructs containing two inserts in the tail-to-tail orientation, located downstream from the *hsp70* promoter and upstream from the *actin 5C* transcription termination and polyadenylation signals, were selected for use in the RNAi experiment.

3.3.5. Germline Transformation

pCaSpeR P elements were introduced into the germline of wild type embryos using the standard protocol (Rubin and Spradling, 1982). Seven independent lines of p*CDmPro1* and eight independent lines of p*CDmPro3* were established as stocks. Further characterisation of the site of insertion was conducted using Inverse PCR.

3.3.6. Induction of dsRNA

At the appropriate development stage, flies were transferred to 1.5 ml eppendorf tubes with a perforated cap, incubated at 37 °C in a water bath for 30 min, transferred into new fly food vials and maintained at 25 °C.

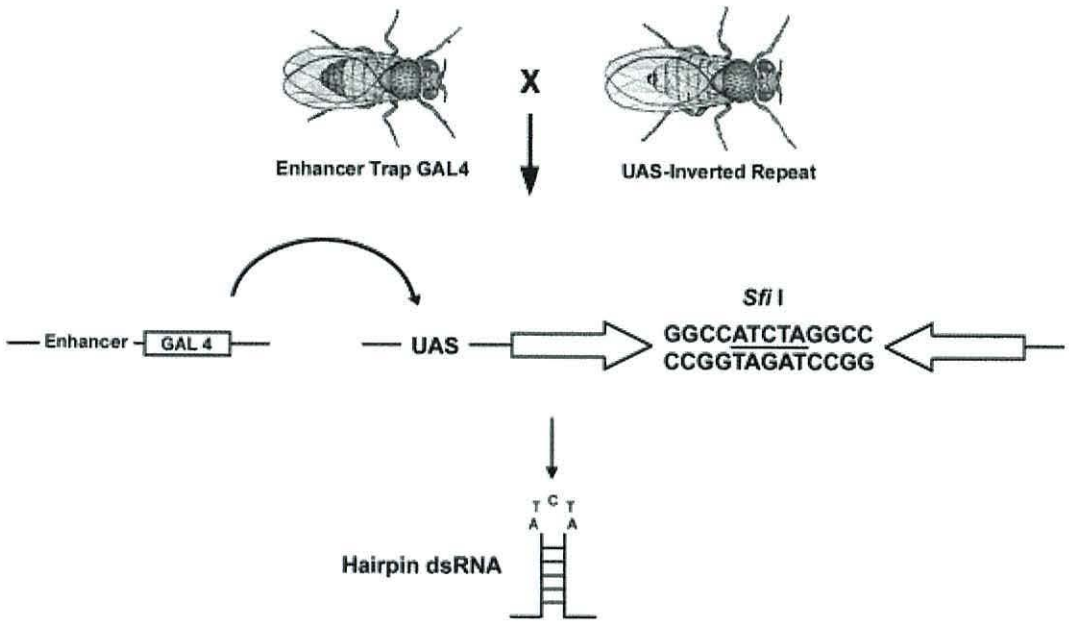


Fig. 20. Following genomic transformation to generate stable transgenic fly lines carrying the pWIZ construct, RNAi may be induced against target genes by setting up mating experiments of transgenic individuals with those carrying a suitable GAL4 driver. The F1 progeny of this cross produces a hairpin dsRNA that triggers the RNAi mechanism. From Lee and Carthew (2003).

The extent of RNAi silencing on the transcript levels of the target genes was verified by RT-PCR analysis using the primers in Table 6, before conducting microscope analysis of the PM phenotype.

3.3.7. Transmission Electron Microscopy (T.E.M.)

Mutant phenotypes were characterised in PM dissected out from larvae and adults. The procedure adopted for the T.E.M. studies of *Drosophila* PM2 is based on the protocol of Reid and Lehane (1984), with a few modifications. All PMs were harvested either from adults 5 d post eclosion or young 3rd instar larvae that had been fed on standard fly medium. The cardia plus the anterior midgut containing the PMs were dissected out from CO_2 and cold anaesthetised adults and larvae respectively in PBS and immediately placed in 1ml chilled fixative 2.5 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, 3 % sucrose, pH 7.2. All changes of solution in which the cardia are bathed were carried out with the tissue samples held within a wetted

porous capsule (TAAB) in a 2 ml glass vial (Scientific Lab. Supplies Ltd.). The tissues were fixed for 1 h in fresh fixative at 4°C, washed in three changes of 0.1 M sodium cacodylate buffer, 3 % sucrose, pH 7.2 at 4 °C, 15 min each, before being postfixed in 1 % osmium tetroxide in 0.1 M sodium cocodylate buffer, 3 % sucrose, pH 7.2 at 4 °C for 1 h. After washing in three changes of distilled water 10 min each, the specimens were stained en bloc overnight in 2 % uranyl acetate (filtered through a millipore filter) at 4 °C in the dark. The tissue was then rinsed in distilled water for 5 min and dehydrated in an ascending ethanol series from 50 % to 100 % for 10 min each, plus two further changes in 100 %, before being placed in 100 % propylene oxide for 2 min. After an additional change of 1:1 mixture of propylene oxide, the tissue was placed in 100% spurrs resin (medium grade), for 20 min with constant agitation before infiltrating with fresh pure spurrs resin overnight with agitation. Finally, tissue samples were embedded in pure spurs resin within a mould and polymerised by heating at 60 °C for 24 h. The blocks were shaped into a face suitable for sectioning on the ultratome using a LKB pyramitome. All sections were cut using a glass knife in a LKB Ultratome III, the thickness of ultra-thin sections being determined by continuous interference and chosen to be between silver and gold, approximately 30nm (300 Å). Transverse sections of the PM were taken in each case. The sections obtained were mounted on pilofilm coated copper hexagonal grids and stained with 2 % uranyl acetate and saturated aqueous lead citrate for 5 min in each stain under a CO_2 free environment, created by KOH, with three SDW washes of 5 min each between stains. The sections were examined on a Philips T. E. M. 301 at 80kV accelerating voltage.

3.4. Results

3.4.1. Effect of deficiencies in the 68E3-4 locus on PM integrity

PM examined from homozygous *D. melanogaster* mutant larvae using T.E.M.

Fig. 21. *Df(3L)vin3* mutant PM phenotypes in homozygous third instar larvae.
A. Note: double headed arrow denotes the complete separation of the PM into two PMs, single headed arrow may show a full thickness break in the equivalent of PM2.
B. Partial thickness break in the PM.
Abbreviations: L1, layer 1; L2, layer 2; L3, layer 3; E, midgut epithelium; and L, midgut lumen side of the PM. Values of scale bars are given.

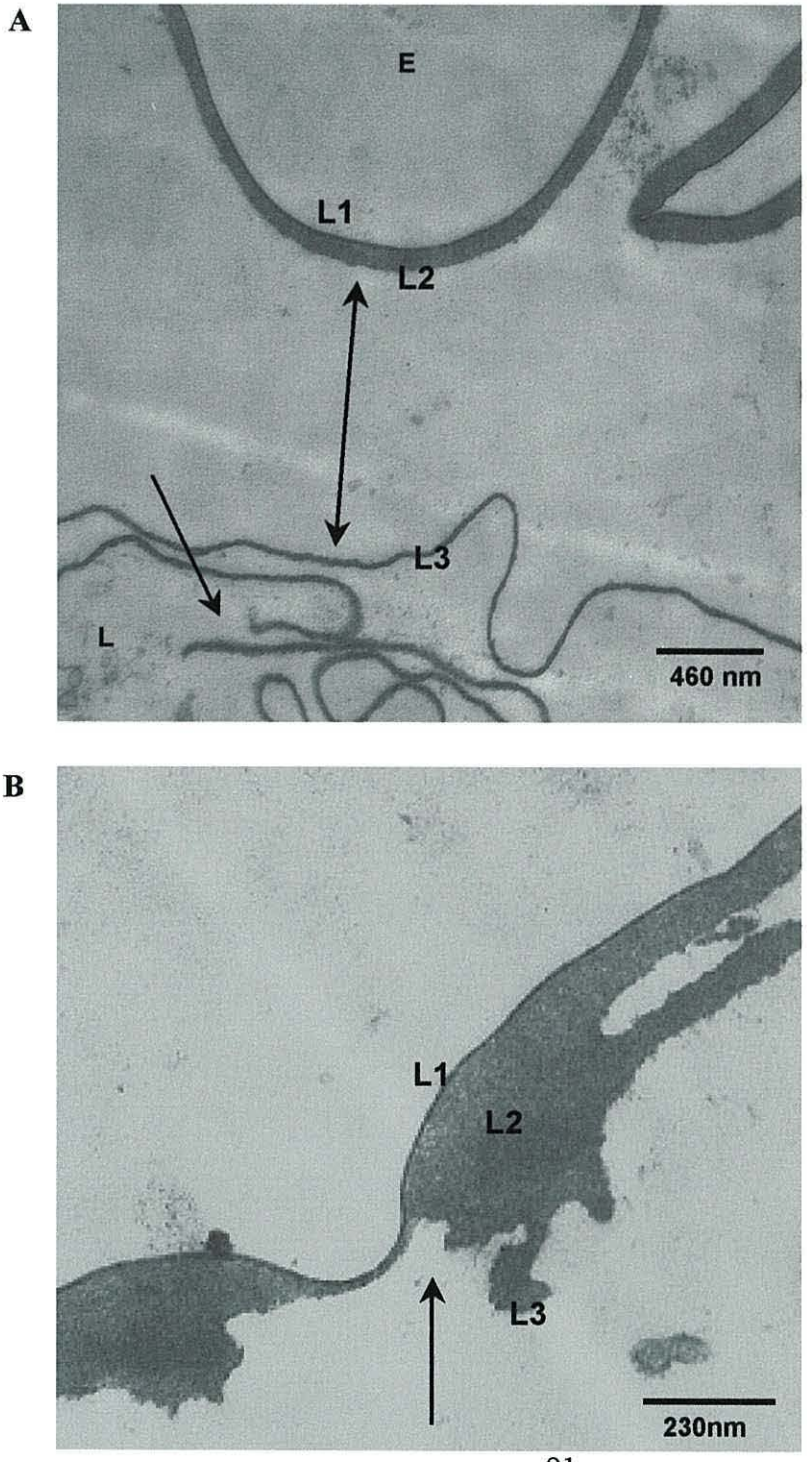


Fig. 22. PM phenotype of third instar homozygous *Df(3L)vin10* mutants. Clearly showing two intact, closely apposed layers.

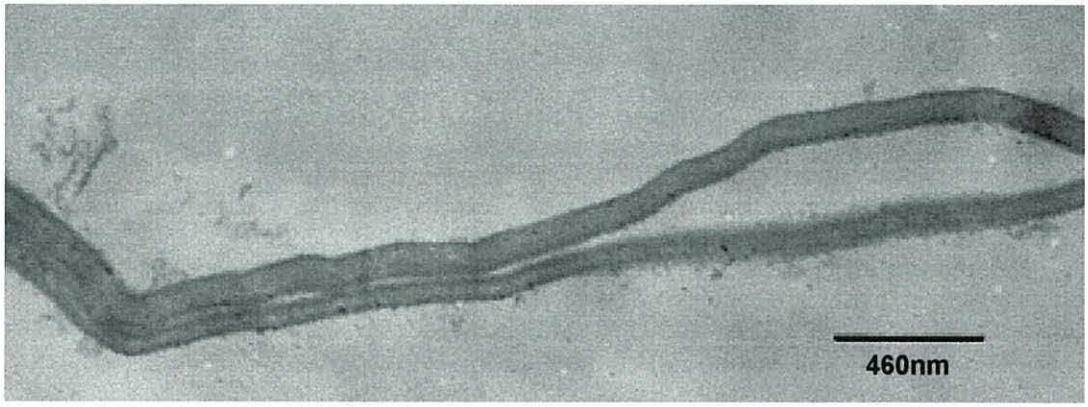


Fig. 23. PM phenotype of third instar homozygous *Df(3L)BK9* mutants. Showing two intact, but closely apposed stretches of the PM, formed from a highly looped PM.

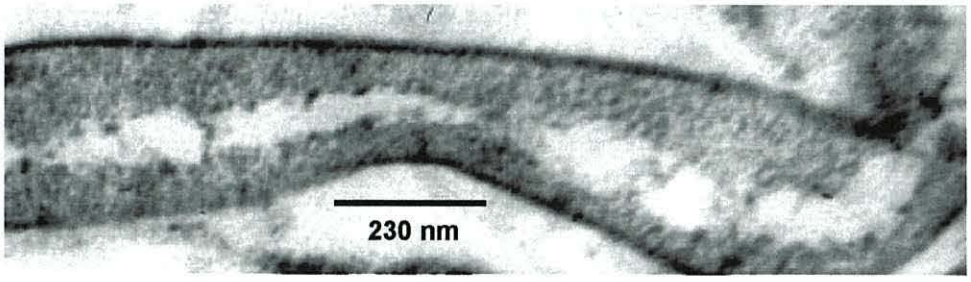
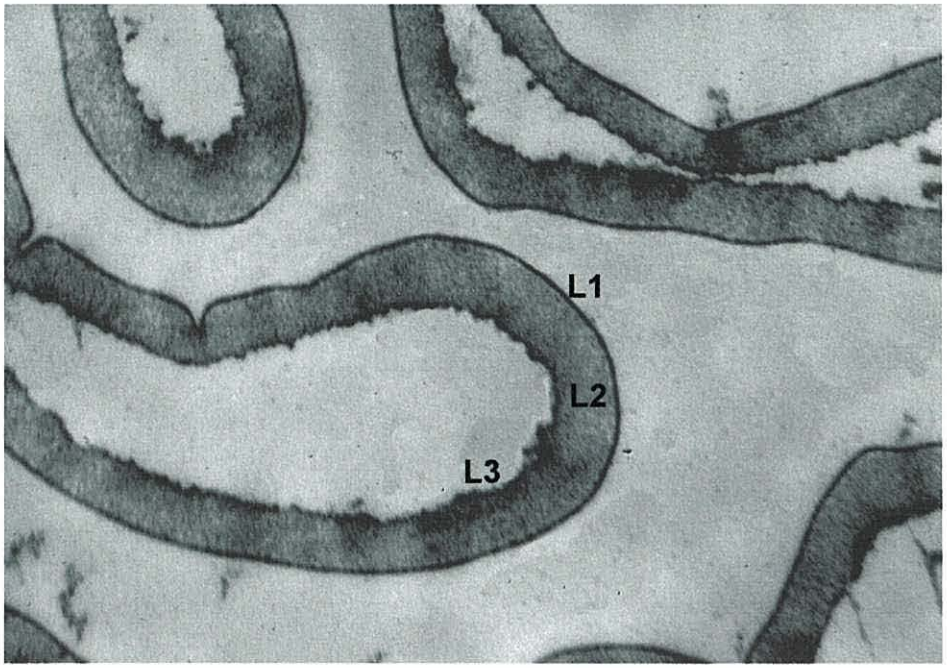


Fig. 24. PM phenotype of control third instar larvae (continued on next page).
A. An intact trilaminate PM clearly showing the electron dense and electron lucent layers.
B. Layer 1, a thicker electron dense layer is distinct, whilst layer 3, the very fine electron dense layer is not very visible.

A



B

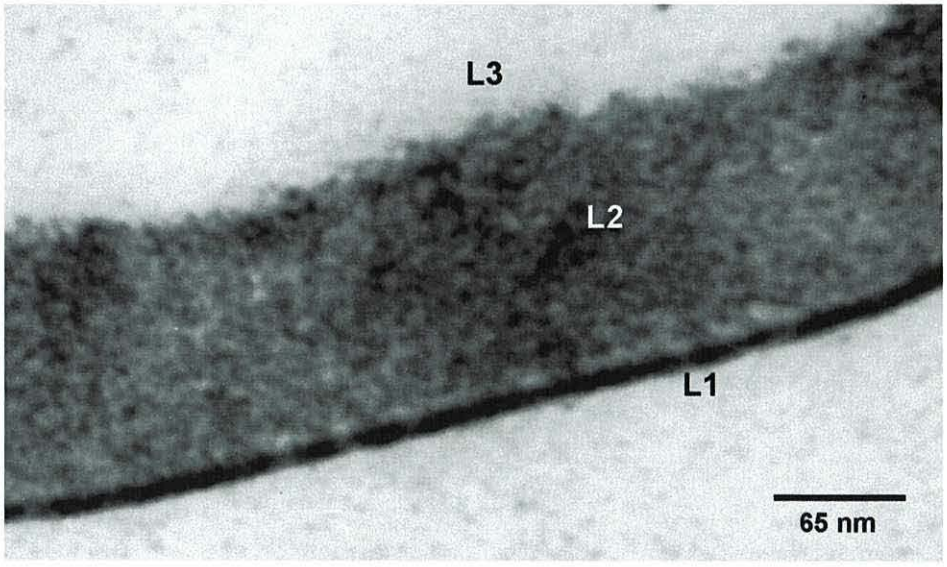
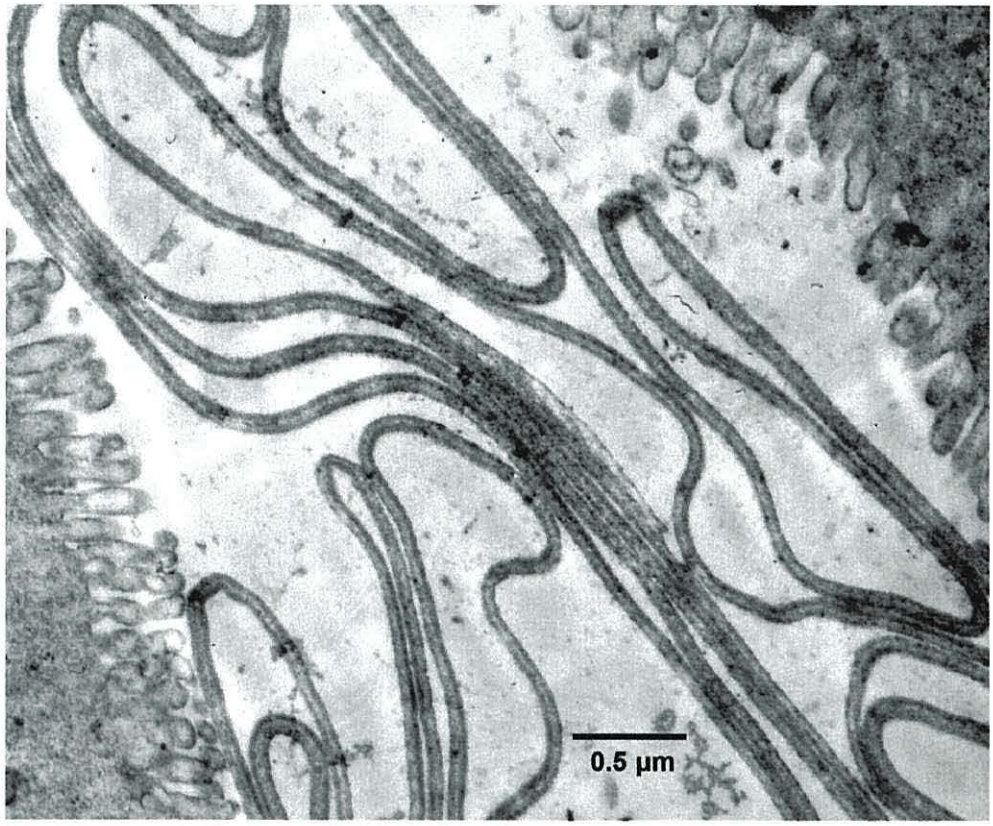


Fig. 25. Continued from previous page.

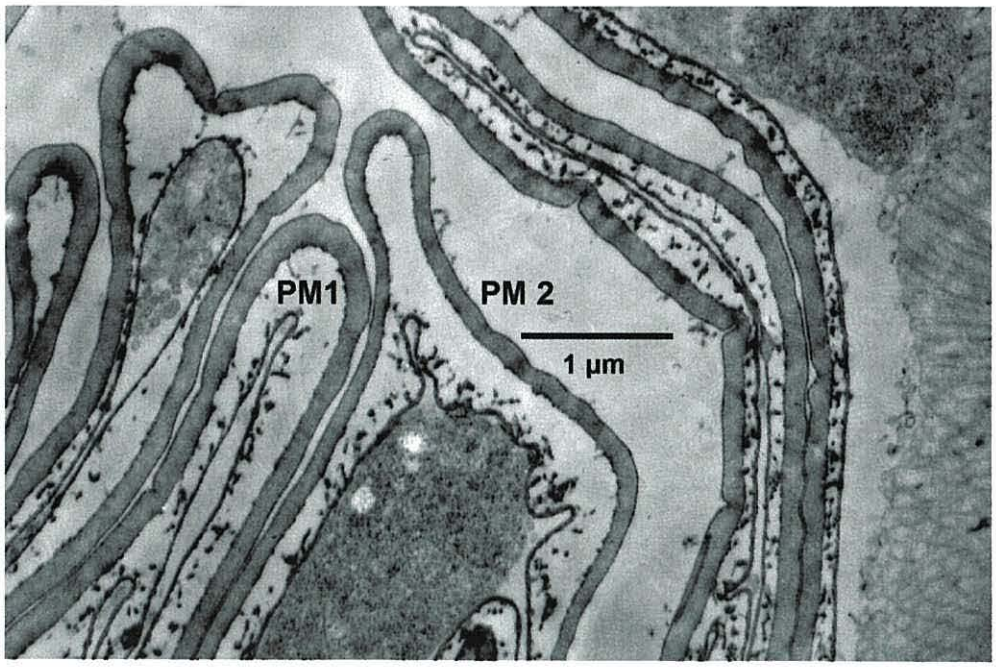
A. The larval PM in wild-type flies. The single PM is intact.

B. The adult PM in wild-type flies. The inner PM (PM1) is thrown into folds, whereas the outer PM (PM2) is more straight.

A



B



Careful examination of the PM from *D. melanogaster* larvae revealed at least two distinct layers in a single PM (Fig. 24B). The layers appear to be tightly apposed in the intact PM of control larvae (Fig. 24A, 24B and 25A). However, in mutant larvae they appear to be separated in damaged PM, the extent of which ranges from light to moderate depending on the mutant strain and the region of the PM examined. The PM of *Df(3L)vin3* (Fig. 21), *Df(3L)vin10* (Fig. 22) and *Df(3L)BK9* (Fig. 23) mutants were examined. Fig. 21A shows the most severe phenotype observed, in which arrows indicate the complete separation of the single PM along the boundary between the third and fourth layers to form two distinct PMs. In addition, there are also instances of the complete break of the fourth layer. Fig. 21B shows the partial break in layers one and three of the PM, and partial separation between layers two and three. The only layer that remains unaffected is layer one, composed of very fine electron-dense material. The PM phenotype observed in *Df(3L)vin10* (Fig. 22) and *Df(3L)BK9* (Fig. 23) mutants resemble that of control larvae, two intact, closely apposed PMs often forming false positive defects. Thus, no additional disruption to the PM structure was observed in either of these mutant strains. The PM of control larvae is fully formed and intact (Fig. 24A, 24B and 25A), as is that of the adult stage (Fig. 19D).

Expression of the genes encoding those peritrophins affected by the deficiency could still be demonstrated in deficient flies since the breakpoints within the 68E3-4 interval result from a frameshift mutation, rather than a deletion, thus the gene loci of the putative peritrophins are present in the genome of deficient strains. However, the genes within the range of the deficiency encode transcripts that should give rise to mature peptides, i.e. peritrophins with a lack of function.

The range of RNAi strategies utilized in this study failed to generate a (detectable ?) PM-compromised phenotype when examined by T.E.M., despite the silencing of various putative peritrophin transcripts.

3.5. Discussion

Previous studies have shown the detrimental effects on the insect of PM disruption through inhibitors of chitin synthesis (Derksen and Granados, 1988; Harper *et al.*, 1998; Wang and Granados, 2000; Pechan *et al.*, 2002; Okuno *et al.*, 2003) (see Chapter 1, section 1.8.1. for details). However, the exact nature of the delicate interaction between the components of the PM remains unclear. PM chitin binding proteins (peritrophins) may prove to be as vital for maintaining the structural integrity of the PM as the chitin component. Peritrophins, as integral components of the PM are tightly associated with the PM (Lehane, 1997) and are reported to

account for up to 55% of the total mass of the PM in most insects (Peters, 1992) implying that they should make an important contribution to the structure and function of the PM. As shown in Fig. 2 and 39, the peritrophins stabilize the chitin fibril network of the PM by forming tight associations with chitin via CBDs.

Ultrastructural studies showed that mutant larvae have an imperfect PM (Fig. 21) when compared to the intact PM of control larvae. There are many gross structural abnormalities in the PM of *Df(3L)vin3* mutants, however they are localised, the majority being characterised by partial loss of PM thickness. In contrast, no PM structural defects were detected in *Df(3L)vin10* and *Df(3L)BK9* mutants when examined by electron microscopy. These results suggest that a null phenotype may only result from the lack of function of multiple peritrophins. This indicates that individual PM components are not absolutely essential to maintain PM integrity and that there is a considerable degree of functional redundancy, so that it is necessary for the loss-of-function of between three and eight larval peritrophins to generate a functional defect in the PM. Moreover, the electron dense layer remains intact, suggesting that the peritrophins disrupted are found predominantly within the electron lucent layers. A null-phenotype was observed in the mutant strain deficient for nine peritrophins (of which eight are larval specific transcripts, which explains why PM structural defects are only in larval, not adult stages of development), being the separation of the single larval PM into two layers (Fig. 21A) compared to control larvae that have an intact PM (Fig. 24A, 24B and 25A). Larval Diptera typically secrete a single PM with a simple electron dense layer on the lumen side, which is believed to be rich in mucins. The total thickness of the PM in *Drosophila* larvae is recorded as 110-130 nm, including an electron-dense layer of approximately 12 nm. While adult *Drosophila* possess two PMs, PM1 being 200 nm, including a 23 nm electron dense layer, and a 45 nm thick PM2 (the PM nearest the gut lumen is denoted as PM1) (Peters, 1976). This mutant phenotype suggests that the single larval PM can be easily separated into two membranes that are normally closely apposed, so that their separation causes the PM to form a double PM that closely resembles that of adults. Furthermore, the adult cardia forms a single multilaminar PM that becomes cleaved distally into PM1, composed of layers 1-3 and PM2, formed by layer 4 only (King, 1989). This suggests that the adult PM lacks the peritrophins present within the larval PM that are necessary to bind layer 3 and 4 together. This may be supported by the detection characterisation of far fewer adult peritrophins compared to larvae (Table 8 and Fig. 25). If this is the case, it would provide a simple means to account for the presence of two separate PMs in other insects that posses a single formation zone, effectively modulating the PM at different stages of insect development by the simple induction or loss of expression of genes encoding key peritrophins.

The results from this null phenotype study, coupled with the ISH localisation of gene expression within the cardia (Appendix) indicate that the peritrophins encoded by the genes of interest are widespread throughout and embedded within the PM 2 of *Drosophila*. This implies that, like most peritrophins they have a structural, rather than protective role in the PM. Conversely, mucins are thought to protect and lubricate the PM, a role which is inferred by their location within the PM, since their large mucin like domains project into the gut lumen, whilst the rest of the molecule is anchored to the PM via CBDs. The limited thickness of the PM imposes a challenge for the PM to perform its role in protection against mechanical, chemical and biological damage. This implies that the PM structure must be ideally suited to meet these demands with the most efficient use of structural components (Fig. 39). The cysteine residues within the CBDs of peritrophins and mucins are thought to facilitate inter-molecular interactions such as those involved in mucin multimerization (Gum *et al.*, 1992; Perez-Vilar and Hill, 1999). It has also been proposed that CBDs form intra-molecular disulphide bridges that are believed to enhance stability of the tertiary structure of peritrophins and mucins in the highly proteolytic environment of the insect gut (Wang and Granados, 1997a and 1997b). Peritrophins are a group of proteins that have been shown to bind chitin, thus contributing to PM structure by cross-linking of chitin fibrils (Shen and Jacobs-Lorena, 1999; Tellam *et al.*, 1999). Thus, it may be inferred that peritrophins containing multiple CBDs (such as *DmPro2*) may have a greater impact on PM structural integrity than those that contain only two CBDs, e.g. *DmPro1*. It would be possible to determine if this is the case by carrying out a systematic gain-of-function (Rorth *et al.*, 1998) study to restore gene function to each of the deficient genes in the *Df(3L) vin3* mutant strain. However, it may be difficult to predict which of the eight peritrophins have the greatest effect on PM structural integrity since this theory assumes that peritrophins are incorporated into the PM as intact proteins. Recently Wang (2004) proposed that high molecular weight peritrophins that are composed of multiple CBDs may be partially degraded into smaller fragments prior to or following their incorporation into the nascent PM, which suggests that peritrophins may have equally long fragments composed of similar numbers of CBDs within the mature PM.

The only PM-compromised phenotype observed in this study (Fig. 21A and 21B) is minor in comparison to the disruption observed in cyclohexamide treated *C. erythrocephala* larvae. Cyclohexamide is an inhibitor of protein synthesis that has been shown to affect both peritrophin and chitin synthase production, which could result in the eventual complete abolishment of PM secretion. Indicating that despite the deficiency for eight peritrophins, there is enough functional redundancy within peritrophin families to stabilize PM structure in the *Df(3L)vin3* mutants, since chitin synthesis is not affected and unaffected peritrophins are still

expressed. Whether PM molecular structure is modulated to compensate for the loss of so many components remains to be studied. The levels of expression of the genes encoding the remaining peritrophins could be measured by Northern blot or Quantitative RT-PCR analysis. Unfortunately, time prevented us from completing these studies.

In contrast to the grossly altered PM structure observed in flies deficient for eight larval peritrophins, there is no evidence for weakened structural integrity of the PM as a result of disruption to individual peritrophins. The generation of a stable hairpin construct was obtainable in pCaSpeR only after numerous unsuccessful attempts. The constructs hpC4 and hpC25 were thoroughly sequenced to verify the orientation of the inserts and the sequence specificity. Nonetheless, a disrupted PM was not detected in fly lines harbouring the pCaSpeR-IR constructs, since the IR constructs were too unstable in the fly lines, resulting in the loss of the IR before balanced stocks could be set up and an RNAi phenotype detected (Lehane and O'Brochta, pers. communication). Thus, pWIZ was used to create IRs, since this improved construct had been specifically developed to efficiently stably express dsRNA in *Drosophila* from a snap-back IR (Lee and Carthew, 2003). However, cloning of an IR was even more problematic using the pWIZ vector, since the gene specific portion of the IR was identical to those incorporated into pCaSpeR, so that the apparent instability of such constructs must be due to the shorter hairpin loop. Surprisingly, the use of a strain of *E. coli* with a recombination deficient phenotype (SURE cells, Stratagene) to propagate the double recombinant clones necessary to create the IR were also repeatedly unsuccessful. The failure of cloning with a bacteria strain defective in *recBC* and *sbcBC* indicates that they were unable to stabilize the secondary structure. The IR scheme of obtaining loss-of-function phenotypes is still far from becoming routine, since this procedure has a number of pitfalls that doom many attempts to failure, including those detailed above. Since the pCaSpeR constructs only targeted *DmPro1* and *DmPro3*, both of which contain just two CBDs, it could be predicted that the PM phenotype that would have been observed may not have been detectable, since *Df(3L)vin10* and *Df(3L)BK9* did not have a null phenotype with a deficiency uncovering two and three larval peritrophins respectively.

Similarly, the injection of dsRNA corresponding to individual peritrophins did not result in a detectable null phenotype, despite a 50-75% reduction in mRNA levels. Possibly due to the limited impact of the loss of a sole peritrophin, when functional redundancy can apparently lead to a PM phenotype that is no different from controls in mutants deficient for 2 larval peritrophins. This suggests that a threshold exists for such loss-of-function phenotypes that require numerous genes to be knocked down in order to achieve a changed phenotype. In conclusion, whilst RNAi is an ideal method for some applications it may not be suitable for those genes that are part of large multigene families, where redundancy is prevalent or those genes that

are transcribed continuously, such as those encoding structural proteins. Insect PMs that are composed of fewer peritrophins may also be more tractable to RNAi, even when redundancy is a factor. Schmid (2002) developed a co-injection approach can be used to simultaneously target up to four genes at a time, thereby overcoming functional redundancy within the neuron receptor gene family RPTP and improving previously weak null phenotypes. However, without modifications to the composition of injection buffer combinational RNAi is restricted to the co-injection of only four dsRNAs due to constraints on the total concentration of dissolved RNA (~6 mg ml^{-1} RNA) within the injection buffer before the crystallization of dsRNA within injection needles. It has been found that the RNAi inducing mechanism does not require perfect homology between the siRNA trigger and the target mRNA, as it is estimated that 60% homology still initiates a response. This implies that additional genes that are sufficiently homologous to the target may be silenced due to cross-talk between genes. However, *Drosophila* peritrophins do not possess sufficient sequence homology (except P-15a and -b) to allow the use of such leaky RNAi to target the whole peritrophin gene family at once. Thus, perhaps the only means of silencing all larval and adult peritrophins at once, albeit transiently (not stably) would involve the delivery of dsRNAs directly into the cardia and the rest of the insect gut via the ingestion of bacterially expressed dsRNA, as has been shown in *C. elegans* (Timmons *et al.*, 2001).

In conclusion, the difficulties encountered during this investigation may be due, in part, to the phenotype being difficult to detect using T.E.M. Thus, it would be desirable to also perform Scanning Electron Microscopy (S.E.M.) on deficient and wild type larvae and adults in order to discern if the findings of this study could be supported (and/ even refuted?) by further electron microscope evidence.

Chapter 4. Comparison of the fitness of peritrophic matrix-compromised and wild-type strains of *Drosophila melanogaster*

4.1. Abstract

The objective of this study was to determine the effect of the abnormal PM phenotype of deficient flies documented in Chapter 4 on the fitness of larvae and adults when exposed to pathogenic bacteria. The results suggest that mortality is correlated with increased permeability of the PM to bacteria and their extracellular products, and thus access to the haemolymph, since deficient larvae are more susceptible to *Serratia marcescens*. The most common route of entry of bacteria and viruses into an insect host is *per* os, the midgut epithelium being the primary site of infection. It has been proposed that the PM serves to protect *Musca domestica* from invasion by bacteria via the oral route. Semi-aseptically reared adults and larvae of wild-type and PM-compromised isogenic lines were exposed to three strains of *Serratia*, and the number of infected dead individuals assessed twice daily for 5 d. Mortality was three to four times higher (p 0.001) in PM-compromised larvae that had fed on *S. marcescens* compared with controls.

4.2. Introduction

The PM-compromised strain of *D. melanogaster* used in this study are deficient for eight separate PM chitin binding proteins (peritrophins) specifically expressed in larvae: *DmPro10*, CG5897, CG6947, CG7248, *DmPro4*, CG9781, CG17824 and *DmPro2* (the latter is also expressed at low levels in adults). All eight genes are located within the 68E3-4 locus of the left arm of the third chromosome, uncovered by the deficiency *Df(3L)vin3* (Fig. 15). Although the PM of PM-compromised larvae was found to be intact, careful examination of the PM using Transmission Electron Microscopy (T.E.M.) revealed numerous defects, manifested as partial thickness breaks (Fig. 21). By contrast, adults of this strain secrete a PM that is not any different morphologically from that of wild-type flies. Thus, they may act as additional controls in survival analyses, serving to verify that larvae are the only stage to be affected by the null phenotype.

In their natural temperate forest environment *Drosophila melanogaster* oviposit on rotting fruit and other vegetation, with larvae completing their development on this substrate. Larvae feed on yeast and other microbes (Gilbert, 1980; Begon, 1982) that commonly colonize such rotting material, in addition to the cadavers of their conspecifics. Thus, *Drosophila* larvae are exposed *per os* to natural infection in the field.

Several species of bacteria have been cited as insect pathogens, for example *Serratia marcescens*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Bacillus thuringiensis* and *B. subtilis*. The genus *Serratia* (Enterobacteriaceae) is commonly found in association with insects. *S.*

marcescens is a gram-negative bacterium that causes disease in both plants and a variety of invertebrate and vertebrate hosts (Grimont and Grimont, 1978). *S. marcescens* biotype A2a is most frequently isolated from insects, perhaps in part at least because it is a distinctive red-pigmented isolate (Grimont *et al.*, 1979; Bollet *et al.*, 1988). *S. marcescens* is capable of killing *Caenorhabditis elegans* following colonization of the gut (Mallo *et al.*, 2002). The isolation of *Serratia* from Coleoptera and Lepidoptera and Diptera is well documented. *S. marcescens* has been isolated from a range of Diptera, including: *Musca domestica*, *Drosophila* and *Dacus dorsalis* by Steinhaus (1959), *Anastrepha ludens* (Kuzina *et al.*, 2001), *Stomoxys calcitrans* (Watson and Petersen, 1991) and *Glossina morsitans morsitans* (Poinar *et al.*, 1979). *S. marcescens* has been regarded as a purely facultative pathogen (Steinhaus, 1959). Recent studies, involving extensive biosassays, have shown that various strains of *S. marcescens* are pathogenic when fed to flies. O'Callaghan *et al.* (1996) documented that *per os* infection of adult blowflies, *Lucilia sericata* with pathogenic strains of *S. marcescens* caused significant mortality.

This study reports on the bioassay screen of three *Serratia* isolates against *D. melanogaster* adults and larvae from both the control and deficient strains. Ingestion of a single dose of each bacterial isolate, and monitoring mortality over five days post ingestion of the bacteria will allow the determination of the physiological effects of a disrupted PM in the PM-compromised fly strain. Adults were held individually in eppendorfs and fed standard fly food coated in bacterial suspensions containing approximately 1x 10^8 bacteria ml^{-1} over a 24 h feeding period; larvae will be fed a semi-liquid fly food and bacterial mixture at ~1x 10^8 cells ml^{-1} for 1 h, before being individually transferred onto fresh fly food.

4.3. Materials and Methods

4.3.1. Experimental Stocks

Survival curves were obtained using the *Df (3L) vin3* deficiency stock of *D. melanogaster* detailed in Chapter 4, while flies with two *y w* chromosomes were used as a wild-type control. Homozygotes for the mutant and control chromosomes were used as the PM-compromised (deficient) fly strain. The two fly strains studied were maintained as "isogenetic" (isofemale) stocks.

4.3.2. Bacterial Strains

Adult and larval *D. melanogaster* were tested for susceptibility to 2 strains of *S. marcescens*, 1 strain of *S. entomophila* and no bacteria (control) in a series of laboratory bioassays. The bacterial cultures were originally isolated from the following insects: House fly, *Musca*

domestica (Diptera: Muscidae); the Tortoise beetle, *Paropsis charybdis* (Coleoptera: Chrysomelidae); and the New Zealand grassgrub, *Costelytra zealandica* (Coleoptera: Scarabaeidae) (Grimont *et al.*, 1979) (Table 15). These particular bacterial strains, chosen for use in the bioassay, are known insect pathogens that have been shown to cause mortality in healthy Dipteran insects upon ingestion. A number of other bacteria spp. were used in preliminary *Drosophila* survival analysis experiments, but were found to have only a limited effect on survival of wild type and deficient flies, in part due to the strains not being dipteran pathogens. These included *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Proteus* spp, supplied by the UK National Culture Collection (UKNCC).

Table 15. Origin of *Serratia* strains used in the Bioassays

Bacteria	Isolate [δ]	Biotype [†]	Host [‡]	Original Source [§]
S. *marcescens*	257	A2a	*Musca domestica*	Steinhaus
S. marcescens	363	A2a	*Paropsis charybdis*	AgResearch
S. entomophila	626	A1	*Costelytra zealandica*	AgResearch

Serratia isolates were kindly supplied by Trevor Jackson (AgResearch Microbial Control/ Insect Pathogen Culture Collection, Lincoln, New Zealand).
[δ] Isolate, denotes the AGR isolate number.
[†] Biotype identified using the biochemical typing system of O'Callaghan and Jackson (1993).
[‡] The species of insect host from which each strain was originally isolated.
[§] The source of the strain obtained.

4.3.3. Preparation of Inoculum

Bacterial suspensions of each culture were produced by aseptically inoculating single colonies of each strain into 10 ml of sterile Luria Bertani (LB) broth and incubating at 30 °C for 24 hr, followed by the enumeration of the number of colony forming units (CFUs) by dilution plating on LB agar. The concentration, checked by measuring A_{600} of the cultures, was typically ~ 3 x 10^8 CFUs (bacteria) ml^{-1}. Bacterial suspensions were prepared in sterile LB broth to a final concentration of ~ 1 x 10^8 CFU ml^{-1}, i.e. viable bacteria, ready for oral inoculation. All three isolates were derived from the original supplied wild type isolate, in contrast to some bioassys that use isolates that are derived from the wild type strain that has also undergone a single passage through the host insect (reisolation from the carcass of a host insect that has died following ingestion of the original wild isolate) prior to use.

4.3.4. Fitness Studies (Bioassays)

A series of four experiments were conducted to determine the pathogenicity of these 3 strains of *Serratia* to *Drosophila* adults and larvae. Each bioassay was independently performed on 50

individuals in triplicate. A number of parameters can influence infection rates, including the temperature, health of the stock and crowding stress. Thus, the protocol of Steinhaus (1959) recently modified by Tzou (2002) was adopted to avoid such effects. In brief, all *Serratia* infections studied thus far in insects have established that they are transmitted by an oral-faecal route (McGhee and Cosgrove, 1980), it is for this reason that flies and larvae used in the bioassays were cultured individually, and transferred regularly to new housing and food within a laminar flow hood.

A total of 150 flies from both fly strains were treated with each bacterial strain, taking care to use equal numbers of male and female flies. Prior to each assay, a batch of 200 newly eclosed adults (collected over 24 h) from each stock were individually placed into eppendorfs (in which two air holes had been made in the base and a 20 x 5 mm piece of filter paper inserted to regulate humidity) and provided with a 3 mm^3 cube of fly food (minus fresh yeast) had been placed ontop of sterile LB agar, that had been pipetted aseptically into the cap. The eppendorfs were held at 25 °C, horizontally in racks for 24 h. 150 actively feeding two-day-old flies that had not inoculated the sterile LB agar with bacteria were selected for the subsequent assay. No attempt was made to surface sterilize the flies to be used in the bioassay, thus bacterial inoculation of the test LB agar could only have originated from the specimen. Adults were individually transferred into new eppendorfs and dehydrated for ~3 h in the absence of fly food, and then treated by replacing the dry cap with one containing a 3 mm^3 cube of fly food pre-treated with 15 µl of 5% sucrose/ bacteria (~ 1 x 10^8 CFU ml^{-1}) inoculum solution at time zero. Untreated controls were dosed with sterile LB broth only by pipetting 15 µl onto the top of a cube of fly food. LB broth, unlike distilled water, will support the growth of bacteria already present in the fly food. All assays were conducted with specimens individually confined and incubated at 25 °C with 16h:8h day:night cycle. The racks of flies fed bacteria were placed adjacent to each other without touching; being isolated from the control flies by being located at least 1.5 m apart in the same room. Following 24 h exposure to the treatments, to ensure individuals ingested sufficient food for delivery of bacteria, flies were transferred to fresh eppendorfs and provided with a fresh uninoculated cube of fly food every 2 days to minimize the effect of regurgitated bacteria on the assay results.

Larvae were harvested from the control and deficient stock using inverted pint plastic beakers secured to petri dishes containing apple juice-agar medium as an oviposition substrate (yeast paste was not used to stimulate oviposition). The humidity of the beakers was regulated by holes ~2 cm in diameter, plugged using small foam bungs. These holes also served as hatches for the introduction of ~25 adult male and female flies from the fly stock. Following a one day oviposition period, the parental flies were discarded and the first instar larvae collected from the

petri dish. Larvae used in the bioassys were thus of approximately the same age (hours post-hatching) at the beginning of the experiment. Natural infections of larvae were performed by placing approximately 100 larvae into a 1.5 ml eppendorf containing a mixture of 200 µl overnight bacterial culture (~ 3 x 10^8 CFUs ml^{-1}) and 400 µl of semi-liquid, autoclaved fly food. The larvae, bacteria and fly food suspension was thoroughly mixed within closed tubes, lacking air holes to prevent contaminated larvae from escaping. The infections were set up in series and allowed to take place for 30 min at 25 °C; the mixture was then transferred to a vial of standard fly food (minus fresh yeast), and incubated at 25 °C for a further 24 h. After which individual larvae could be retrieved, briefly washed in 70% EtOH, rinsed in sterile distilled water and placed individually on fresh fly food within eppendorfs as in the adult set-up. Larvae were transferred to fresh eppendorfs and provided with a cap filled with fresh, autoclaved, uninoculated fly food, which was replaced every 2 days. The larvae were considered to be alive if they responded to touch by moving, even if they had not developed significantly.

All treatments consisted of 50 individuals, assayed in triplicate, and cumulative mortality recorded. The viability of the flies and larvae was assessed, twice daily at 22 °C over a period of 5 d, after which time the experiment was terminated. The mortality rate of larvae was corrected for pupation. Single adults and larvae were removed upon death and the entire alimentary tract, including PM with luminal contents was dissected out in PBS on slides so that it could be immediately examined as live, wet preparations for the presence of bacteria by phase microscopy.

4.3.5. Statistical Analysis

Mortality data was analysed using Statistica, release 5.5 (StatSoft, Inc., Tulsa, U.S.A., available at: http://www.statsoft.com). Initial analyses were conducted using basic statistics (Table 17). Mortality data were statistically analyzed separately for each fly strain, life stage and bacterial treatment using multivariant (three way) ANOVA and *post hoc* analysis (Table 16). The data in each group were subjected to tests for normality (K-S and Lillifers test for normality) and homogeneity of variances (Levene's test) to confirm that the data obeyed the two basic assumptions of ANOVA prior to the analysis. The Tukey honest significant difference (HSD) test was employed for *post hoc* analysis of the data.

The results of this analysis were confirmed by further analyses using the parametric models: Weibull survival distribution and Cox's proportional hazards regression (Cox, 1972; Cox and Oakes, 1984) (Table 18), using the expression: $p(t) = \exp (- (\alpha t)^{\beta})$, the parameters being: t, time; α, the scale parameter (shape of the distribution); β, the intercept (rate of death). The factors were defined using dummy variables to describe: the variance of strain within adults

(wild type or mutant) where D1 = 0 if adults tested were wild type or D1 = 1 if the adults were deficient; the variance between life-stage of the insects (larvae or adults) where D2 = 0 if they were larvae or D2 = 1 if they were adults; the variance between the strain of larvae (wild type or mutant) where D3 = 0 if larvae were wild type or D3 = 1 if the larvae were deficient. In addition the results were categorized into four groups, depending on the bacterial isolate they had been fed (AGR 257, 363 and 626 or non = control) where v3 = 0 if the sterile LB contained no bacteria, v3 = 1 if the bacteria were *S. marcescens* AGR 257, v3 = 2 if the bacteria isolate was *S. marcescens* AGR 363 and v3 = 3 if the bacterial strain was *S. entomophila* AGR 626.

Logarithmic regression analysis was used as an approximation technique to take into account the shape of the entire mortality curve and so accurately determine the parameter values required for Probit analysis. The regression analysis was conducted using a user specified, nonlinear estimation, with the loss function: $Mt = Mm \cdot (1 - e^{-kt})^{n}$, where Mt, lethal time; Mm, maximum mortality; e, exponential; k, constant; t, time; and n, to the power n). The graphs in Fig. 26 also provided a more precise graphical representation of the data in the form of a mortality curve, when compared to traditional survival box plot diagrams. The LT_{25} (lethal time for 25 % mortality) was calculated for each treatment based on the regression model, since LT_{50} could not be used as a measure for all the groups in the study as some treatment groups did not reach 50 % mortality by the end of the experiment.

4.4. Results

According to *post hoc* analysis (Table 16) the mortality of deficient larvae fed *S. marcescens* 257 was significantly greater than both wild-type larvae fed the same isolate ($p = 0.0002$) and control deficient larvae ($p = 0.0002$). Conversely, there was no significant difference in the mortality of wild type larvae and wild type and deficient adults when compared to controls ($p = 0.14$). The *S. marcescens* strain 363 caused both deficient larvae and adults to die significantly ($p = 0.0002$) more rapidly than untreated, control flies. Moreover, the analysis showed that there was a significant difference in the susceptibility of wild-type and deficient larvae ($p = 0.0003$) treated with *S. marcescens* 363. There was no significant difference in mortality caused by *S. marcescens* 363 between wild type and deficient adults ($p \geq 0.99$). Similarly, there was no difference in susceptibility of wild type and deficient adults treated with *S. entomophila* 626 ($p \geq 0.99$). Furthermore, there is no significant difference in mortality between wild type and deficient larvae fed *S. entomophila* 626 ($p = 0.71$) or any group treated with *S. entomophila* 626 in comparison to controls ($p \geq 0.57$), except deficient larvae for which there was significantly less mortality in comparison to the control group ($p = 0.003$). Adults and larvae of both strains

showed no difference in susceptibility to LB treatment in control assays ($p \geq 0.99$). Overall, bacterial treatments incur significant mortality for larvae only. The results were confirmed by further statistical analyses using Cox's proportional hazards model.

Table 16. Comparisons with an indication of significance levels for survival at 97 h using ANOVA and *post hoc* analysis

Treatment	Stage	Strain	Mean % M_{97} †	Case	Relevant Pairwise comparisons ‡
Control	Ad	Wt	40.00	a1	
		Df	41.34	a2	
	La	Wt	39.34	a3	
		Df	38.66	a4	1. all within a : $p \geq 0.99$
Sm 257	Ad	Wt	30.00	b1	2. b1 vs b2 : $p = 0.43$
		Df	38.00	b2	**3. b3 vs b4 : $p = 0.0002$**
	La	Wt	49.34	b3	4. b1 vs a1 / b2 vs a2 / b3 vs a3 : $p \geq 0.14$
		Df	69.34	b4	**5. b4 vs a4 : $p = 0.0002$**
Sm 363	Ad	Wt	62.66	c1	6. c1 vs c2 : $p \geq 0.99$
		Df	64.66	c2	**7. c3 vs c4 : $p = 0.0003$**
	La	Wt	57.34	c3	**8. c1 vs a1 / c2 vs a2 / c4 vs a4 : $p = 0.0002$**
		Df	75.34	c4	**9. c3 vs a3 : $p = 0.0003$**
Se 626	Ad	Wt	44.00	d1	10. d1 vs d2 : $p \geq 0.99$
		Df	46.00	d2	11. d3 vs d4 : $p = 0.71$
	La	Wt	24.66	d3	12. d1 vs a1 / d2 vs a2 / d4 vs a4 : $p \geq 0.57$
		Df	31.34	d4	13. d3 vs a3 : $p = 0.003$

† Mean % mortality at t = 97 h; based on 3 replicates counting 50 individuals each
‡ Based on *post hoc* analysis (Tukey honest significant difference test) upon 3-way ANOVA (treatment + stage + strain); significance levels (p-values) indicated in bold if $p < 0.05$. Relevant pairwise comparisons are given; the remainder are not relevant to this analysis.

The results of statistical analysis using Cox's proportional hazards model corroborates those obtained from the aforementioned *post hoc* analysis, in that it suggests that the *S. marcescens* strains (257 and 363) caused deficient larvae and adults to die significantly ($p \leq 0.003$) more rapidly than untreated, control flies. Within the assays, flies treated with pathogenic *S. marcescens* strains had an LT_{25} ranged from 7 to 56 h (Table 17). The non pathogenic *S. entomophila* strain 626 produced LT_{25} of 42 to 105 h, not significantly different from control LT_{25} of 43 to 75 h. Moreover, deficient larvae were three to four times more susceptible to *S. marcescens* 257 and 363 than wild type larvae. Statistical analysis of the bioassay results showed there was a significant difference in the susceptibility of wild-type and deficient larvae ($p = 0.001$) treated with *S. marcescens* 257. There was no significant difference in mortality of wild-type and deficient adults fed *S. marcescens* 257 ($p = 0.09$). There was no significant difference in mortality caused by *S. marcescens* 363 between wild type and deficient

Fig. 26. Cumulative mortality (%) data with regression analysis of first instar and adult *D. melanogaster* infected *per os* with three strains of *Serratia* and LB control. Note: 150 individuals per treatment, time (h) after feeding. Abbreviations: *Sm*, *S. marcescens*; *Se*, *S. entomophila*; 257, AGR strain 257; 363, AGR strain 363; 626, AGR isolate 626; Df, deficient flies; Wt, wild-type flies; Ad, adults; La, larvae.

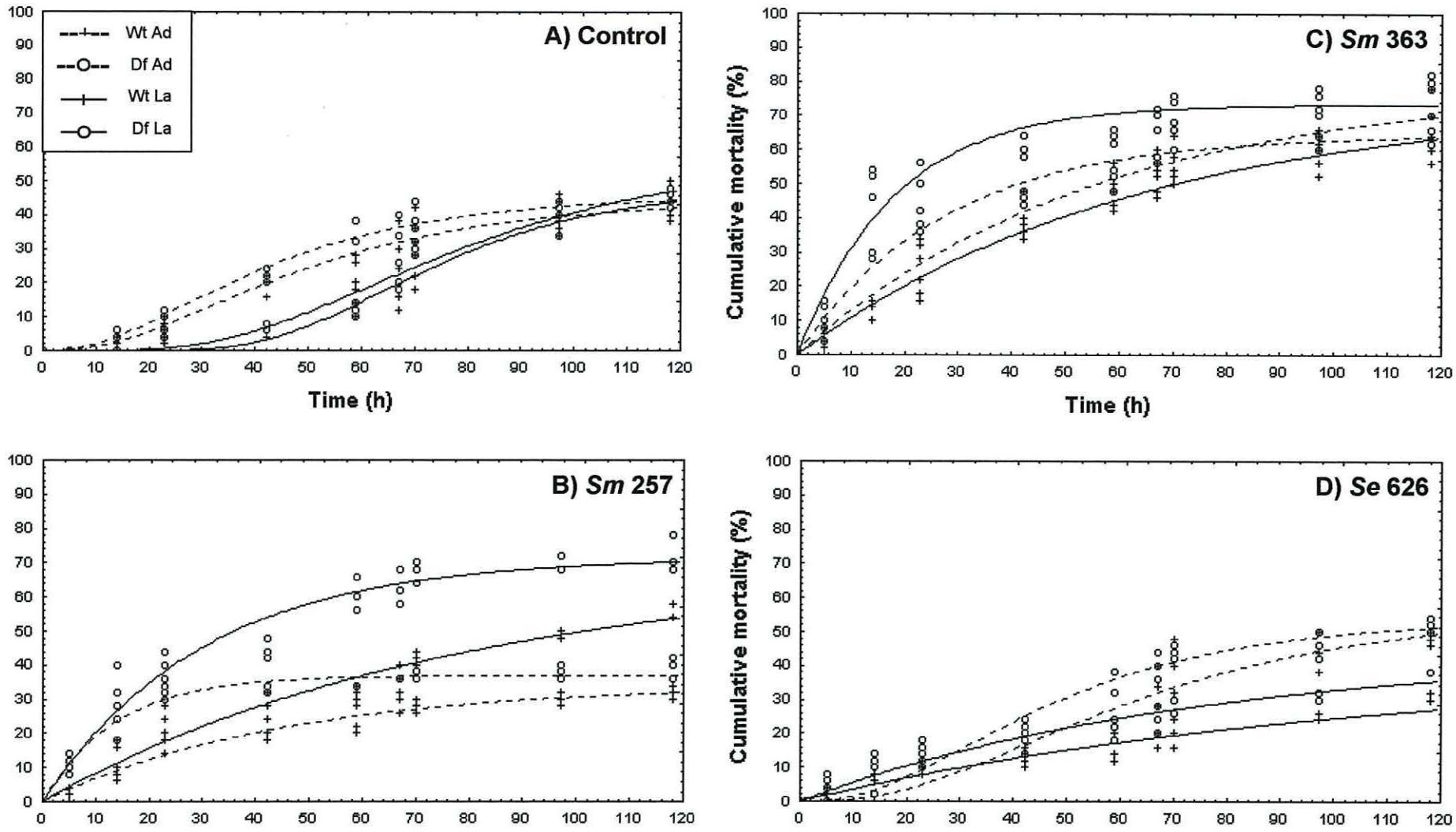


Table 17. Comparative overview of the main statistics.

Treatment	Stage	Strain	LT_{25} (h)	Mean survival time (h) ± SE	No. Surveyed	No. Censored	Mm (%)	k	n	R value
Control	Ad	Wt	53	86.1 ± 3.4	150	89	44	0.003	2.8	0.98
		Df	43	82.1 ± 3.6	150	86	46	0.037	2.6	0.991
	La	Wt	75	93.0 ± 2.6	150	84	48	0.042	14.5	0.975
		Df	73	91.2 ± 2.7	150	80	57	0.029	6.2	0.978
Sm 257	Ad	Wt	34	90.2 ± 3.6	150	102	34	0.022	1	0.977
		Df	15	77.9 ± 4.3	150	91	37	0.072	1	0.983
	La	Wt	56	74.6 ± 3.8	150	67	68	0.013	1	0.975
		Df	12	51.1 ± 3.9	150	42	72	0.033	1	0.977
Sm 363	Ad	Wt	20	59.5 ± 3.7	150	44	79	0.018	1	0.983
		Df	13	56.0 ± 4.0	150	57	64	0.036	1	0.983
	La	Wt	25	66.2 ± 3.8	150	51	75	0.015	1	0.985
		Df	7	40.9 ± 3.8	150	39	73	0.055	1	0.969
Se 626	Ad	Wt	42	85.2 ± 3.1	150	78	56	0.027	3.2	0.065
		Df	54	78.6 ± 3.5	150	103	54	0.035	2.9	0.991
	La	Wt	105	97.1 ± 3.0	150	72	40	0.0095	1	0.958
		Df	60	89.1 ± 3.5	150	93	45	0.013	1	0.956

Note: LT_{25}, lethal time to 25 % cumulative mortality (in hours) and mean survival time (in hours). The regression approximation of cumulative mortality (Mt, in %) as a function of time (t, in hours). General function: $Mt = Mm \cdot (1 - e^{-k \cdot t})^{n}$, where Mm, maximum mortality; k, slope of curve; and n, shape of curve.

Table 18. Results of Cox's Proportional Hazards Model.

Treatment	Dummy Variable	Chi ^{2}	p	T value	b ± SE	Exponent β	Wald Statistic	p	Sig.
Control	D1	1.37	0.7127	0.698	0.124 ± 0.177	1.132	0.488	0.485	NS
	D2			-0.426	0.076 ± 0.179	0.927	0.182	0.670	NS
	D3			0.258	0.045 ± 0.175	1.046	0.067	0.796	NS
Sm 257	D1	51.34	0.0000	1.709	0.332 ± 0.194	1.394	2.919	0.088	NS
	D2			3.482	0.631 ± 0.181	1.881	12.126	0.001	***
	D3			6.522	1.136 ± 0.174	3.114	42.539	0.000	***
Sm 363	D1	18.12	0.0004	0.237	0.033 ± 0.140	1.034	0.056	0.813	NS
	D2			-1.279	0.182 ± 0.142	0.834	1.635	0.201	NS
	D3			2.938	0.393 ± 0.134	1.481	8.630	0.003	**
Se 626	D1	16.01	0.0011	0.931	0.152 ± 0.164	1.165	0.867	0.352	NS
	D2			-2.791	0.524 ± 0.187	0.592	7.787	0.005	**
	D3			-1.432	0.245 ± 0.178	0.776	2.051	0.152	NS

General function $p(t) = \exp(-(\alpha t)^{\beta})$ where the parameters are: t, time; α, the scale parameter (shape of the distribution); β, the intercept (rate of death). Abbreviations: *Sm*, *S. marcescens*; *Se*, *S. entomophila*; NS, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Three dummy variables were created in order to distinguish between the four classes of flies tested during statistical analysis: D1 = variance between wild type and deficient adults; D2 = variance between adults and larvae; D3 = variance between wild type and deficient larvae.

adults. Similarly, there was no difference in susceptibility of wild type and deficient adults treated with *S. entomophila* in the susceptibility of wild type and deficient larvae when exposed to *S. marcescens* 363. No significant difference in mortality was found in cases where wild type and deficient larvae were fed *S. entomophila* 626. Adults and larvae of both strains showed no difference in susceptibility to LB treatment in control assays ($p \geq 0.5$).

4.5. Discussion

This study provides the first direct evidence in support of a protective function for the PM. Mortality was three to four times greater in deficient larvae compared to wild-type larvae ($p \leq 0.005$) when infected *per os* with S. *marcescens* 257 or 363 (Table 16 and 18). No significant difference in mortality occurred between larvae infected with 626 and the controls ($p \geq 0.99$). The majority of data from bioassays in which flies were fed either only LB (control) or the non pathogenic strain of *Serratia*, 626 assumed a sigmoidal regression line (Fig. 26). Whereas data fitted from bioassays involving pathogenic *Serratia* strains produced logarithmic regression lines. This reflects the general effect of feeding pathogenic and non pathogenic bacteria to flies, the former resulting in higher mortality, particularly early during survival analysis. Sick, experimentally infected larvae presented symptoms characteristic of bacterial infection (septicaemia); they became flaccid, lethargic, with a marked loss of appetite (personal observation).

In nature, *Drosophila* larvae and adults feed on bacteria, some of which are destroyed and digested, but others persist, forming either the midgut microbiota or pathogens. The pathogenicity of *Serratia* varies greatly among specific species and strains of bacteria (Huber *et al.*, 1991; Suzuki *et al.*, 1998) and insect hosts. *Serratia* spp. are known bacterial pathogens of most insects, however *S. entomophila* 626 produced similar adult mortality to those in the control group, for both wild-type and deficient adults ($p \geq 0.57$). Moreover, 626 appears to enhance survival of both wild type and deficient larvae in comparison to controls (Fig. 26A and 26D), since survival is significantly enhanced ($p = 0.003$). Other strains of *S. entomophila* have been previously identified as having a positive influence on nutrition (Fitt and O' Brien, 1985; O'Callaghan *et al.*, 1996) and it seems probable this is the effect we are seeing here. Similarly, Lysyk (1999) found that *Stomoxys calcitrans* larvae fail to develop when reared on a bacteria-free diet, often mortality occurring during the first instar, highlighting the need for bacteria as a food source during development. However, this particular insects larva requires mixed bacterial populations to enhance development and reduce mortality, as single strain inoculations of *S. marcescens* and *Aeromonas* sp. produced greater mortality.

However, these results are not conclusive as to the exact nature of the mechanism of *S. marcescens* pathogenicity (agent causing fly mortality), since this was not determined in this study. It could equally have been due to the production of an extracellular toxin rather than pathogenicity of live bacteria. To determine the mode of pathogenicity of *S. marcescens* 257 and 363 it would be necessary to expose flies to either heat-killed bacterial cells or purified culture supernatant (minus live bacterial cells). Provided there is no mortality of treated flies (corrected for control mortality), it could be concluded that pathogenicity is a result of direct infection with viable bacteria as opposed to the action of their extracellular enzymes and toxins, such as β-hemolysins, elastases and chitinases (Lysenko, 1976; Suzuki *et al.*, 1998). In addition, the effect of growth of the bacterial cultures on different growth media can be assessed to determine the effect of such growth substrates on the production of toxins, and thus mortality on feeding. Indeed, Lysyk (2002) found that the mortality of adult *S. calcitrans* was enhanced by growth of the isolate on egg yolk media in comparison to nutrient broth, indicating greater pathogenicity due to the production of toxins. In addition, further work could include more extensive bioassays, carried out to further support the data and particularly refine the LT_{25} results, including testing a range of concentrations of inocula, to produced dose, mortality response curves. In this study the bacteria cultures were used as supplied following passage through LB media, whilst a freshly insect passed culture may have a dramatic effect on mortality rates. *Drosophila* larvae are gregarious feeders, that have been previously shown to be reared successfully on cadavers of conspecifics (Gregg *et al.*, 1990). Thus, providing it would be possible to ensure that only the intended strain of bacteria were involved, cadavers of adult or larval *Drosophila*, killed following ingestion of bacteria strain to be used in the bioassay could be used to more efficiently transmit infection via the *per os* route. A similar protocol has been used recently to expose *Drosophila* larvae to flagellate trypanosomatids (Trypanosomatidae: Kinetoplatida) (Ebbert *et al.*, 2003). Moreover, bacteria that passed through the host insect prior to use in survival analysis may have enhanced pathogenicity in comparison to the wild strain (O'Callaghan *et al.*, 1996). Alternatively, the susceptibility of deficient compared to wild-type larvae and adults could be tested against other biological agents, ranging from the *Bacillus thuringensis* (Bt) toxin to various parasites including *Plasmodium gallinaceum* (Schneider and Shahabuddin, 2000).

Chapter 5. Quantitative Real-Time RT-PCR analysis of peritrophin transcription levels in *D. melanogaster*

5.1 Abstract

PM chitin binding proteins (peritrophins) are believed to play key roles in PM structure and function. Although PMs are constitutively secreted in type 2 PM producing insects, and we hypothesize that genes encoding peritrophins are differentially expressed so that PM production, although constitutive, can be modulated in order to respond to varying challenges. A two-step, RT-PCR approach was used to accurately quantify peritrophin transcripts based on iCycler assisted QPCR in combination with the construction of external cRNA standards, with identical sequence as the target mRNAs. This study reveals some interesting dynamic changes in peritrophin gene expression in response to age, with a two to three fold upregulation in the expression of *DmPro1*, *DmPro2* and CG14645 in response to starvation in comparison to newly emerged flies, whereas no dynamic changes were detected between fed and unfed, age matched flies. Furthermore, the expression profiles of peritrophins in response to bacterial challenge indicate that transcription of *DmPro3* and *DmPro4* is increased in the presence of infection, whilst the majority are not immune sensitive. The significance of these findings is discussed with discussion of the putative roles of these peritrophins in immunity.

5.2. Introduction

The hypothesis is that peritrophins have important roles in PM structure and function. If this is so, then insects that produce a type 2 PM may well differentially regulate peritrophin gene expression in response to infection. To test this hypothesis I will accurately quantify the transcription of peritrophin genes in order to profile their expression in response to feeding and bacterial infection. This study could act as a foundation for other studies in which more specific hypotheses can be tested in order to further investigate the molecular basis of PM structure and function. I have concentrated on the expression of four adult and four larval peritrophins in this study, the sequences tested were selected based on their being a representative sample of the putatitve peritrophins investigated in this thesis.

Reverse-trancription polymerase chain reaction (RT-PCR) has become the gold standard in gene expression analysis, especially of low abundance mRNAs. In comparison to other methods that are currently used to quantify transcription levels, e. g. Northerns (Parker and Barnes, 1999), RNAse Protection Assays (Hod, 1992; Saccomanno *et al.*, 1992), *in-situ* hybridisation (Tautz and Pfeifle, 1989) and cDNA arrays (Yang *et al.*, 1999), RT-PCR is a much more sensitive technique for the analysis of low-abundance mRNA, often obtained from minute or valuable samples. However, due to the exponential nature of the amplification step during

PCR, even small variations in the efficiency of the reaction (that result from problems inherent in PCR) will significantly affect the data. Thus, conditions must be tightly controlled so as to minimise any variation in efficiency during the RT and PCR step.

The introduction of Quantitative, Real-Time RT-PCR has allowed the accurate quantification of starting amounts of mRNA. First demonstrated with the non-specific reporter ethidium bromide over a decade earlier (Higuchi *et al.* 1993), SYBR® Green I is now the reporter of choice (Morrison *et al.*, 1998) for such assays since it fluoresces 50 times stronger than ethidium bromide when bound to dsDNA. The fluorescence of the reporter molecule increases as products accumulate during successive rounds of amplification due to the reporter binding and intercalating to double-stranded DNA, measured in real time during the extension step of the assay (Fig. 27). The threshold cycle (C_T), defined as the fractional cycle number at which the fluorescence rises appreciably above the background (usually 10x the standard deviation of the baseline) is determined and the relative gene expression levels quantified. The linear relationship between log of the starting amount of template and its corresponding threshold cycle during real-time PCR allows the construction of a standard curve by plotting the values for known starting amounts of the nucleic acid. The standard curve can then be used to determine the starting amount for each unknown template based on its threshold cycle. Since intercalating dyes, such as SYBR Green I indiscriminately bind to all doube-stranded DNA products, including primer-dimers and secondary products, the increase in fluorescence may not accurately reflect the sole amplification of the desired amplicon. It is thus necessary to carefully optimise the reaction, since specificity of the PCR dictates the sensitivity and reliability of real-time PCR quantification. This requires the careful choice of primers, target sequence and reaction conditions that will allow the robust and efficient amplification of the target amplicon, in the absence of secondary PCR products and primer dimers. In real-time PCR assays it is important to amplify the target gene at high efficiency for its accurate quantification. An amplification efficiency of between 90% and 100% typically denotes optimal assay results. Proper primer design should ensure that the primers are specific for the target sequence, produce an amplicon of the optimum length for RT, bind at positions that avoid any secondary structure, and minimize primer-dimers. It is possible to quantify initial template concentrations over a dynamic range of six or more orders of magnitude using a well-designed QRT-PCR assay.

Melt curve analysis should be used to confirm the specificity of primers and reveal primer dimers. The analysis is performed immediately following the completion of the amplification protocol with 80-120 cycles of 0.5 °C increments (10 sec each) beginning at 55 °C (data collection step). A melt curve profile is generated by recording the fluorescence in real-time as a function of temperature change. A significant change in the rate of altering fluorescence during

the melt curve reaction, dF/dT, denotes the melting temperature (T_m) of the double-stranded PCR product (Ririe *et al.*, 1997). Primer dimers, being much smaller than the amplicon, usually melt at much lower temperatures, whilst secondary products may melt at a higher or lower

Fig. 27. Schematic diagram of SYBR Green activity in the iCycler assay, during:

A. Denaturation, SYBR Green is unbound since it is released by denaturation of the DNA, causing minimal fluorescence signal (background).
B. Annealing, a few SYBR Green molecules bind to the dsDNA primer/ target hybrid, resulting in some fluorescence emission.
C. Elongation, an increasing number of SYBR Green molecules bind to the minor groove of the newly synthesized DNA double helix, fluorescence is greatly enhanced and the signal monitored in real-time.

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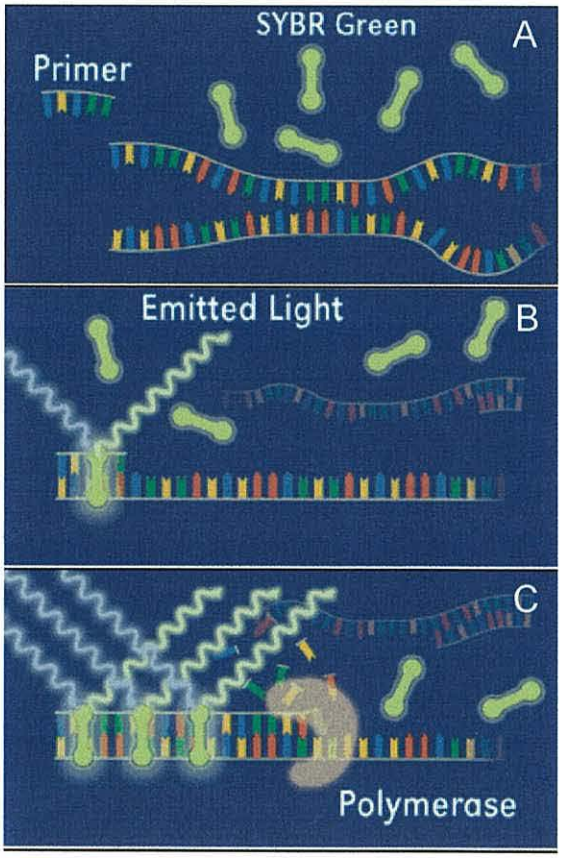


temperature dependent on their length and composition. The common problems encountered during RT PCR assays such as: an amplification efficiency of greater than 100 % (resulting in a poor correlation coefficient), difficulty in resolving the least concentrated dilutions, and amplification in the negative control are usually caused by the generation of more than one product during the reaction. In most instances, the undesired products are primer dimers. The most effective way to avoid primer dimers is to simply redesign the primers, since assays run in the presence of significant primer dimers will yield inefficient and thus unreliable data, despite

taking various measures to reduce their impact on the reaction. However, a single peak in a melt curve does not necessarily mean that a single product was amplified. Since, the T_m of each product is formed from both the length and the GC content of the sequence, resulting in some products that can't be resolved from others by the melt curve alone. The best method to reduce secondary product formation is to optimise the annealing temp for amplification of the desired product, since this will increase primer specificity and so eliminate secondary products.

Usually, relative quantitation of gene expression between treatments is sufficient. In which the expression of the target gene is given as a ratio to that of a housekeeping gene (HKG), before and after experimental treatment. However, RNA probes are required if absolute quantitation of gene expression is necessary. For which synthetic ssRNA probe (identical to the target amplicon) are first synthesized, reverse transcribed, and then co-amplified and detected with the endogenous target gene. The sense RNA is used to create a concentration curve to which the endogenously expressed mRNA is compared, so as to obtain an absolute measurement of the copy number of the transcript under study.

5.3. Materials and Methods

5.3.1. Treatment of Drosophila adults and larvae

Three different sample groups of *Drosophila* were obtained from the wild-type stock (Bloomington number 2057). Fed flies were maintained on standard fly food (minus live yeast) that was autoclaved, but the specimens were not maintained axenically. Prior to setting up infection experiments baseline transcription levels of genes encoding peritrophins were determined for three groups of adults: newly eclosed, termed basal (< 24h post eclosion), and age matched (2 d post eclosion) unfed and fed flies. For infection studies cardia were dissected from 3 d old flies, 2 d post ingestion of either: standard fly medium and LB only, for untreated controls or standard fly medium supplemented with 15 µl of 5% sucrose/ *S. marcescens* strain AGR 363 ~ 1 x 10^8 CFU ml^{-1} (using the protocol described in Chapter 4, section 4.3.3.) in the infected flies. A comparison between expression in first instar larvae, 2 d post ingestion of either: semi-liquid autoclaved fly food in untreated controls or a mixture of 200 µl overnight culture of *S. marcescens* AGR 363 (~ 3 x 10^8 CFUs ml^{-1}) and 400 µl of semi-liquid, autoclaved fly food for infected larvae.

5.3.2. Sample Preparation: Isolation and Purification of RNA Template

Pfaffl and Hageleit (2001) found that high background concentrations of eukaryotic RNA suppress the cDNA synthesis rate up to 6.5 times, with an even greater reduction caused by

insect RNA. Thus, cDNA was synthesized from the RNA derived from 30 cardia (~ 50 ng μl^{-1}) per sample (eight samples per treatment group), which were immediately stored within RNAlater (Ambion) following harvest, prior to RNA extraction. Cardia were pulverised in 350 µl lysis buffer (lysis buffer supplied in Rneasy Kit plus 2-mercaptoethanol diluted 1:100) within individual micro tubes using a micropestle before isolating total RNA from the tissue lysate using RNeasy kit (Qiagen) according to the manufacturer's protocol. The integrity and purity of the RNA was verified by an OD_{260}/OD_{280} nm absorption ratio higher than 1.85/1. To remove genomic DNA contamination, isolated RNA samples were then treated with 40 units DNase 1 (Ambion) for 1 hour at 37°C in the presence of 120U of RNase OUT (Invitrogen) RNase inhibitor.

5.3.3. Preparation of cDNA template for Real-Time RT-PCR

RT reactions were performed according to the manufacturers instructions (SuperScript First-strand Synthesis System for RT-PCR, Invitrogen). First strand cDNA was synthesised from constant amounts of total RNA (50 ng μl^{-1}) in each RT reaction: 1 µl random hexamers (50 ng μl^{-1}), 1 µl dNTP mix [(10 mM) 10 mM each dATP, dCTP, dGTP, dTTP] and DEPC-treated water in a total reaction of 10 µl were incubated at 65 °C for 5 min, and held at 4 °C for 1 min, before proceeding to set up the remainder of the reaction, by the addition of: 2 µl 10x RT buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 4 µl $MgCl_2$ (25mM), 2 µl DDT (0.1M) and 1 µl RNase OUT (Recombinant Ribonuclease Inhibitor) to a total reaction volume of 19 µl, mixed gently and incubated at 25 °C for 2 min. Next, 1 µl Superscript II reverse transcriptase, RNase H^- (50 U μl^{-1}) (Invitrogen) was added, and the samples incubated at 25 °C for 10 min, 42 °C for 50 min and 70 °C for 15 min to terminate the reaction, followed by RNase H treatment (1 µl of 40 u μl^{-1}) at 37 °C for 20 min to remove the RNA template. The controls: minus RT and RNA template were used during first-strand cDNA synthesis in order to determine the presence, and level of contaminating genomic DNA following DNase 1 treatment. No PCR products were detected when RNA samples were used, in contrast to their respective cDNA templates, provided that the RNA samples had sufficient DNase I treatment. The levels of the reference housekeeping gene, GAPDH were used to normalise the cDNA template concentration to control for possible differences in RNA extraction between samples, so that equivalent amounts of cDNA are used per reaction.

5.3.4. Synthesis and Purification of Run-Off Transcripts as Reference RNA Templates for the Calculation of Copy Number in the Real-Time RT-PCR Assay

Two types of standard were used during this study to further improve the sensitivity of this two-step real-time RT-PCR assay. In addition, this allowed the absolute numbers of molecules to be calculated in a technique referred to as 'absolute quantification'. An endogenous (internal) RNA expressed in the cell, such as mRNA from HKG was used to determine the accuracy of the QRT-PCR and to compare expression levels of the target genes (normalize the data). Three HKGs were tested: β-actin, β-tubulin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The advantage of endogenous standards is that they underwent simultaneous reverse transcription along with the other mRNAs to be assayed to create the same cDNA pool, from which parallel real-time PCR reactions were setup. The second type of standard used was an artificial exogenous (external) recombinant RNA template (cRNA). Since the molecular weight of this cRNA standard is known, fixed molar concentrations were serially diluted to create a precise calibration curve. By creating a series of calibration dilutions (5x 10^9 to 5x 10^4 copies), the iCycler outputs for the standards using this method were used to calculate copy number of the unknowns within the samples.

cRNA standards were prepared in the form of short run-off transcripts, created using Lig'nScribe and T7 Megashortscript kits (Ambion). Lig'nScribe was used in two sequential reactions to perform non-directional ligation of a T7 promoter adaptor onto a PCR product and a second PCR to amplify those products ligated to the adaptor that can be used as a template in the transcription reaction.

5.3.4.1. Pre-ligation PCR

The template for the ligation reaction was prepared using long product primers to synthesize the amplicon corresponding to the real-time RT-PCR primers (see Table 6) plus a portion of adjacent template. The template for the reaction was provided by cDNA derived from an RT reaction in which RNA, extracted from 50 dissected cardia (~ 50 ng μl^{-1}) was used as template. The cDNA is used as a template for PCR amplification using the specific primers detailed in Table 19, and the conditions: 95 °C for 3 min; 30 cycles of: 94 °C for 0.5 min, 58 °C for 0.5 min, 72 °C for 1 min; final extension 72 °C for 10 min. The reactions contained 5 µl 10x PCR buffer, 5 µl $MgCl_2$, 5 µl dNTPs, 3 µl sense primer (25 pM μl^{-1}), 3 µl reverse complement primer (25 pM μl^{-1}) and 0.2 µl Taq DNA polymerase (5u μl^{-1}) (Promega) with a total reaction volume of 50 µl. In most cases the purity of the initial PCR product was sufficiently high for use directly as a template for the ligation reaction. Alternatively, the target fragments were purified by electrophoresis on a 2 % agarose gel, from which bands were excised and the DNA purified

using QIAquick Gel Extraction Kit (Qiagen), according to the manufacturers instructions and eluted in 30 µl DEPC water in the instances when the PCR yields both the desired PCR product and nonspecific products.

5.3.4.2. Ligation Reaction

The ligation reactions were composed of the following, assembled in nuclease-free eppendorfs: 1 µl of 10x ligation buffer (Ambion, composition unknown), 1 µl of T7 promoter adapter, 1–7 µl of template (typically 10 - 25 ng of DNA) and 1 µl of T4 DNA ligase, in a total reaction volume of 10 µl. The reaction was mixed by pipetting and incubated at R.T. for 15 min. Remove a 2 µl aliqot of the ligation reaction for use in the Lig'nScribe PCR reaction, and store the remainder at ⁻20 °C.

5.3.4.3. Short (Nested) PCR

A nested PCR was then performed on the ligation product by assembling the following on ice: 2 µl ligation reaction product, 5 µl 10x PCR buffer, 4 µl dNTPs (2.5 mM), 1.25 µl PCR Adapter primer 1 (5'-GCTTCCGGCTCGTATGTTGTGTGG-3') or 2 (5'-TATGTTGTGTGGAAGCGGAAGA-3') (10 µM), 1.25 µl gene specific (user specified) primer, nuclease-free water to a volume of 49.5 µl, and 0.5 µl thermostable polymerase (5 U μl^{-1}). The following thermal cycling parameters were used: 94 °C for 3 min, plus 30-40 cycles of: 94 °C for 0.5 min, 58 °C for 0.5 min, and 72 °C for 1 min. A 5 µl aliquot of the PCR product was run on a 2 % EtBr agarose gel to accurately determine the size of the PCR product. Adaptor 1 consistently gave a better PCR product in comparison to adaptor 2, which commonly appeared as a single, discrete band of 64 bp longer than the T7 PCR product generated by the initial PCR reaction. The desired product was isolated by excision from a 2 % EtBr agarose gel on occasions where the product consisted of multiple bands.

5.3.4.4. T7 MEGAshortscript reaction

Lig'nScribe reaction products that consisted of a single band of the desired length and a concentration >10 ng μl^{-1} were used directly as a template for the MEGAshortscript transcription reaction (Ambion). The transcription reaction was performed according to the manufacturer's instructions for calculating the optimum quantity of PCR generated DNA template required to maximize the transcript yield. The following reaction was assembled at R.T.: 2 µl 10X reaction buffer (Ambion, composition unknown), 8 µl rNTPs [2 µl each rATP, rCTP, rGTP, rUTP (75 mM)], 100-200 ng template DNA, 2 µl T7 Megashortscript enzyme mix , and DEPC UPW in a

total reaction volume of 20 µl. Next, the reaction was mixed gently by flicking, briefly spin to bring the reaction mixture to the bottom of the eppendorf, and incubated at 37 °C for 2 - 4 h.

5.3.4.5. Removal of DNA template

For downstream applications involving RT-PCR it is necessary to degrade the template DNA, even though it is present at very low concentrations relative to the RNA product, since this could interfere with the accurate determination of copy number of the standards generated. Since DNA/ RNA hybrids are not easily degraded by DNase I, it is necessary to remove cations and denature the reaction prior to DNase I treatment in order to maximise the efficiency of the enzyme. Firstly, the transcription product was treated with 5 µl of "DNA-free" sludge (Ambion) prior to DNase I treatment, mixed and incubated at R.T. for 2 min with intermittent gentle flicking, prior to centrifugation at 10,000xG for 2 min, and recovery of the supernatant. The treated transcription product was incubated at 95 °C for 3 min and snap cooled on ice prior to the addition of: 2 µl 10x DNase buffer (Ambion) and 2 µl (2 U μl^{-1}) RNase-free DNase I (Ambion), and incubated at 37 °C for 1 - 4 h. DNase I was inactivated by the addition of 5 µl DNase I Inactivation Agent (Ambion), following a repeat of the sludge procedure. Next, each sample containing ~ 20 µl purified RNA was precipitated at -80 °C for 20 min following the addition of 2 µl NH_4OAc (5M) and 66 µl (3x vol) 100 % EtOH, and centrifuged at 19000xG for 30 min at 4 °C. The supernatent was aspirated and the pellet washed in 100 µl 70 % EtOH by centrifugation at 19000G for 15 min at 4 °C, before being dried and resuspended in 20 µl RNase-free water.

5.3.4.6. PolyAcrylamide Gel Electrophoresis (PAGE) Purification of RNA standards

It is desirable to PAGE gel purify transcripts of the expected size so as to generate cRNA standards that produce a single narrow peak during melt curve analysis, which in turn allows the more accurate determination of copy number of experimental samples. Mini gel plates (Mini-PROTEAN 3, Biorad) were assembled following the thorough treatment of all glassware and other components (especially plates, spacers and combs) with RnaseZap (Ambion) before being rinsed in DEPC water and dried. Two 5 % resolving gels, composed of: 4.98 ml 30 % acrylamide solution, 18.8 ml dH_20, 6 ml 5x TBE, 420 µl fresh 10 % ammonium persulphate (Sigma) and 10.5 µl TEMED (Sigma) in a total volume of ~30 ml, were poured before being allowed to polymerise for 1 – 2 h. The rig was filled with running buffer (0.5x TBE) so that the upper reservior was filled to the brim, and the level of the lower reservoir was 2/ 3 way up the cassette. The wells were rinsed with running buffer and the gel pre-run for 15 – 30 min at 125 – 150 V prior to loading the samples. Meanwhile, the samples were prepared, with the addition of 5 µl denaturing formamide gel loading buffer/ dye (95 % deionised formamide, 0.5 mM EDTA,

0.025 % xylene cyanol, 0.025 % bromophenol blue, 0.025 % SDS) (Ambion) to each 2 µl aliquot of the RNA, before being mixed and heated to 95 °C for 3 – 5 mins in a heatblock. Similarly, 1 µl of RNA markers (0.28 – 6.58 kb, Promega) were treated, and the samples loaded onto the gel whilst still denatured. The samples were run for ~1 h, until the bromophenol blue band just runs out of the gel. One of the glass plates was removed carefully, and the remaining plate inverted in 0.5x TBE so as to float off the gel, before the addition of 0.5 µg ml^{-1} EtBr. The gel was stained for 10 min and the bands containing transcripts of the correct size excised on a 300 nm wavelength transilluminator using an RNase-free razor blade. Excess gel was trimmed from the excised slice and transferred to a fresh RNase-free eppendorf, before being immersed in 300 µl Elution Buffer (Ambion) overnight at R.T. to extract the purified RNA. The eluate was transferred to a fresh eppendorf and precipitated by the addition of 3x volumes 100 % EtOH, incubated at ˉ80 °C for 20 min, centrifuged at 19000 rpm at 4 °C, aspirate and wash pellet twice with 70 % EtOH, dried and resuspended in 20 µl RNase-free UPW. Aliquots of the stock solution of each cRNA were stored at ˉ80 °C in RNase-free microtubes that had been silanized (5% dimethysilane in toluene, 3 rinses in toluene, followed by a final rinse in MeOH and baked at 50 °C for 3 h) to reduce adsorption of RNA over time.

5.3.4.7. Determination of Purity and Quantification of RNA standards

A series of RT reactions were run in order to verify that the RNA samples were free of contaminating DNA, by diluting 1 µl aliquots of purified RNA in RNase-free UPW to a total volume of 10 µl, before testing 1 µl aliquots of the diluted sample by positive and negative RT reactions. 1 µl of each first strand reactions were used as a template for separate PCR reactions, using the thermal cycling conditions of: 95°C denaturation for 3 min, 50 cycles of 10 sec at 95°C, 45 sec at 55-60°C (data collection step); melt curve steps were 95°C denaturation for 1 min, 80 cycles of 0.5°C increments (10 sec each) starting at 55°C (data collection step). The PCR products were also electrophoresis on a 2% agarose gel. The purified RNA was DNase I treated further in cases when bands were visible for the negative RT samples.

5.3.4.8. Use of cRNA Standards to Generate Standard Curves

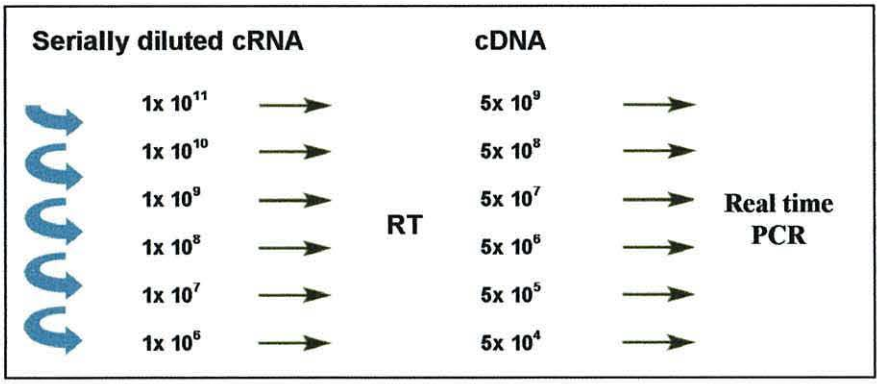
A standard curve was generated for each amplicon from a stock of ssRNA, which were accurately quantified using the RiboGreen RNA quantification assay (Molecular Probes, Invitrogen). Firstly, RNA concentration was accurately measured (1000x more sensitive than UV absorbance A_{260}) on the Wallac Victor 2 fluorescence microplate reader (Perkin-Elmer). Copy number was calculated using the formula: $MW = (A_n \times 328.2) + (U_n \times 305.1) + (G_n \times 344.2) + (C_n \times 304.2) + 159$, taking into account the extra 10 nt (5'-CCCUCUCCUC-3',

equilivalant to 3144 Da) added to transcripts during the transcription reaction. Since 1 Mole = 6.022 x 10^{23} molecules, and M = mass/ MW, the copy number of each cRNA could be calculated. The cRNA standards were then diluted to 1x10^{11} copies μl^{-1}, 1 µl used in the RT reaction. Accurate calibration curve cRNA aliquots were prepared, from a dilution series of 1x10^{11} down to 1x 10^{6} copies μl^{-1}. Aliquots of the cRNA dilution series were stored in silanized RNase-free microtubes at -80 °C until use in experiments.

5.3.5. Reverse-transcription of cRNA standards

1 µl aliquots of each cRNA dilution were used as template in separate RT reactions, as previously described (section 5.3.3.), in a parallel procedure to the total RNA samples (Fig 2.). Finally, 1 µl aliquots of the serially diluted cRNA derived cDNAs were run as copy number standards in conjunction with cDNA from the total RNA samples on each experimental real-time RT-PCR plate.

Fig. 28. The synthesis of cDNA standards from serially diluted cRNA.



5.3.6. Primer Design

The primers (Table 19) were carefully designed such that the annealing temperatures of the primers were ~ 60 °C and the amplicons were < 200 bp, using Beacon Designer, version 3 (Premier Biosoft International, available at: http://www.premierbiosoft.com/ molecularbeacons/index.html. This program allowed the characterisation of the targets amplified by the primer pairs by accurately calculating the melting temperature (Tm), performing searches on the M fold server (http://bioinfo.hku.hk/Pise/mfold.html) to control against presence of secondary structures at primer binding sites, and conducted homology searches of oligos and templates against all the nucleotide redundant databases available at NCBI to verify the specificity of the primers designed, so as to optimise the primer pairs for each target cDNA sequence. Primers were synthesised by Invitrogen (Paisley, Scotland) and desalted when purified as HPLC purification was found to be unnecessary.

5.3.7. Performing QRT-PCR Analysis on the iCycler

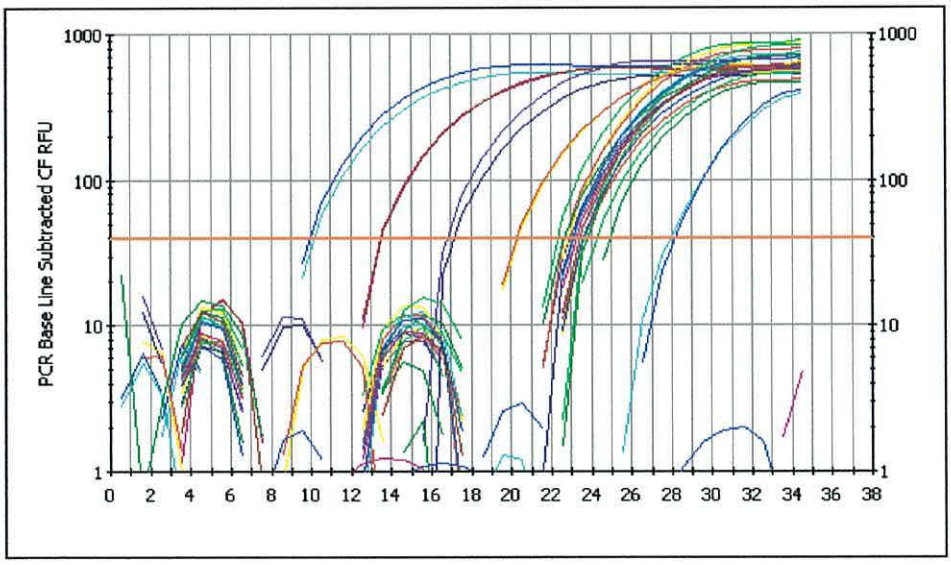
Real-time RT-PCR analysis was performed on the iCycler™ (Biorad), software version 3.0a, using natural coloured 96 well PCR plates (Abgene) sealed with iCycler iQ Optical Quality Sealing Tape (Biorad) with the aforementioned primers. During the experiments cDNA from the cRNA standards and biological samples was amplified together with a no template negative control. All samples to be compared were run in the same assay. Master mix was prepared according to the manufacturer's instructions, with the exception that all volumes were halved to give a final reaction volume of 25 µl: 12.5 µl Platinum Quantitative PCR Supermix-UDG (Invitrogen), 8.5 µl UPW, 1.5 µl sense primer (10 µM), 1.5 µl antisense primer (10 µM), and 1.0 µl cDNA template per 25 µl reaction. Sufficient master mix was prepared for three replicates per plate, per sample. Plates for each assay were subjected to similar amplification and melt curve protocols, differing only in relative annealing temperatures for each primer pair. The amplification steps were: 95°C denaturation for 3 min, 50 cycles of 10 sec at 95°C, 45 sec at 55-60°C (data collection step); melt curve steps were 95°C denaturation for 1 min, 80 cycles of 0.5°C increments (10 sec each) with a start and end temperature of 55°C and 95°C respectively during the data collection step. In addition to the melt curve analysis, PCR reactions were run out with Amplisize Molecular Ruler (50-2000bp, Biorad) to accurately confirm amplification of the correct product, since both are required for reliable quantification. To check the dynamic range of the real-time PCR assay, the amplification of each mRNA in serial dilutions of each cDNA sample is measured. A broad dynamic range is depicted by the threshold cycles of each replicate group being separated by exactly 3.32 cycles. Thus, in theory, the R value of the slope should be < 1, because this would imply that the efficiency of the PCR > 1 (100 %). Since it is impossible for more than twice as many amplicons to be synthesised per PCR cycle, this situation could only be accounted for by primer dimers and or the formation of secondary products.

Table 19.
Sense (S) and reverse complement (RC) primers used in Real-Time RT-PCR analysis:

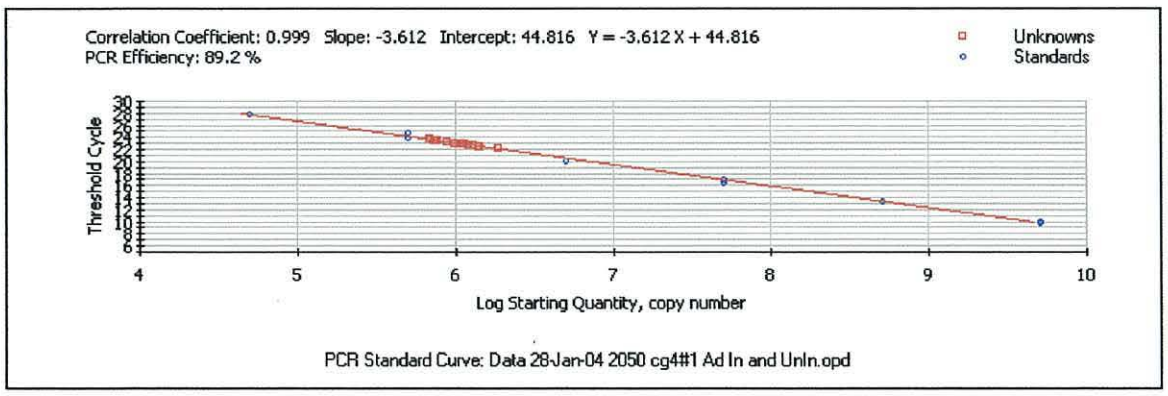
Name	Sequence (5'-3')	Tm (°C)	Amplicon length (bp)
DmPro1 (S)	GTCCTTGCGAAATGGTGCCTATG	63	189
DmPro1 (RC)	GCCAGTAATGCTCCGTGTGC	64	
DmPro3 (S)	GCTGCAATGAAGTGTAAGTACCTG	60	103
DmPro3 (RC)	GCAAGGACATCAACATCCAGAC	62	
DmPro2 (S)	GCCCAATCCAGAACTAAACACTATG	61	131
DmPro2 (RC)	GCACTTGACGATCCCATAAAGAG	59	
CG14645 (S)	CATTGCCCTCGCCTATGATGG	64	191
CG14645 (RC)	GTTGACGCAGTTCTTGAGACTATCC	63	
DmPro6 (S)	TGTTCCGACCATCGAGCGAGAG	64	97
DmPro6 (RC)	ACGGCAGGCGCATTCCCTG	64	
DmPro4 (S)	TACAACGCCACCTTAGACCAGTG	64	164
DmPro4 (RC)	CTCGACGGCGCAGTTGAAGTTAC	63	
DmPro5 (S)	GCAGCTGTGCCGCAACGAGAC	66	213
DmPro5 (RC)	TTGGCCGCCTGGATGGACTG	64	
Actin 5C (S)	CCCAAGGCCAACCGTGAGAAG	63	105
Actin 5C (RC)	ACCGGAGGCGTACAGCGAGAGC	63	
β-tubulin (S)	GTGCGTTGTGCGGTGAAGTTG	62	162
β-tubulin (RC)	GAAGGCTAAGGCTGGAATCGGATC	65	
GAPDH 2 (S)	TACCGATTTCCTCAGCGACACC	62	168
GAPDH 2 (RC)	GTTCCACATTTAATCCTTGCTCTGC	64	

Fig. 29. A set of representative data from this study, in which the expression of *DmPro1* is assayed in cDNA derived from cRNA and total RNA from infected and uninfected samples, amplified with SYBR Green 1:

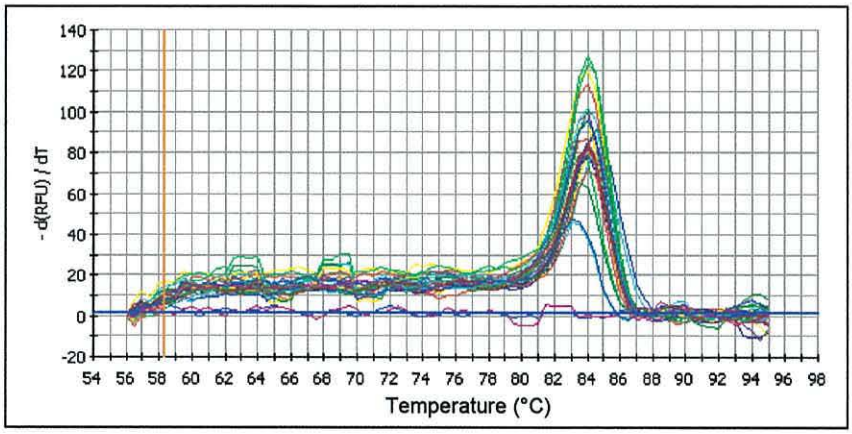
A. Amplification plot of log PCR Amplification vs cycle data. The cDNA derived from *DmPro1* cRNA at varying RNA concentrations (10-fold dilutions from 5x 10^9 to 5x 10^4 copies) and total RNA samples was used as template for the reaction. The horizontal bar represents the user defined threshold cycle (C_T) during the linear phase of the PCR. PCRs with higher initial cDNA concentrations cross the C_T at an earlier cycle number, with subsequent dilutions crossing the C_T at later cycles. The cRNA and RNA samples produced near exact amplification plots, in terms of the slope. These results verify that the PCR was optimized, essentially achieving the theoretical gap of 3.33 cycles per 10-fold dilution of cRNA.



B. PCR Standard Curve data created by the cRNA standards, on which the unknowns are plotted. The calibration curve is used to calculate the copy number of the unknowns, since the copy number of the cRNAs are known (varying from 5x 10^9 to 5x 10^4 copies). The standard curve is constructed from the amplification profile in Fig. 29A. The logarithmic plot relates starting cDNA concentration (copy number) versus C_T for the cDNA samples. Replicate values almost perfectly overlay each other (e.g. cRNA standards, plotted as circles), indicating reproducibility. A similar curve was generated for each of the genes in the study from the cDNA derived from the serially diluted cRNA.



C. Melt curve analysis, performed immediately following PCR, in which the software calculates the Tm of the PCR products from a plot of the negative first derivative (-dF/ dT) of the fluorescence intensity. This allows the accurate identification of the desired amplicon based on the Tms. For each iCycler run a melt-curve was acquired for the products of the cRNA and native mRNA RT-PCR reactions, to allow their direct comparison. Note that no amplification products were detected in the no-template control (NTC) reactions after 35 cycles; usually some amplification occurs after 40 cycles.



5.3.8. Quantification of gene expression

In order to accurately determine the total amount of peritrophin transcripts in 50 ng μl^{-1} total cardia RNA the RNA from samples were reverse-transcribed and amplified in parallel with the cRNA standards used to calculate copy number (Fig. 28). The copy number of peritrophin transcripts was calculated using the iCycler software to extrapolate from the C_T value using the standard curve as described previously (Fig. 29C). The level of tubulin transcription was used as an endogenous RNA control in order to obtain a normalized value for copy number of peritrophins and minimize errors derived from different samples (Table 20).

5.3.9. Statistical Analyses of the Data

The levels of gene expression were quantitated by calculating the mean amplification cycle at which the PCR product of interest was first detected (C_T), prior to being normalised to the expression level of the endogenous reference in each sample. The results are shown as mean (with SD) for 9 different PCR reactions. The *t* test for independent samples was used to statistically analyse the results of these studies, after verifying the normalization of data (at least approximately normally distributed). Both tests were performed using Statistica for Windows, release 5.5 (StatSoft, Inc., Tulsa, U.S.A., available at: http://www.statsoft.com).

5.4. Results

The reliability of QRT-PCR assays was evaluated by comparative amplification efficiencies of the cRNA used to construct the standard curve and the native mRNA in the samples. The slopes of the traces of the QRT-PCR amplifications are parallel when viewed with the y-axis on the log scale (see e.g. in Fig. 29A), indicating that the individual efficiencies of cDNA derived from native mRNA and cRNA are approximately equilivant. In addition, standard curves (see e.g. in Fig. 29C) were constructed from separate cDNA pools prepared from individual RT reactions of a dilution series of cRNA. The proportion of cDNA transcripts synthesized by each cRNA dilution were consistent at all the concentrations of template tested, resulting in the reliable and sensitive with high linearity of standard curves with a PCR efficiency (correlation coefficient close to 1, 0.999) over six orders of magnitude from $5x\ 10^4$ to $5x\ 10^9$ cRNA standard molecules. To verify QRT-PCR products derived from cRNA or sample RNA, melt curve analysis on the iCycler (Biorad) and gel electrophoresis were performed. Products showed no primer dimers, a single sharp peak, with identical melting points and expected length in gel electrophoresis.

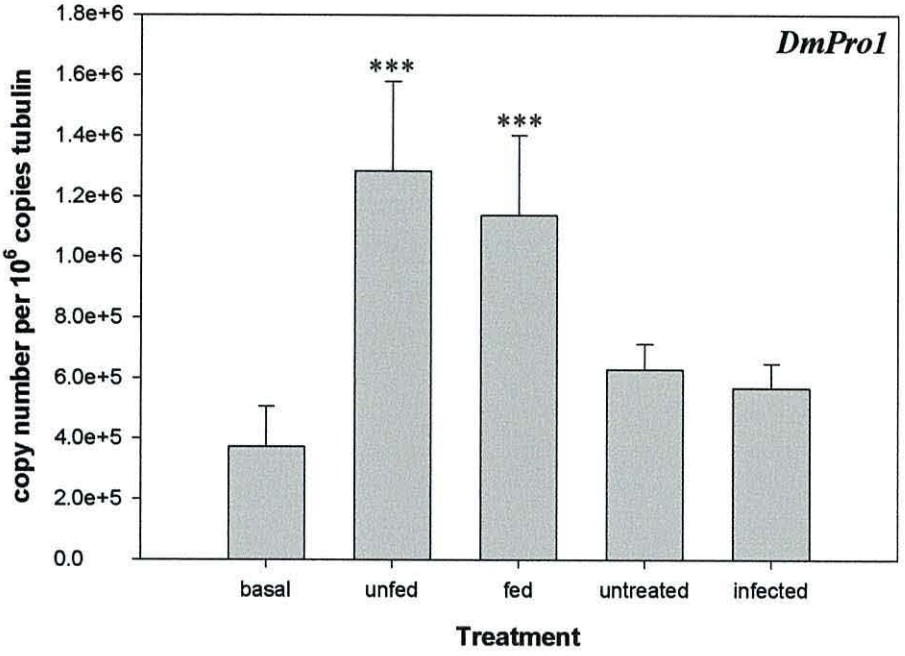
Upon validating the suitability of the control genes, prior to measuring the expression of the genes of interest it was found that *Glyceraldehyde-3-phosphate dehydrogenase 2* (Gapdh 2) (Genbank accession number NM080714) transcription varied between samples within the same treatment group. Such fluctuation in the level of internal standards in cardia RNA samples would lead to erroneous quantification results, rendering it unsuitable as an internal reference. Similarly, Gapdh 2 expression has been found to vary considerably between biological samples and treatments in numerous studies (Bustin, 2000; Ke *et al.*, 2000; Bustin, 2002; Dheda *et al.*, 2004). In contrast, genes encoding the cytoskeletal components: Actin 5C (accession number NM167053) and β-tubulin (accession number NM079118) were essentially invariant between treatments, and thus were suitable reference genes to control for error between samples.

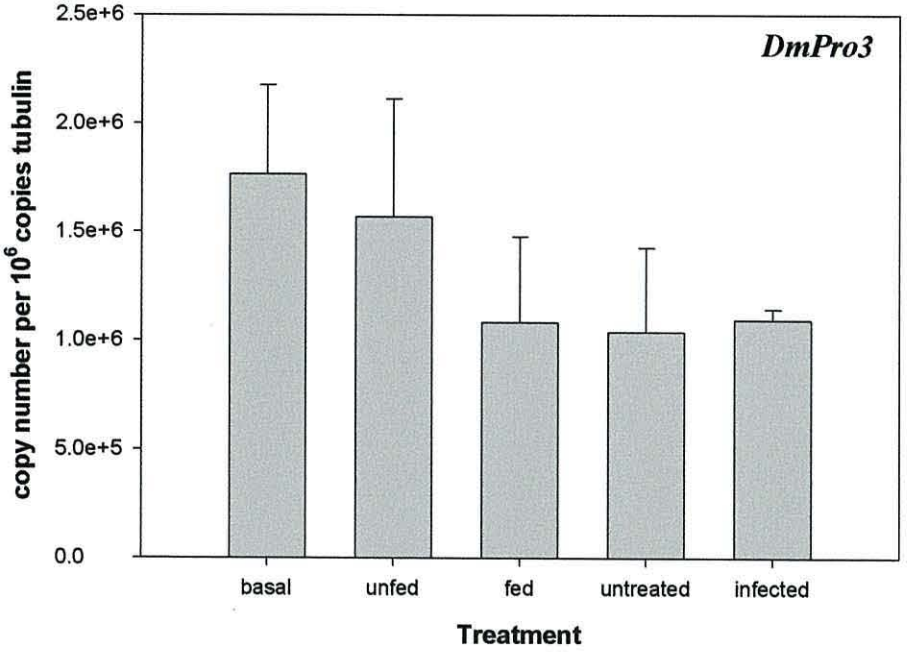
The total number of peritrophin transcripts was determined using the standard curve as described. The relative amount of peritrophin transcripts ranged from $1.4x\ 10^5$ to $7.4x\ 10^6$ copies/ 10^6 copies of tubulin. As shown in Table 20 mRNA expression of *DmPro1*, *DmPro2* and CG14645 increased by 3.4-fold ($p<0.001$), 2.3-fold ($p<0.01$) and 1.8-fold ($p<0.05$) respectively in the absence of stimulation by feeding, in newly eclosed vs 2 d old flies. Similarly, transcription of *DmPro1* and CG14645 was significantly upregulated 3.0-fold ($p<0.001$) and 1.9-fold ($p<0.01$) respectively in fed 2 d old flies vs unfed newly emerged flies. However, only *DmPro2* showed a significant induction in expression of 1.8-fold ($p<0.05$) in response to feeding, in comparison to age matched, unfed flies. Transcription of the majority of

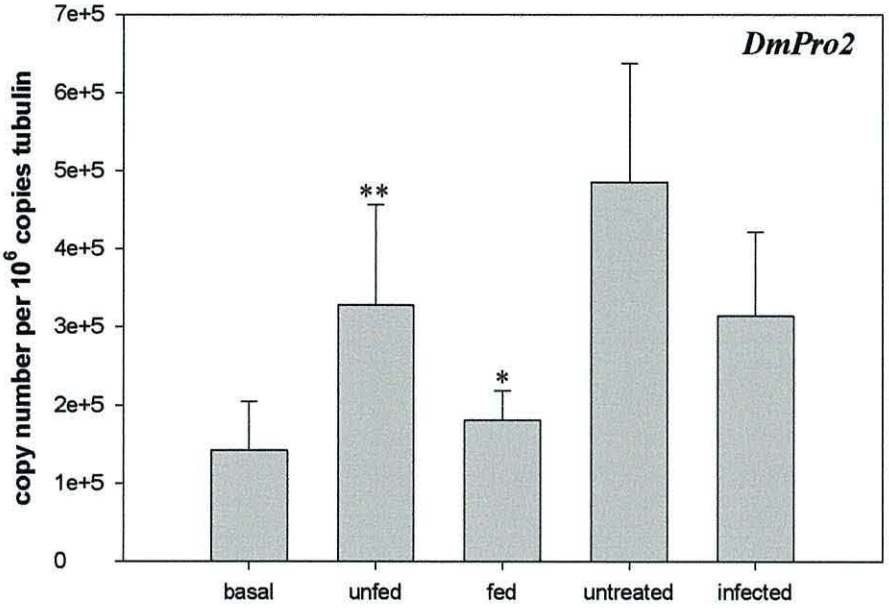
Table 20. Data represent the mean copy number of mRNA for each gene, the absolute copy number of β-tubulin being given in each case and Peritrophin gene expression data are expressed as copy number per 10^6 β-tubulin molecules. Treatments were compared to the relative controls and statistical analysis performed using t-test of independent variables. Abbreviations: §, first treatment variable; ¶, second treatment variable; Ad, adult; La, larvae; NS, not significant; asterisks represent p-values: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The first four sections represent data from the adult QRT-PCR study, the last being that from the larval study, in which expression of larval *DmPro* sequences were determined. The main results are shown as a graphical representation in Fig. 30.

Treatment	Gene	Mean*	Mean¶	t-value	df	p	Sig.	Std.Dev.*	Std.Dev.¶	F-ratio variances	p variances
Ad Basal* + Unfed¶	tubulin	25085483	17524700	0.552	10	0.59331	NS	27564747	19165264	2.069	0.44400
	DmPro1	373174	1284762	-6.889	10	0.00004	***	13352	295366	4.893	0.10622
	DmPro3	1763612	1568571	0.489	10	0.63555	NS	812552	543305	2.237	0.39763
	DmPro2	142762	328000	-3.181	10	0.00980	**	61809	12855	4.326	0.13385
	CG14645	522776	954952	-2.847	10	0.01734	*	188220	320695	2.903	0.26703
Ad Basal* + Fed¶	tubulin	25085483	38259067	-0.634	10	0.54046	NS	27564747	42808257	2.412	0.35607
	DmPro1	373174	1138381	-6.341	10	0.00008	***	13352	263720	3.901	0.16148
	DmPro3	1763612	1082681	1.846	10	0.09461	NS	812552	394768	4.237	0.13907
	DmPro2	142762	181027	-1.295	10	0.22441	NS	61809	37656	2.694	0.30066
	CG14645	522776	998695	-3.320	10	0.00775	**	188220	296479	2.481	0.34125
Ad Fed* + UnFed¶	tubulin	38259067	17524700	1.083	10	0.30429	NS	42808257	19165264	4.989	0.10236
	DmPro1	1138381	1284762	-0.906	10	0.38648	NS	263720	295366	1.254	0.80965
	DmPro3	1082681	1568571	-1.772	10	0.10677	NS	394768	543305	1.894	0.50019
	DmPro2	181027	328000	-2.688	10	0.02279	*	37656	12855	11.654	0.01748
	CG14645	998695	954952	0.245	10	0.81116	NS	296479	320695	1.170	0.86739
Ad Untreated* + Infected¶	tubulin	35237500	33735938	0.23	14	0.82111	NS	9414265	15847633	2.834	0.19277
	DmPro1	629966	568317	1.518	14	0.15120	NS	82481	79918	1.065	0.93575
	DmPro3	1039728	1094158	-0.395	14	0.69881	NS	386579	49778	60.313	0.00002
	DmPro2	485244	314464	1.314	14	0.21001	NS	35174	106941	10.819	0.00554
	CG14645	489501	373148	1.211	14	0.24598	NS	128678	239388	3.461	0.12360
La Untreated* + Infected¶	tubulin	3287500	2717500	0.832	14	0.41955	NS	1733178	868245	3.985	0.08844
	DmPro6	4649962	4947978	-0.109	14	0.91437	NS	582340	503583	1.337	0.71110
	DmPro2	4088146	6751838	-1.738	14	0.10411	NS	118089	417049	12.473	0.00358
	DmPro4	3356383	5661765	-3.831	14	0.00184	**	144841	894092	2.624	0.22633
	DmPro5	4385258	7475919	-1.176	14	0.25928	NS	201076	715761	12.671	0.00341

Fig. 30.
Effects of age, nutrition and bacterial infection on transcription of genes encoding peritrophins in adults. Graphical representation of the Real-Time RT-PCR data after normalization for input total RNA levels using β-tubulin. Data for the genes of interest are expressed as copy number per 10^6 copies tubulin. Note: asterisks represent significance: none, not significant; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, p values refer to comparison within the two groups: basal vs fed/ unfed and infected vs uninfected, df = 10 (see Table 20). Statistical analysis was conducted on expression levels in basal vs unfed/ fed flies, and untreated vs infected flies. Error bars indicate standard deviation. Quantification of tubulin and peritrophin copy number was performed by iCycler PCR.






DmPro2
copy number per 10^6 copies tubulin
7e+5
6e+5
5e+5
4e+5
3e+5
2e+5
1e+5
0
**
*
basal
unfed
fed
untreated
infected

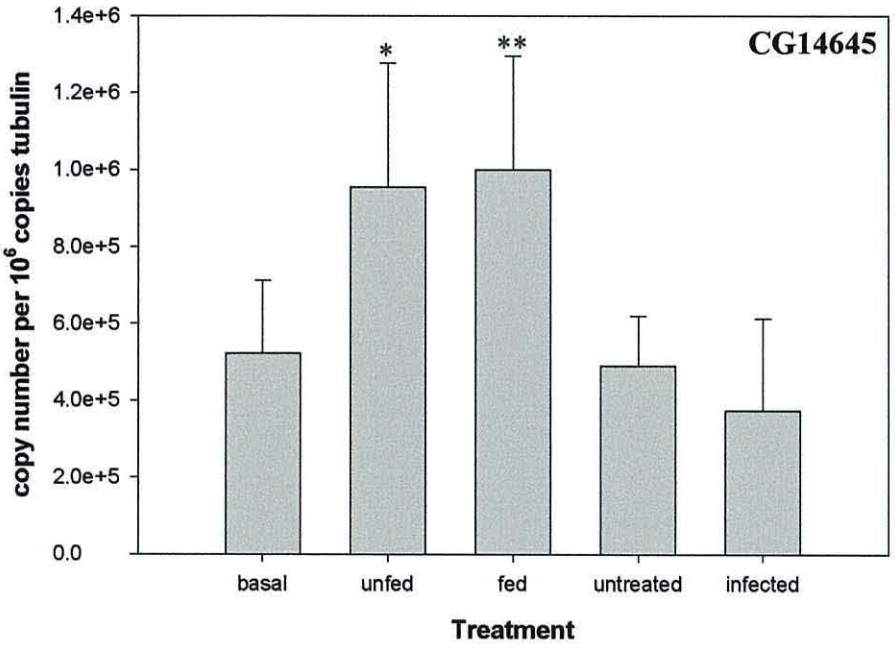
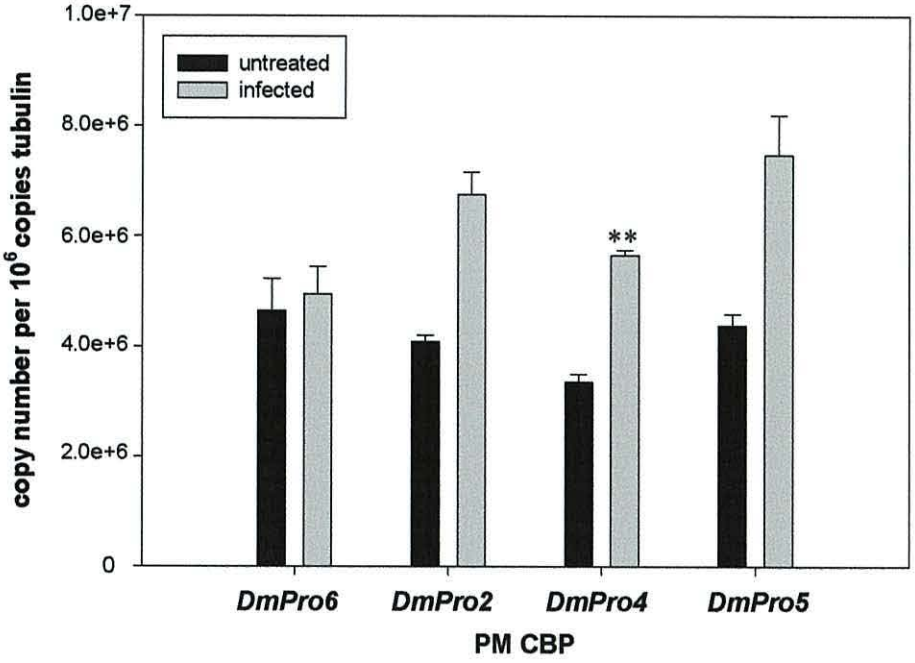

CG14645
copy number per 10^6 copies tubulin
1.4e+6
1.2e+6
1.0e+6
8.0e+5
6.0e+5
4.0e+5
2.0e+5
0.0
*
**
basal
unfed
fed
untreated
infected
Treatment

Fig. 31.
Effects of bacterial infection on transcription of genes encoding peritrophins in larvae. Graphical representation of the Real-Time RT-PCR data after normalization for input total RNA levels using β-tubulin. Data for the genes of interest are expressed as copy number per 10^6 copies tubulin. Note: asterisks represent significance: none, not significant; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, p values refer to comparison within the groups: infected vs uninfected, df = 14(see Table 20).



adult peritrophins was repressed following bacterial infection. *DmPro3* expression was slightly responsive, although not significantly, with only a 1.1-fold increase in untreated vs infected adults. By contrast, all larval peritrophins were upregulated 1.1- to 1.7-fold in response to bacterial infection, but only *DmPro4* transcription levels varied significantly ($p<0.01$) (Fig. 31).

5.5. Discussion

This QRT-PCR data supports the previously reported theory that type 2 PMs are constitutively produced. In addition, it provides some tentative evidence in support of the physiological regulation of peritrophins in the adult PM. Although, there is little evidence in support of an immunological response in the larval PM, since only *DmPro4* showed some upregulation in response to infection. In adults, *DmPro3* and *DmPro2* transcripts appear to constitute the dominant and minor (least abundant) class of peritrophins from the adult PM respectively (Fig. 30), whereas, the levels of *DmPro2* are comparable to the other larval sequences studied (Fig. 31), in accordance with the results of the previous (F)ISH study (Appendix). Maximal changes

in transcription were observed in *DmPro1* after a period of 2 d in fed and unfed vs newly emerged flies (3 to 3.4-fold change in copy number respectively). However, the relative expression of peritrophins is approximately equal in fed vs unfed flies (Fig. 30), indicating that the expression of peritrophins is not regulated by the nutritional status of the fly or that post-transcriptional regulation may be responsible for upregulation of peritrophin transcription and thus PM secretion in response to feeding. The upregulation of *DmPro1*, *DmPro2* and CG14645 may indicate that the secretion of these putative peritrophins in *Drosophila* occurs in a responsive manner, in that production may be upregulated when adults are unfed so that stores of these PM components are available for rapid secretion on feeding.

The down-regulation of peritrophins observed in infected adults in comparison to untreated controls could indicate that there is a malaise response in the midgut of adults, such as that previously reported for genes encoding lysozymes in *D. melanogaster* (Daffre *et al.*, 1994). This may also be caused -in part- by an immune reaction, as Hao and Aksoy (2002) recently demonstrated an immune responsive component of the type 2 PM of *Glossina*, *GmPro1*, characterised by a similar down-regulation in expression in response to infection. *DmPro3* was the only adult peritrophin in which expression was upregulated (although not significantly) in response to bacterial infection, with a one to two fold induction in comparison to controls. Infection of larvae appeared to result in an immune response for all the larval peritrophin genes in the study in terms of relative expression, but only *DmPro4* was significant ($p<0.01$), with a one to two fold induction (Fig. 30). *DmPro4* encodes five tandem CBDs, and is hence likely to act as a molecular linker that connects the proteinaceous matrix to the chitin meshwork of the PM and/or cheletes chitin so as to protect the fibrils from the action of endo-chitinases. Thus, the larval PM is probably reinforced by the formation of comparatively more links between PM components, providing a tighter matrix, in which the closed framework could be more resistant to degradation. Moreover, alternatively the mature *DmPro4* peptide could be cleaved to form five fold more peptides containing a single CBD, capable of capping the ends of chitin, suggesting that the larval PM is capable of forming a more structurally sound matrix on bacterial induction than the adult PM. The data suggest that *D. melanogaster* larvae may employ a PM synthesis regulating mechanism that allows the modulation of PM secretion in response to bacterial challenge. Previous studies have shown that upregulation of genes involved in PM synthesis can regenerate PM secretion in response to disruption caused by calcofluor in *T. ni* (Wang and Granados, 2000). Larvae are gregarious feeders that develop in an environment in which they are constantly exposed to microbes, suggesting that they may have developed defences that allow them to resist infection (Gregg *et al.*, 1990).

Many parasites, including those species that affect humans and livestock, must survive the harsh environment of the insect midgut in order to complete their life cycle. Hence, there are immediate practical implications for understanding the protective mechanisms against such parasites that exist within the insect vector. Mechanical protection consists mainly of structures such as the PM and lectins. The results suggest a novel and tactical response of peritrophins to bacterial infections, demonstrating the complexity of host-parasite interactions. The PM plays a key role in parasite survival, limiting the number of parasites that develop successfully in the insect midgut (Billingsley and Rudin, 1992), a theory which is reinforced by the observation that parasite chitinase activity has a key role in determining virulence of some pathogens that infect insects via the PM. This is evident in the parasite-vector interactions: *Plasmodium falciparum-Anopheles gambiae* (Shahabuddin *et al.*, 1993), *P. gallinaceum-Aedes aegypti* (Sieber *et al.*, 1991), *Babesia microti-Ixodes dammini* (Rudzinska *et al.*, 1982), *Trypanosoma brucei rhodesiense-Glossina morsitans morsitans* (Welburn *et al.*, 1993) and *Brugia malayi-Aedes aegypti* (Perrone and Spielmann, 1986). In addition it was previously shown that baculovirus enhancin chemically disrupts PM integrity by specifically degrading insect intestinal mucin (IIM), a major protein constituent of the PM in *T. ni* (Brandt *et al.*, 1978; Wang and Granados, 1997a; Peng *et al.*, 1999).

The high sensitivity and specificity of iCycler QRT-PCR analysis implies that this method is extraordinarily useful to quantify peritrophin mRNA levels and study the molecular mechanisms involved in the regulation of gene expression. The transcription levels recorded in this study suggest a defined *in-vivo* modulation of composition of the PM in response to age, nutrition and infection status of the fly. The data support the theory that the type 2 PM of *Drosophila* may act as a facultative barrier (Lehane, 1997). However, this study does not provide conclusive evidence since QRT-PCR failed to detect significant transcriptional regulation in response to bacterial challenge of more than one gene of interest for both adults and larvae. This study best serves as a preliminary study on the expression of peritrophins, which should also be investigated at the proteomic level, since evidence of some regulation in the expression of a gene does not automatically imply that this will translate into secretion of similar levels of the mature peptide.

Chapter 6. Cardia-specific cDNAs characterised from the Tsetse: *Glossina morsitans morsitans*, *G. pallidipes*, and *G. palpalis*

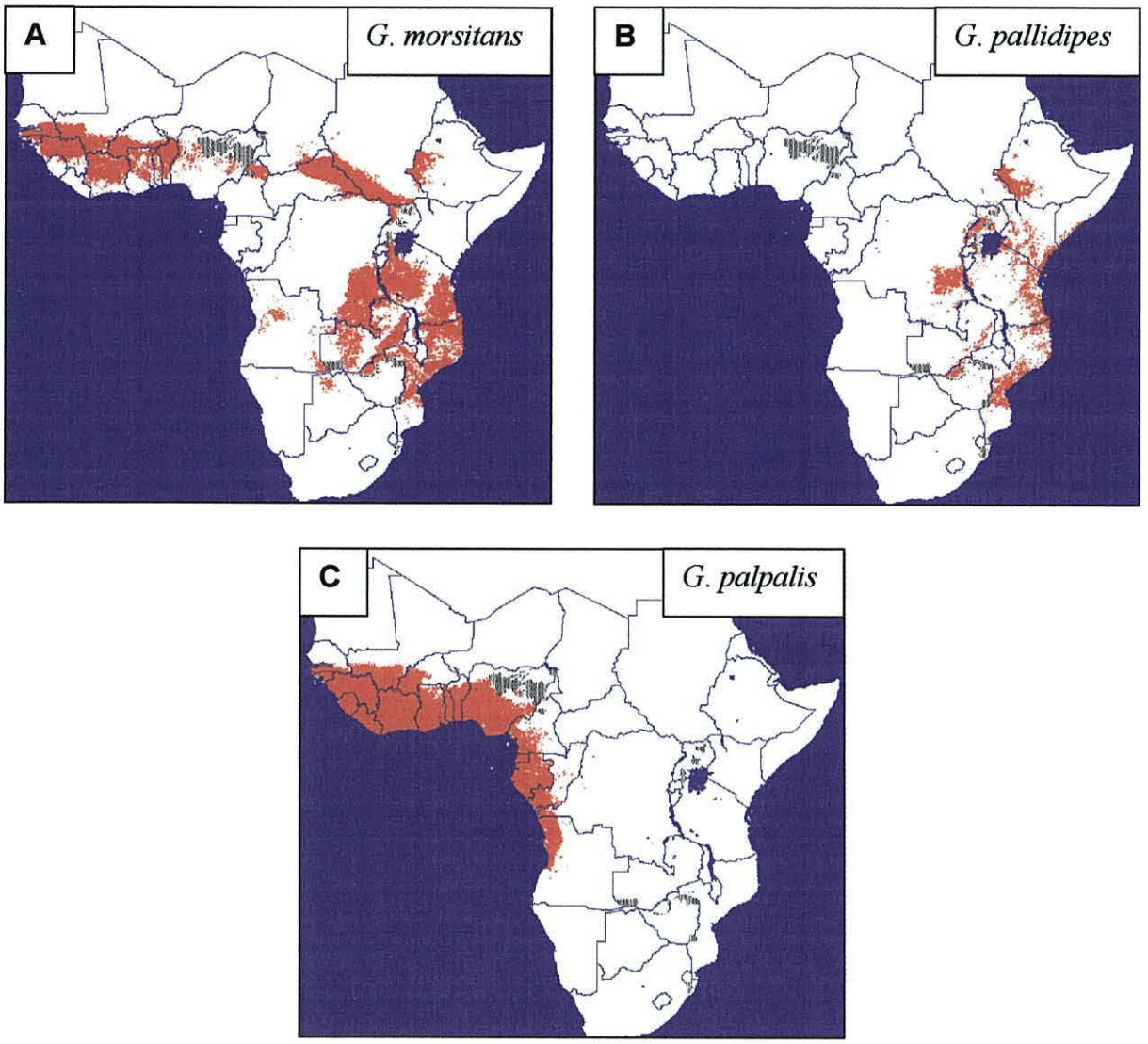
6.1. Abstract

The insect midgut has been shown to play a key role in vector-parasite interactions. The data provided by this study provides a valuable insight into possible roles of the PM by characterizing the genes encoding a further five putative peritrophins. The implications of which may prove to be pertinent to the further study of tsetse-trypanosome interactions at the molecular level. The outcome of infection in tsetse is dependent on the tsetse-trypanosome interaction, which can be influenced by the PM barrier. Using a bioinformatics based approach eight putative peritrophins were identified from the *Glossina* midgut expressed sequence tag (EST) database. DNA sequence analysis indicates that GmPro4-8 share similarities with known PM proteins, including *Pro2* from *G. morsitans morsitans* and *DmPro6*, *DmPro2* and *DmPro3* from *Drosophila melanogaster*. RT-PCR analysis indicates that transcripts for all five cDNAs are preferentially expressed in the cardia in *G. m. morsitans*, *G. pallidipes* and *G. palpalis*. The developmental expression profile of these genes indicates that *GmPro5* is abundant in the larval PM, and transcribed at extremely low levels in adults. The levels of the transcripts were not modulated in response to infection 21 d post infection with procyclic trypanosomes. Sequencing analysis of the homologs in *G. pallidipes* and *G. palpalis* has indicated that variability in the sequences between the three species is limited to hypervariable regions within the sequences, the conserved cysteine and aromatic regions that are characteristic of CBDs remain unaffected, which suggests that they are all functional peritrophins.

6.2. Introduction

Tsetse flies (Diptera: Glossinidiae) are the sole vector of African Trypanosomiasis, a disease also known as Sleeping Sickness in humans, of which there are half a million cases per annum (Welburn and Maudlin, 1999). Trypanosomiasis in animals (Nagana) continues to act as a considerable burden to agricultural development in affected areas (Jordan, 1986). The genus *Glossina* is composed of three subgenera: *morsitans* (savannah group), *palpalis* (riverine group) and *fusca* (forest group). The species *Glossina morsitans morsitans* and *G. pallidipes* transmit *Trypanosoma brucei*. Both are members of the *morsitans* group, although *Glossina m. morsitans* infests a large expanse of sub-Saharan Africa (Fig. 32A) in comparison to the limited range of *G. pallidipes* (Fig. 32B), whilst *G. palpalis* is a Central African species that transmits *T. gambiense* (Fig. 32C). *T. brucei* and *T. gambiense* are the major trypanosome pathogens of humans.

Fig. 32. Geographical distribution of the three *Glossina* spp. used in this study predicted in 1999 based on predicted presence models [original data from Ford and Katondo (1977)]. Taken from http://ergodd.zoo.ox.ac.uk/tseweb.htm.



In contrast to many haematophagous diptera that produce a type 1 PM, secreted by the entire midgut epithelium in response to a blood meal (Peters, 1992) adult tsetse produce a type 2 PM, present prior to taking the first blood meal (Moloo *et al.*, 1970). Electron microscopy reveals that adult tsetse possess a single 310-330 nm thick PM, composed of three layers rich in glycosaminoglycans, glycoproteins and chitin (Lehane and Msangi, 1991; Lehane *et al.*, 1996). Relatively little is known about the composition of the type 2 PM in adult dipterans in comparison to that of the larval type 2 PM (Tellam *et al.*, 1999). Moreover, the exploration of tsetse-trypanosome interactions, in which the PM is a key factor, is at an embryonic stage, with remarkably little molecular data currently available. The adult tsetse midgut expressed sequence tag (EST) project provides the first opportunity to identify and study the constituents of the type 2 PM in tsetse (Lehane *et al.*, 2003). The results of the molecular analyses of a number of

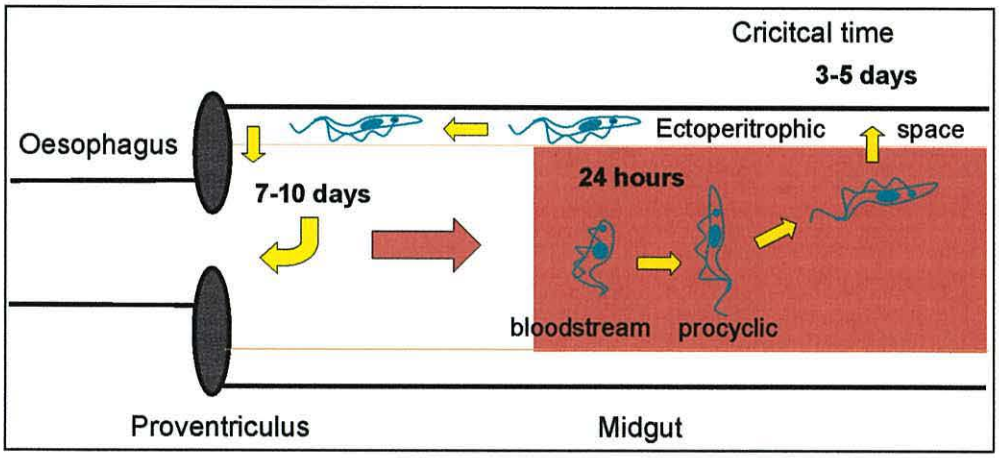
putative peritrophins reported here will provide further information on type 2 PM of adult Diptera and give a more thorough understanding of tsetse-trypanosome interactions. In addition, the PM of intra-uterine larvae has not been previously investigated.

Besides synthesizing the PM, the cardia is also believed to play a role in immunity in adult tsetse (Hao *et al.*, 2003). The cardia and the PM are intimately associated with parasite development within the vector, which suggests that establishment of infection within the midgut is dependent upon their interaction with these two structures in order to successfully migrate between the endo- and ecto-peritrophic space. The life cycle of parasitic protozoan African trypanosomes is complex (Fig. 33), and is initiated with the tsetse vector taking a blood meal from an infected mammalian host. Their initial development within the adult tsetse is rapid, since the short-stumpy form of the parasite, pre-adapted for the environment of the tsetse midgut rapidly differentiates into the procyclic, infective form within the midgut lumen. However, they are retained within the endoperitrophic space by the PM for the first 72 h post ingestion (Gibson and Bailey, 2003), since the type 2 PM is constitutively produced, the parasites have no opportunity to bypass the PM barrier, as is the case in type 1 PM producing insects. Nonetheless, within 6 d post infection the ectoperitrophic space is packed with trypanosomes, although their exact route used to escape the confines of the PM has been debated for many years. It is believed that they either penetrate the PM directly or migrate to the posterior, open end of the PM at the midgut-hindgut boundary (Welburn and Maudlin, 1999). Indeed, recent evidence provided by Gibson and Bailey (2003) support previous T.E.M. studies (Ellis and Evans, 1977; Evans and Ellis, 1983) that show the active penetration of the PM by *T. brucei*. There also remains the contentious issue of the route of trypanosomes from the midgut to the salivary glands, where they mature into metacyclics, ready to be injected into a mammalian host on taking the next blood meal. It is thought that they either migrate across the PM, back into the gut lumen, gaining access to the salivary glands via the cardia and the mouthparts, or that they cross the epithelial barriers of the midgut and salivary glands. The former theory is supported by evidence provided by FISH (Gibson and Bailey, 2003) and light microscopy (Van den Abbeele *et al.*, 1999) studies, in which huge numbers of parasites were observed within the cardia and anterior midgut 6 d post infection, with very low numbers in the posterior midgut. These observations support the hypothesis suggested by Yorke (1933) that trypanosomes may penetrate the soft, newly secreted PM within the cardia in order to migrate through the cardia, and thus gain access to the anterior midgut and salivary glands. However, it must be emphasized that under field conditions tsetse flies are often refractory to trypanosome infections, with typical infection rates of $< 10\%$, of which only a small proportion develop the mature infections necessary to become infective. Clearly, the nature of the physical and physiological barriers to

infection within tsetse require further study in order to gain a clearer understanding of the interactions between the various factors involved.

Since the PM barrier is involved in establishing trypanosome infection in tsetse flies, putative peritrophins were identified from the tsetse midgut EST library in order to further elucidate the role of the cardia in the synthesis of the PM and immunity in tsetse.
A number of contigs were selected for further study, in which their cDNAs were further characterized by determining their tissue-specific transcription profiles in response to blood meal ingestion and trypanosome challenge in all three tsetse species. Such studies are of importance, since disruption of the PM would have major implications for the vectoral capacity of the fly.

Fig. 33. A diagrammatic representation of the development of *Trypanosoma brucei* in tsetse midgut. The route of passage of trypanosomes through the fly gut in which they enter and leave the ectoperitrophic space via the PM. From Wendy Gibson (University of Bristol).



6.3. Materials and Methods

6.3.1. Origin and maintenance of flies

Three Tsetse species were used in this study: *Glossina morsitans morsitans*, *G. pallidipes*, and *G. palpalis*. Puparia were supplied by: The Tsetse Research Laboratory, Langford, Bristol, UK (*Glossina. m. morsitans*) and from The International Atomic Energy Agency Laboratories, Seibersdoft, Vienna, Austria (*G. pallidipes*, and *G. palpalis*). These and adult flies were maintained at 25 °C +/- 2 °C, with 55-60 % relative humidity and a light:dark cycle of 12:12. Flies were fed every two days on sterile defibrinated horse blood (Cat # HB033 TCS Biosciences Ltd, Botolph, Claydon, Buckingham) using an artificial membrane system. For each replicate of the RT-PCR to determine tissue specificity ~ 40 experimental flies were fed a regular blood meal (sexes separate) and dissected 48 h post blood meal (PBM).

6.3.2. Bioinformatics

Genes encoding putative peritrophins were initially retrieved from the *Glossina* midgut EST database [(Lehane *et al.*, 2003), available at: ftp://ftp.sanger.ac.uk/pub/pathogens/Glossina] using the TBlastN function trained on known peritrophins (peritrophins and mucins). The full-length cDNA sequence of each contig was used to search the non-redundant protein sequence databases using the computer program BLASTX (http://www.ncbi.nlm.nih.gov) and SRS (http://srs.sanger.ac.uk/srs5) in order to determine the nearest homolog. Homologues retrieved were used to assign putative function. Further sequencing of partial contigs allowed the functional characterisation of the putative open reading frames (ORFs) encoded by all the genes of interest. Comparative sequence analysis was carried out using DNAStar software programs (Lasergene, Madison, USA). Signal peptide and potential *N*- and *O*-linked glycosylation sites were determined using the SignalP 3.0 (Bendtsen *et al.*, 2004; Nielsen *et al.*, 1997), NetNGlyc (Blom *et al.*, 2004) and NetOGlyc 3.1 Servers respectively (Julenius *et al.*, 2004) (Center for Biological Sequence Analysis, Technical University of Denmark, available at: http://www.cbs.dtu.dk/services/SignalP, http://www.cbs.dtu.dk/services/NetNGlyc and http://www.cbs.dtu.dk/services/NetOGlyc) respectively.

6.3.3. Expression analysis by RT-PCR

Firstly, RT-PCR expression analysis was performed to investigate the tissue-specific expression profiles of putative peritrophins and other peritrophins of interest (chitinases and serine proteases). The cardia, anterior portion of the midgut and fat body tissues were dissected in chilled 0.9 M NaCl solution from cold anaesthetised two-week old flies, 48 h post ingestion of a regular blood meal and were flash-frozen immediately on liquid nitrogen. Total RNA was prepared from ~ 5 mg of frozen cardia, anterior midgut (excluding the cardia), fat body and carcass samples (the latter was first ground under liquid nitrogen using a mortar and pestle) using a rotor-stator homogenizer and the RNeasy® Mini Kit (Qiagen) according to the manufacturer's instructions. The isolated RNA samples were purified by subsequent treatment with an RNase-free DNase 1 (Ambion), following the manufacturer's protocol, prior to checking the concentration and integrity of the isolated RNA by UV absorbance (A_{260}) and running a sample run out on a formaldehyde/agarose/EtBr gel. Approximately 5 μg total RNA was isolated from each sample and between 0.5 μg and 2 μg of mRNA purified from cardia, midgut, fat body and carcass tissue using the Dynabead mRNA Direct Kit (Dynal). cDNA was prepared by first strand cDNA synthesis using mRNA derived from each of these tissues and oligo (dT) primers, according to the manufacturer's instructions (SuperScript First-strand Synthesis System for RT-PCR, Invitrogen). The cDNA was used as a template for PCR analysis using the gene-specific

primers detailed in Table 21, and the thermal cycling conditions: 95 °C for 2 min; 25-30 cycles of: 94 °C for 1 min, 53-55 °C for 1 min, 72 °C for 1 min; final extension 72 °C for 10 min. The reactions contained the following: 2.0 µl 10x PCR buffer, 2.0 µl $MgCl_2$, 2.0 µl dNTPs, 0.25 µl sense primer (20 pM μl^{-1}), 0.25 µl reverse complement primer (20 pM μl^{-1}), 1.0 µl cDNA template and 0.2 µl Taq DNA polymerase (5 U μl^{-1}, Promega) in a total reaction volume of 20 µl. Tsetse *tubulin* cDNA (GenBank accession number AF330159) was used as an internal control for RNA integrity and quantification. The *tubulin* gene specific primers S (5'-ACGTATTCATTTCCCTTTGG-3') and AS (5'-AATGGCTGTGGTGTTGGACAAC-3') were used for normalization of templates synthesized from samples to be compared, prior to running the RT-PCRs with experimental primer sets (see Table 21 for details). Images of the amplification products were captured and quantified following electrophoresis on 2% agarose/EtBr gels using Kodak 1D Imaging software. In each experiment, the number of PCR cycles was chosen empirically to avoid saturation (measured during the exponential rather than plateau phase) and thus attain comparable quantities of PCR products for the different primer sets. Whereas the PCR cycle number was constant in a given experiment for a particular target sequence in the multiple samples analysed.

Further RT-PCR analysis was conducted on a few sequences following determination of their tissue-specific expression profiles in *G. morsitans*. Genes with cardia specific expression patterns were selected since they may potentially encode novel peritrophins. To this end a similar set of tissue-specific expression experiments were conducted with two other Tsetse species for a more meaningful comparison: *G. pallidipes* and *G. palpalis*. In addition, their expression patterns were determined in second (immature) and third instar (mature) intra-uterine larvae dissected from *G. morsitans*. Furthermore, to investigate gene expression in response to immune challenge, three groups of teneral flies (~ 40 each) corresponding to the three tsetse species were given a procyclic trypanosome (YTAT1.1 strain, 10^6 cells ml^{-1}) containing blood meal, supplemented with 0.06M D(+)-glucosamine to enhance the rate of infection. Flies that failed to feed were discarded, the remainder subsequently being maintained on regular blood meals. On day 21, all flies were dissected and scored for midgut parasite infection as either infected (+) or recovered (-), prior to harvesting cardia, anterior midgut and fat body tissues from flies belonging to the two experimental groups. In each case, RT-PCR analysis was performed twice, with total RNA prepared from two independent experiments, and showed the same results as those presented in the results.

Table 21. The primer sets used during RT-PCR analyses. Abbreviations: S, sense; RC, reverse complement.

Name	Sequence (5'-3')	Expected product (bp)
Gmm-3262 (S)	GGGCAGCTTGTGACGGTTTAG	303
Gmm-3262 (RC)	TGCTCTGTGCACACAGATCATTG	
Gmm-2452/2745 (S)	GCTGCTCCATGATAGACGGTTG	384
Gmm-2452/2745 (RC)	ACTTCTGGGCAGCTATCACAAC	
Gmm-3093 (S)	AGAACGACACCACTTCCAACAG	304
Gmm-3093 (RC)	GCATGACACGCAATACCTGAAG	
Gmm-757/1329 (S)	GCGGATGATTCGGATGATTC	184
Gmm-757/1329 (RC)	GCTGTCCGCTTTAGCAACTG	
Gmm-1891 (S)	CAGAGCGTAATAACGGCATGAG	164
Gmm-1891 (RC)	ACGGATTACTCCAAACCCAGTC	
Gmm-1083 (S)	GGCGATGGCAAACCGGAG	120
Gmm-1083 (RC)	CTTTAAGCTTGTTCTCTGGTTCAC	
Gmm-2445 (S)	ATCCTACGCATTATTGGGAATG	150
Gmm-2445 (RC)	CTTTAAGCTTGTTCCGGACAAG	
Gmm-2768 (S)	GACGTGATCCTAGCGGTGTTTG	132
Gmm-2768 (RC)	GCGACATTGGCAAAGACATCAG	
Gmm-7528 (S)	CGATGCAATCATTGGACATG	233
Gmm-7528 (RC)	TTTACTGGAACGTTGCCACAG	
Gmm-8806 (S)	CATGTCACGCCTTTGGTTTC	243
Gmm-8806 (RC)	GTTTCATCCGAACCATCCGTAC	
Gmm-2409/2386 (S)	CGCGTACCGCCCGTCTTATG	343
Gmm-2409/2386 (RC)	TTTCGAGCCCTCATTCCATCCAC	
Gmm-2843 (S)	TGTAGGTATCGTTGTCGGTACTG	462
Gmm-2843 (RC)	GTTCCAACGACATCACCAAC	
Gmm-2709/8983 (S)	GCGGGAAATAGAAACACCAACTAC	303
Gmm-2709/8983 (RC)	CATAGAGCAGTCGGAAGTGCTC	
Gmm-10788 (S)	TTAGCTGGAGCCATGTTATGGTC	274
Gmm-10788 (RC)	TGCGTCGTACAATAAACCGGGAG	
Gmm-3356 (S)	TACTACGTTCCGGACGTGTTG	416
Gmm-3356 (RC)	CACTTTTTTCCTGAGCTTGCTC	
Gmm-3256 (S)	GTCTGCGAACGTTTGAATGAC	358
Gmm-3256 (RC)	GATATGGGAACTGATGTGGTTG	
Gmm-10538 (S)	GTAACGTGTTTAGAAGATGCCAAG	312
Gmm-10538 (RC)	CGGTAAAGCAGTAGAATCAGTTG	
Gmm-10245 (S)	ACCGTTGCTGTGTTGCTTCTTG	148
Gmm-10245 (RC)	TCACGCACTTCCAATAGGCTG	

6.3.4. Cloning and Sequencing of Tsetse Homologs

Comparative sequence analysis of the *G. m. morsitans* homologs in *G. pallidipes* and *G. palpalis* was carried out by firstly obtaining full length ORFs, PCR-amplified from 100ng μl^{-1} genomic DNA with the specific oligonucleotide primers detailed in Table 22, designed to prime the adjacent 5' and 3' UTRs. The PCR amplification conditions were 95 °C for 3 min; 30 cycles: 94 °C for 1 min; 55 °C for 1 min; 72 °C for 2 min; final extension of 72 °C for 10 min.

Table 22. Primers used to obtain full-length sequences of the ORFs, plus a portion of the adjacent UTRs for sequencing of each peritrophin homolog. Abbreviations: *GmPro*, *Glossina morsitans* midgut protein; UTR, untranslated region; S, sense; RC, reverse complement.

Name	Sequence (5'-3')	Expected product (bp)
GmPro1 5' UTR (S)	TGTGGTGCTGTGGAACTTC	416
GmPro1 3' UTR (RC)	TACACAGGGTGTTTATTTTGTG	
GmPro2 5' UTR (S)	GAAGTCGACCGAAGCCTATAAC	345
GmPro2 3' UTR (RC)	CTGAATAAACTCTTACATTGGTTTG	
GmPro4 5' UTR (S)	ACGAGGCGACACTTTGAC	273
GmPro4 3' UTR (RC)	CACTGTGTATTAATCGTACGGA	
GmPro5 5' UTR (S)	GGACGCATATTTGAAATACTTG	717
GmPro5 3' UTR (RC)	GACATTTGCACAGAAAATTAACAC	
GmPro6 5' UTR (S)	AAGAAGTTGAAATAAATTCATAG	668
GmPro6 3' UTR (RC)	TCTATCCTTCAATAGCAGTG	
GmPro9 5' UTR (S)	AAGAAAAGAGCGCAAAACACAG	432
GmPro9 3' UTR (RC)	CGTTTAAAGTCAGATAATGCCGTC	

The amplification products were either used directly or purified by gel band excision using a gel extraction kit (Qiagen) before being directionally cloned. A 2 µl or 4 µl aliquot of each PCR product or gel purified PCR product, respectively was inserted into the pCR II cloning vector using a T/A cloning kit (Invitrogen). The pCR II vector inserts were screened using M13 F (-20) and R primers, under identical thermal cycling conditions to the primary PCR: 95 °C for 3 min; 30 cycles: 94 °C for 1 min; 55 °C for 1 min; 72 °C for 2 min; final extension of 72 °C for 10 min. A 8 µl sample of each screening reaction was analysed by 2% agarose/EtBr gel electrophoresis in 1x TBE buffer to positively identify those clones that contained the insert. Plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen) and accurately quantified using the PicoGreen dsRNA protocol (Molecular Probes, Invitrogen) prior to setting up sequencing reactions. Sequencing reactions were set up according to the manufacturer's instructions (DTCS Quick Start Kit for Dye Terminator Sequencing (Beckman Coulter Ltd.), as quarter reactions which comprised of: 1 µl CEQ Buffer (Beckman Coulter Ltd.), 1 µl DNA (~165 ng μl^{-1}), 9 µl UPW, 5 µl M13 Sequencing Primer (2 pM μl^{-1}) and 4 µl CEQ DTCS Master Mix in a total

reaction volume of 20 µl. The thermal cycling parameters: denaturation of template at 96 °C for 1 min prior to setting up the remainder of the sequencing reaction; 40 cycles: 96 °C for 20 sec; 50 °C for 20 sec; 60 °C for 4 min. Sequencing was carried out on the Beckman CEQ 2000XL capillary sequencer, using CEQ 8000 Genetic Analysis System software (Beckman Coulter Ltd.), with a minimum of two sequencing reads of both the sense and antisense strands. Nucleotide sequences were initially trimmed, assembled and analysed using the DNA Star software programs: SeqMan and MegAlign (Windows 32, version 5, 2001, DNA Star Inc). The nucleotide sequence was aligned by the ClustalW method, with final analysis of conserved mutations within the amino acid sequence.

6.4. Results

6.4.1. Bioinformatics

Putative functions were ascribed to fifteen of the eighteen contigs identified from the *Glossina* midgut EST database by sequence homology. Of which seven contigs were identified as having significant sequence homology to known peritrophins and eleven had the most significant match in the *Drosophila* database (Table 23). The high degree of homology between these two species means that the depth of information available for the *Drosophila* genome is a valuable resource for the study of homologous genes in *Glossina*. Moreover, it implies that the genes encoding chitin binding proteins in *Glossina* and *Drosophila* bear considerable similarities, and thus any immune response involving chitin binding proteins (peritrophins, chitinases or serine proteases) may have similar properties. Full length cDNAs were recovered for twelve of the contigs during the initial screen of the EST database, further sequencing was necessary to obtain the entire open reading frame (ORF) of the remaining six contigs.

Three main groups of peritrophins were identified: homologs of known or putative peritrophins, serine protease proteins and chitinases, the latter two groups of proteins were retrieved due to bias caused by the presence of CBDs within the sequences. Gmm-2452/2745, Gmm-3093 and Gmm-3262 are homologs of putative peritrophins from *Drosophila DmPro6*, *DmPro2* and *DmPro3* respectively (see Chapter 2). Furthermore, Gmm-2445 is a homolog of P-15, a peritrophin from *Chrysomya bezziana*. Of particular interest are the two putative peritrophins Gmm-1329 and Gmm-1891, that are homologs of the cardia specific cDNAs from *G. morsitans morsitans*, *GmPro1* and *GmPro 2* respectively, since the former is believed to be involved in immunity and the latter is an abundant integral PM protein (Hao and Aksoy, 2002). In addition, Gmm-1083 is a homolog of *ICHIT* an *Anopheles gambiae* gene, transcribed at high

levels in the adult midgut and induced in response to microbial challenge (Dimopoulos *et al.*, 1998). Furthermore, Gmm-3356 is a homolog of tsetse EP protein, which is highly expressed in the adult midgut. Those genes found to be specifically expressed in the adult cardia of *G. m. morsitans* were chosen for further study and thereby re-defined as *G. m. morsitans Proventriculus protein 4-9*, in accordance with the notation adopted by Hao and Aksoy (2002).

Three genes encoding putative serine proteases were also retrieved, including Gm-2768, a homolog of *GmPro3* from *G. morsitans*, a serine protease-like protein with a cardia specific expression profile that shares significant sequence similarity with Ssp3, an immune-related protein in *Stomoxys calcitrans* (Hamilton *et al.*, 2002). Gm-7528 is homologous to Tequila, a serine protease from *Drosophila*; Gmm-8806 is a homolog of the trypsin-like serine protease encoded by CG31217, from *Drosophila virilis*. Serine proteases have been implicated in the midgut immune response, as integral components of the protease-activated cascades. Similarly, four genes encoding chitinase-like proteins were also identified, including Gmm-10788, Gmm-2409/2386, Gmm-2709/8983, and Gmm-2843, homologous to the Chitinase 1 precursor found in *Glossina* and the *Drosophila* chitinases CG9357, CG12726 and CG33265 respectively. Three further genes encoding peritrophins that could be putative members of any of the previous three groups were also identified.

SignalP analysis of the amino acid sequences of the peritrophins suggest that the leader sequences of these peptides consist of the first 20-27 amino acid residues (Table 23). When the amino acid sequence of the putative peritrophins were subjected to analysis by N- and O-glycosylation site prediction software (Hansen *et al.*, 1998) the majority of sequences were positive. The number of residues predicted to be N- and O-glycosylated are shown in the Table 23, with details of their position within the amino acid sequence. peritrophins are characteristically N-glycosylated glycoproteins. Indeed, potential N-linked glycosylation sites were found in *GmPro5*, *GmPro6*, *GmPro8*, *GmPro9*, Gmm-1083 and Gmm-3356, at positions 81, 110, 91, 52, 32 and 233 respectively, at sites distant from O-linked glycosylation sites within the amino acid sequence. Mucin domains within mucins or mucin-like peritrophins are characterized by highly O-glycosylated regions that may function as adhesive and, or repulsive domains within molecules that have been implicated in defence against toxins from bacterial pathogens, such as hemomucin in *Drosophila* (Theopold *et al.*, 1996). Thus Pro/Thr rich domains within the putative peritrophins were expected to be highly O-glycosylated. One putative peritrophin, *GmPro5* did contain many threonine residues that were predicted to be O-glycosylated: aa no.19-56, and 117. Similarly, Gmm-2709/8983 contains 21 threonine and 10 serine residues with predicted O-glycosylation. Moreover, the unknown peritrophins: Gmm-

3256 and Gmm-10538 contain 14 threonine plus 6 serine residues and 13 threonine plus 2 serine residues with potential O-linked glycosylation respectively.

6.4.2. Expression Analysis by RT-PCR

Firstly, the expression of the cDNAs from cardia, anterior midgut and fat body tissues was analyzed by semi-quantitative RT-PCR in adult *G. m. morsitans* 48 h post blood meal (Fig. 34). Representatives from all 4 groups of peritrophins identified from the EST database were screened during this initial study. The cDNAs Gmm-1891 and Gmm-2452/2745 are members of the peritrophin-like cDNAs that were found to have cardia specific expression profiles. Whereas, Gmm-757/1329, Gmm-2445 and Gmm-3262 were also detected at low levels in anterior midgut. Gmm-3093 was not expressed in any of the tissues in the assay, but was chosen for further study based on it homology to *DmPro2*, a peritrophin in *Drosophila* (Chapter 2). Hence, these six cDNAs were chosen for further study, and subsequently denoted as *GmPro4* (Gm-1891), *GmPro5* (Gm-3093), *GmPro6* (Gm-3262), *GmPro7* (Gm-2445), *GmPro8* (Gm2452/2745), and *GmPro9* (Gm-757/1329). Fat body tissue did not express transcripts corresponding to these sequences. Gmm-1083 and Gmm-3356 were the only cDNAs from this group for which expression was detected in all tissues. Both members of the putative serine protease group investigated (Gmm-7528 and Gmm-8806) were found to be expressed in all tissues. Whereas, the chitinase-like cDNAs (Gmm-2409/2386 and 2709/8983) were expressed exclusively within the cardia. Likewise, Gmm-10245 showed cardia-specific expression, an unknown cDNA containing CBDs. Whereas Gmm-3256 and Gmm-10538 were expressed in the fat body and the cardia, and all tissues respectively (Fig. 34).

Transcript abundance of *GmPro 4-8* was also analysed from RNA derived from the gut of immature and mature larvae. The expression of *GmPro4* and *6* cDNA was found to be equally abundant in all stages of development. By contrast *GmPro5* appears to be specifically expressed in larvae, since there is no detectable expression in adults. Conversely, *GmPro 7* and *8* transcription products are more abundant in the adult than the larval gut (Fig. 35).

Table 23.
Putative functions were determined by homology identified by TBlastX of the full length cDNA sequence against the NCBI GenBank database in conjunction with information retrieved from the SRS database. Contig or EST numbers identify portions of the entire genome within the EST database. The closest homologues along with the score and E value of the homology are listed. Higher score and lower E value indicates high homology. Abbreviations: *Aedes aegypti* (A.a.), *Anopheles gambiae* (A.g.), *Drosophila melanogaster* (D.m.), *Drosophila virilis* (D.v.), *Glossina morsitans* (G.m.), *Chrysomya bezziana* (C.b.). The presence and location of the signal peptide, predicted using the SignalP 3.0 server is given both as the exact position within the amino acid sequence from the start codon and the identity of flanking amino acids. The position of *N*-linked glycosylation sites and mucin type GalNAc O-glycosylation sites were predicted using the NetNGlyc and NetOGlyc 3.1 Server. Those contigs re-defined as *GmPro* numbers were chosen for further study.

Putative function		Contig/EST number	*GmPro* number	Homologue	Length (bp)	Score	E value	Predicted Signal Peptide (aa)	Potential O-linked glycosylation (aa)	Potential N-linked glycosylation (aa)
PM Chitin binding	A	1891	*GmPro4*	Pro2 (G.m.)	677	77	3e-13	21/22 (TFA-AE)	None	None
		2445	*GmPro7*	P-15 (C.b.)	385	106	8e-23	27/28 (TSA-VR)	None	None
	B	757 + 1329	*GmPro9*	Pro1 (G.m.)	544	205	4e-52	27/28 (VES-TF)	None	52
		1083		ICHIT (A.g.)	661	159	1e-07	20/21 (VLA-CD)	None	32
		2452 + 2745	*GmPro8*	*DmPro6*-PA (D.m.)	1,164	253	e-120	22/23 (TAA-CC)	None	91
		3262	*GmPro6*	*DmPro3*-PA (D.m.)	891	54	4e-06	19/20 (LQC-QE)	None	110
	C	3093	*GmPro5*	*DmPro2*-PA (D.m.)	1,046	64	8e-09	21/22 (CYA-IT)	19-56, 117	81
		3356		tsetseEP protein (G.m.)	1,234	376	e-103	19/20 (VAA-LS)	306, 310	233
Serine protease		2768		Pro3 (G.m.)	556	229	3e-66	18/19 (AKA-SH)	None	43, 132
		7528		Tequila (D.m.)	563	211	6e-54	20/21 (AKA-SH)	173	131, 148
		8806		CG31217 (D.v.)	488	115	3e-25	28/29 (VIA-QQ)	None	None
Chitinase		2409 + 2386		CG9357-PA (D.m.)	397	160	6e-39	23/24 (AEA-KE)	None	101, 448
		2709 + 8983		CG12726 (D.m.)	569	74	2e-02	20/21 (VRA-YA)	43-124	None
		2843		CG33265-PA (D.m.)	1,061	61	4e-08	33/34 (VVG-AV)	None	None
		10788		Chit1 precursor (G.m.)	584	313	1e-84	24/25 (IEA-AN)	None	26
Unknowns		3256		CG16762-PA (D.m.)	1,008	141	2e-32	20/21 (SLA-DY)	187-224, 234-239	None
		10245		CG32950-PD (D.m.)	527	71	1e-11	25/26 (AVA-KP)	None	None
		10538		CG33265-PA (D.m.)	766	44	0.005	22/23 (GNA-VP)	145-171	143

In addition, the *G. m. morsitans* primers (Table 21) were used to successfully amplify the desired product in *G. pallidipes* and *G. palpalis* for only *GmPro 4-8*, since there was no amplification with *GmPro9* primers. The tissue expression patterns of *GmPro 4-8* within *G. pallidipes* and *G. palpalis* (Fig. 36) reflects that detected in *G. morsitans* (Fig. 34) in that the fat-body tissue did not express any transcripts corresponding to the cDNAs investigated. The similarity is further emphasized by the expression of transcripts corresponding to *GmPro4, 5* and *8* solely in the cardia and *GmPro6* and *7* by the cardia and anterior midgut at equal levels. Nonetheless, whilst *GmPro7* was expressed at low levels in the anterior midgut of *G. pallidipes* in agreement with *G. morsitans*, it was shown to be transcribed at a much higher level in *G. palpalis*. Moreover, *GmPro5* which appeared to be not present in cDNA from adult *G. morsitans* was transcribed at very low levels in adults of *G. pallidipes* and *G. palpalis*.

Transcripts of all 5 genes encoding the peritrophins of interest were found to be transcribed at similar levels in infected and uninfected flies (Fig. 37). This suggests that there is no response, at least at the level of the transcriptome by peritrophins to immune challenge by trypanosomes. This is consistent with the expression of *GmPro 1 , 2 and 3* in *G. morsitans* 20 d post ingestion of an infected blood meal, since they were also found to be expressed at similar levels in uninfected flies (Hao and Aksoy, 2002). The only sign of modulation of gene expression in response to the presence of trypanosomes was a significant downregulation of *GmPro1* transcription, observed during the first 8 hours following infection.

6.4.3. Cloning and Sequencing of Tsetse Homologs

The primers used to amplify the ORF successfully amplified the expected product in each case. The entire ORF of each gene of interest was sequenced to reveal how homologous the sequences were between the three species. The alignment of the deduced amino acid sequences for each species indicates that there are wide differences between the number of mutations occurring in the genes studied, ranging from 2 – 20 % of the sequence length (Fig. 38). The residues affected by mutation of the nucleotide sequence are confined to the hypervariable regions of the proteins in each case, with the cysteine and aromatic residues of the chitin binding domains being conserved.

Fig. 34. RT-PCR analysis of tissue-specific expression in *G. m. morsitans*. Shown are cDNA samples derived from RNAs from cardia (lane 1), anterior midgut (lane 2) and fat body (lane 3), with genomic DNA (lane 4) and no template (lane 5) positive and negative controls respectively. Tsetse *tubulin* was used as an internal RT-PCR and loading control. Note: no expression was found in carcass (data not shown), asterisks denote contigs of interest, on which further work was conducted during this study.

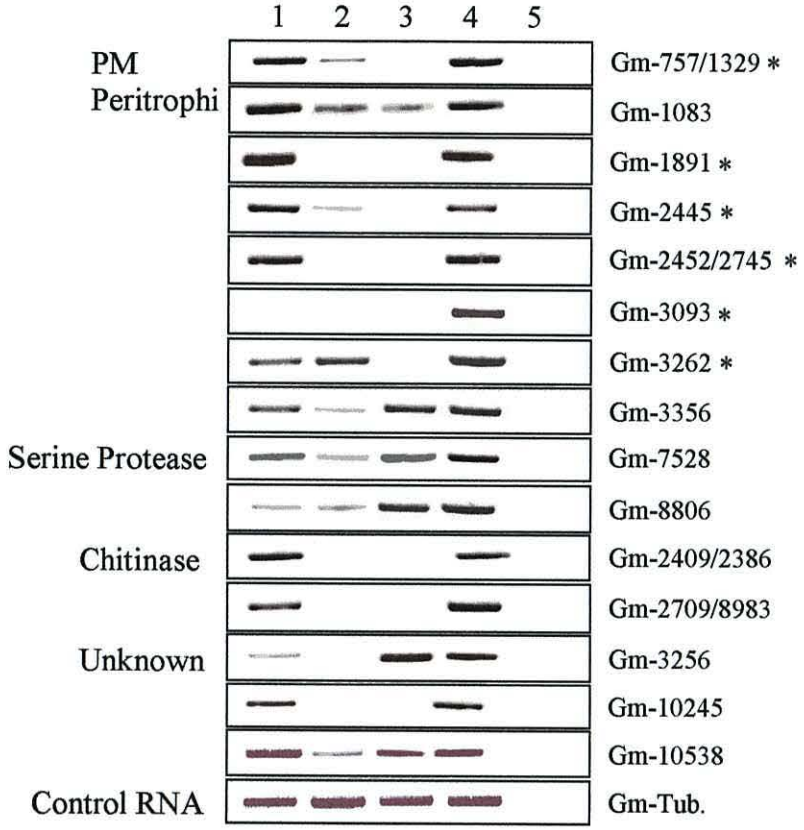


Fig. 35. Agarose gel showing RT-PCR assayed developmental expression profiles of *GmPro4-8* (see text for previous Gmm- numbers) in the gut of second (lane 1) and third instar larvae (lane 2) in comparison to cardia of adult (lane 3) *G. morsitans*, with genomic DNA (lane 4) and no template (lane 5) positive and negative controls respectively. All cDNA templates were normalized using tsetse *tubulin* gene- specific primers. Note: asterisks denote genes of particular interest. *GmPro5* is is exclusively expressed in young and old larvae, *GmPro4* and *6* are expressed at all stages at equal levels, whereas *GmPro 7* and *8* are expressed at higher levels in the adult stage.

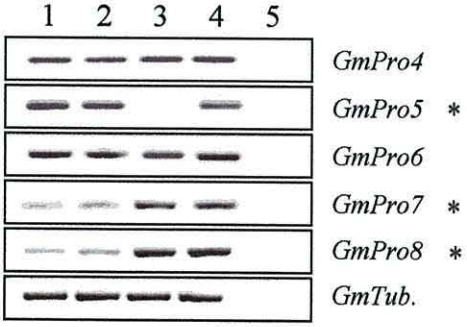


Fig. 36. Agarose gel showing RT-PCR assayed tissue-specific expression of putative Peritrophins in: Fig. 36A *G. pallidipes*, and Fig. 36B *G. palpalis*. Shown are cDNA samples derived from RNAs from cardia (lane 1), anterior midgut (lane 2) and fat body (lane 3), with genomic DNA (lane 4) and no template (lane 5) positive and negative controls respectively. Tsetse *tubulin* was used as an internal RT-PCR and loading control. Note: no expression was found in carcass (data not shown). The tissue-specific expression reflects that observed for *G. morsitans* (Fig. 33) in that the fat-body tissue did not express any transcripts corresponding to the cDNAs investigated. Furthermore, *GmPro4* and *8* were expressed solely in the cardia, *GmPro6* was expressed in the cardia and anterior midgut at equal levels, whereas *GmPro7* was expressed at low levels in the anterior midgut in *G. pallidipes* in agreement with *G. morsitans*, but not *G. palpalis*. *GmPro5*, not detected in cDNA from adult *G. m. morsitans* was present at very low levels in *G. pallidipes* and *G. palpalis*.

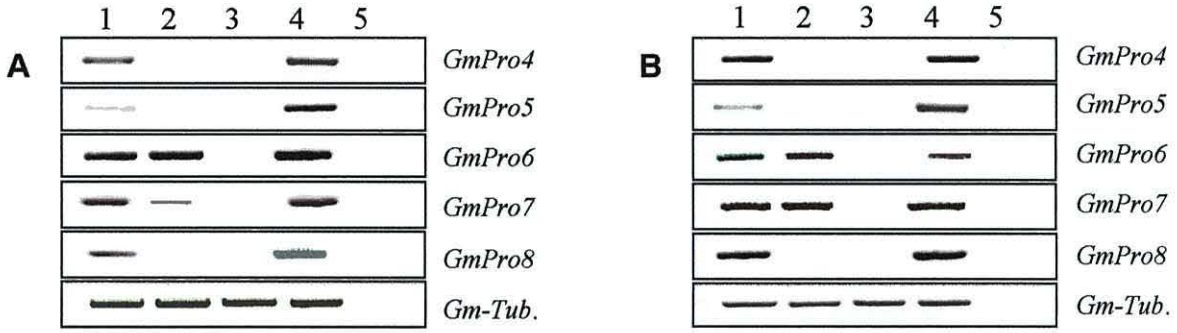


Fig. 37. Agarose gel showing RT-PCR analysis of expression profiles of *GmPro4-8* in response to immune stimuli in adult: Fig. 37A *G. m. morsitans*, Fig. 37B *G. pallidipes*, and Fig. 37C *G. palpalis*. The cDNA temeplate was dervived from cardia RNA 20 d post ingestion of a blood meal containing procyclic trypanosome. Parasite infected (lane 1) and uninfected/ recovered adults (lane 2), are compared with genomic DNA (lane 3) and no template (lane 4) positive and negative controls respectively. Tsetse *tubulin* was used as an internal RT-PCR and loading control for all RNAs. All 5 genes were found to be transcribed in infected flies at similar levels to those detected in uninfected flies.

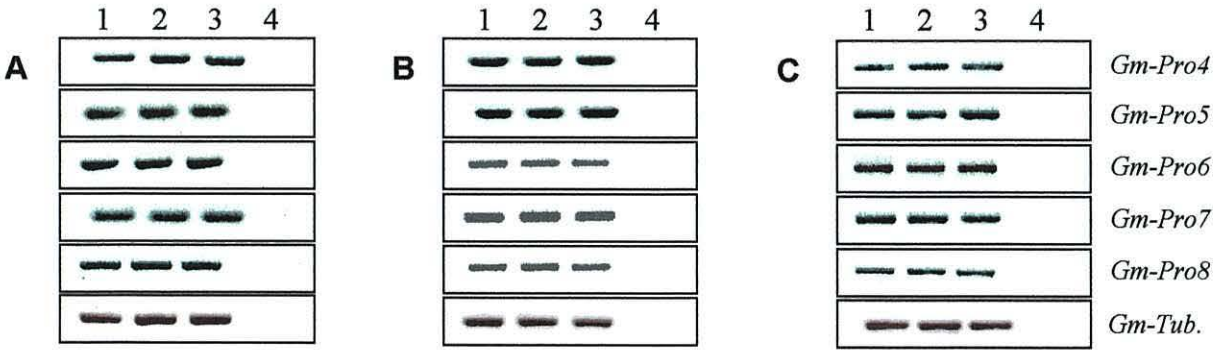


Fig. 38. Alignment of deduced amino acid sequences of peritrophins characterised from *G. morsitans* and their homologs in *G. pallidipes* and *G. palpalis*. Abbreviations: GmPro, *G. morsitans Proventriculus protein*; Gmm, *Glossina morsitans morsitans*, Pall, *G. pallidipes*, Palp, *G. palpalis*. The sequences analysed include: *G. morsitans Proventriculus* 1, *GmPro1* (GenBank accession number AF468865) and *G. morsitans Proventriculus* 2 (AF468866), *GmPro2*, in addition to four of the clones characterised in this study: *GmPro 4*, *GmPro 5*, *GmPro 6* and *GmPro 9*. The number indicates the number of amino acid residues distant from the start codon of the predicted protein. Conserved cysteine residues are indicated in grey boxes and mutations are shown in boldfaced red. The similarities are described in the text. Primers used to generate the sequences were designed to the 3' and 5' UTRs of the sequences.

A. *GmPro1*

```
            1          11         21         31         41         51         61
GmPro1-Gmm  MKYYLCLIVL IAFAVASVKA IAGRSACRDP DELGQTYPHH WDPSKYWYCE KLNENALEMD CPKGQAYMHH
GmPro1-Pall MKYYLCLIVL IAFAVASVKA IAGRSACRNP VELGQTYPHH WDPSKYWYCE KLNEDALEMD CPKGQAYMHH
GmPro1-Palp MKYYFCLIAL IAFAVASVKA IAGRSACRDP VELGQTYPHH WDPSKYWYCE KLNEDALEMD CPKGQAYMHH

            71         81         92
GmPro1-Gmm  LKTCIPWPNW IWKQPQDPPT LA.
GmPro1-Pall LKTCIPWPNW IWKQPQDPPT LA.
GmPro1-Palp LKTCIPWPNW IWKKPQDPPT LA.
```

B. *GmPro2*

```
            1          11         21         31         41         51         61
GmPro2-Gmm  MKAAFCLIVC LAVALSCVLA CDPHGDGKPE CNSSNVNVKQ RNFWDPTHYW ECANAGGEPE NKRCPDSFLF
GmPro2-Pall MKAAFCLIVC LAVGLSCVLA CDPHGDGKPE CNSSNVNVKQ RNFWDPTHYW ECASAGGEPE NKRCPDSFLF
GmPro2-Palp MKAAFCLTVC FAVALSCVLA CNPDGDGKPE CNSSNVNVKQ RNFWDPTHYW ECENATGEPE NKRCDDGLLF

            71         81          93
GmPro2-Gmm  LTEKGDCVIW SEWVWTPPCP EQA.
GmPro2-Pall LSEKGDCVIW SEWVWTPPCP EQA.
GmPro2-Palp SSDKADCIPL GEWVWTPPCP EKL.
```

C. *GmPro4*

```
            1          11         21         31         41         51         61
GmPro4-Gmm  MSKKLIIYLA ILFLAFVCTF AAEDCNPESD GKPICSERNN GMRFRNYWDP TKYWSCNDLK AISNGCQPSK
GmPro4-Pall MSKELIIY-A ILFLAFVCTF AAEDCNPESD GKPICSERNN GMRFRNYWDP TKYWSCNDLK AISNGCQPSK
GmPro4-Palp MSKKLIIYLA ILFLAFVCTF AAGDCNPESD GKPICSGRNN GMRFRNYWDP TKY-SCNDLK AISNGCQPSK

            71         81         91     100
GmPro4-Gmm  LYDEKSQDCI NWYDWVWSNP CPELSAGEIF.
GmPro4-Pall LYDEKSQDCI NWYDWVWSNP CPELSAGGIF.
GmPro4-Palp LYDEKSQDCI NWYDWVWSNP CPELSAGEIF.
```

D. *GmPro5*

```
            1          11         21         31         41         51         61
GmPro5-Gmm  MKFNMKISIL FLALRVSACY AITCPPTPTC PPPTDCLPAV TCPPCTTQNP LPIRTTPLP- TGPSSIYCNE
GmPro5-Pall MKLNMKIYIL LLALRLSACY AITCPPTPTC PPPTNCTNVV TCPPCTTVKP GPIKTTPPPP TGPSGIYCNE
GmPro5-Palp MKFNMKISIL FLALRLSACY AITCPPTPTC PPPTNCTNVV TCPPCTTVKP VPIKTTPPP- TGPSGIYCNE

            71         81         91         101        111        121        131
GmPro5-Gmm  NEMCVGQPDG TMFPDNGSNG YIVCQCECDI PVPCPPGTAF DDGNKVCDHI PTPCPPPPEC LECPQQECPP
GmPro5-Pall SEKCVDQPDG TMFPDNESTG YIFCQCGCDI LLPCPPGTAF DDHNKVCDHI PSPCPSPPAC AECPQQECPP
GmPro5-Palp SEKCVDQPDG TMFPDNESTG YIVCQCGCDI LLPCPPGTAF DDHNKVCDHI PSPCPSPPAC AECPQQECPP

            141        151        161        171        181        191        201
GmPro5-Gmm  CNEHSEVGPS GIACHASKHC VGQPDGATFP LEGKNGFIVC QCECDIERPC AEGTAYDSDL KTCAFINQNY
GmPro5-Pall CDGHSEVGPS GIACHASKHC IGQPDGAMFP LDEKNGFIVC QCECDIERPC AEGTAYDSDL KTCALINQTN
GmPro5-Palp CDGHSEVGPS GIACHASKHC IGQPDGAMFP LDEKNGFIVC QCECDIERPC AEGTAYDSDL KTCALINQTN
```

```
            211  218
GmPro5-Gmm  KRLSQVYH.
GmPro5-Pall RRLS-VYH.
GmPro5-Palp RRSV-NYH.
```

E. *GmPro6*

```
            1          11         21         31         41         51         61
GmPro6-Gmm  MSILLRVICC FLLIEALQCQ EDQTPNIRAW AACDGLVGAM LPHPNDCQRY HVCMNGLAKV EWCPTNLHWN
GmPro6-Pall MSILLRVICC FLLIEALQCQ EDQTPNIRAW AACDGLVGAM LPHPNDCQRY HICMNGLAKV EWCPTNLHWN
GmPro6-Palp MSILLRVICC FLLIEALQCQ EDQTPNIRAW AACDGLVGAM LPHPNDCQRY HVCMNGLAKV QWCPTNLHWN

            71         81         91         101        111        121        131
GmPro6-Gmm  ALASRCDPPE VAKCENNNHK IQVTKFRKVL QPHLSVNEEN LTKQNDCQLC NDLCAQSNKM YLPYPDDCHK
GmPro6-Pall ALASRCDPPE VAKCENNNHK IQVTKFRKVL QPHLSVNEEN LTKQNDCQLC NDLCAQSDKM YLPYPDDCHK
GmPro6-Palp ALASRCDPPE VAKCENNNHK IQVTKLRKVL QPHSSVNEEN LTKQNDCQLC NDLCAQSNQM YLPHPDDCHK

            141        151        161        173
GmPro6-Gmm  YIQCNGNIGY INTCHDLYWN AQLNTCYGYC VPN.
GmPro6-Pall YIQCNGNIGY INTCHDLYWN AQLNTCYGYC VPN.
GmPro6-Palp YIQCNGNIGY INTCHDLYWN AQLNTCYGYC VPN.
```

F. *GmPro9*

```
            1          11         21         31         41         51         61
GmPro7-Gmm  MQFNTLKIIN GIGLWLILAG CLSVVESTFV CPVQQADDSD DSFVMLYPSP TNCSEFYECV RGEALLYACP
GmPro7-Pall MQFNTLNIIN GIGLWLIVAG CLSVVESTFV CPVQQADDSD DSFVMLYPSX TNCSEFYECV RGEALLYACP
GmPro7-Palp MQFNALNMIN GIGLWLIVAG CLSVVESTFV CPVQQADDSD DSFVMLYPSP TNCSEFYECV RGEALRYACP

            71         81         91   97
GmPro7-Gmm  VDLHFNTRRK VCDYPQRAKC QLLKRTA.
GmPro7-Pall VDLHFNTRRK VCDYPQRAKC QLLKRTA.
GmPro7-Palp VDLHFNTRRK VCDYPQRAKC QLLQRTA.
```

6.5. Discussion

The PM is a suitable target for vector control strategies since it is the first line of defence in any insect against ingested pathogens. A possible role for peritrophins in midgut immunity is implicated by its intimate relationship with ingested trypanosomes. All the putative *Glossina* peritrophins appear to be secreted based on the presence of a signal peptide, consistent with their status as putative constituents of the acellular PM, however this remains to be seen since the proteins they encode have not yet been localized to the PM.

Chitin-binding domains (CBDs) are believed to have a role in binding to carbohydrates on the surface of microbes, a theory supported by the finding that the antimicrobial peptide Tachycitin has chitin binding activity (Kawabata *et al.*, 1996). Moreover, mucin domains are highly O-glycosylated, and thus have the potential to function as adhesive domains (lectins). Of particular interest is hemomucin, a protein identified in *Drosophila* that contains mucin domains, localized to many barrier structures, including the PM, that are believed to be involved in antimicrobial defence (Theopold *et al.*, 1996). *ICHIT*, a protein that is transcriptionally induced in response to both bacterial and malaria parasite challenge may also prove to be localized to the

PM (Dimopoulos *et al.*, 1998). The predicted O-linked glycosylation of *GmPro5*, Gmm-3256 and Gmm-10538 suggests that they may contain mucin domains. The occurrence of single predicted N-linked glycosylation sites within the amino acid sequence of *GmPro5*, *GmPro 6*, *GmPro 8*, *GmPro 9*, Gmm-1083 and Gmm-3356, in regions distinct from proline and threonine rich regions is comparable to the domain structure identified in P-55 (Tellam *et al.*, 2003).

The implications of the presence of glycosylated peritrophins in Tsetse PM, if these predicted glycosylation sites are occupied by oligosaccharide side chains include an adhesive role, with the possible implications for the attachment of trypanosomes to the PM surface. In this context peritrophins may be involved in providing the recognition and attachment sites necessary for the association of trypanosomes with the PM, since attachment must precede penetration of the PM (Sordat, 1990). Tsetse midgut lectin activity has a central role in determining refractoriness to infection in tsetse flies. Natural infection rates are typically < 1 %, whilst 100 % infection rates can be achieved when flies are fed specific lectin-inhibitory sugars with the infective blood meal (Maudlin and Welburn, 1987). Moreover, older flies, with a mature PM are just as susceptible as teneral flies when fed the correct inhibitory sugar, indicating that the PM is not a barrier to establishment of midgut infections in tsetse. The lectin-trypanosome interaction is mediated by the procyclin coat of the trypanosome, specifically *N*-linked glycosylation act as ligands for lectin binding, with normal levels of lectin activity inhibiting establishment of midgut infections (Welburn *et al.*, 1994). The mode of refractoriness has been investigated by *in vitro* experiments in which programmed cell death in procyclic culture forms (PCF) of *Trypanosoma brucei rhodesiense* was induced by treatment with the lectin Concanavalin A (ConA) (Welburn *et al.*, 1996; Murphy and Welburn, 1997).

The tissue-specific expression analysis of the peritrophins in adult *G. m. morsitans* showed that transcripts of most putative peritrophins, and one of unknown function were abundant in the cardia (Fig 34). While a low level of transcription of *GmPro 7* and *9* was also detected in the midgut. Transcripts corresponding to Gmm-1083 and 3356 were expressed in fat body tissue, indicating that they are unlikely to be PM constituents. The expression profiles of the remaining peritrophins are consistent with their predicted function (Table 23).

The adult specific expression pattern in *G. pallidipes* and *G. palpalis* closely reflects that of *G. m. morsitans*, with the exception of the much lower levels of GmPro7 transcription in the midgut of *G. pallidipes* in comparison to *G. m. morsitans* and *G. palpalis*. However, these differences may purely reflect the effect of using non species specific primers on RT-PCR efficiency.

Very little is known about the structure and composition of the tsetse intra-uterine larval PM. This is the first report on the molecular characteristics of their type 2 PM, in which the

expression of five genes encoding putative peritrophins were characterized. *GmPro5* mRNA was only detected in larval gut, being absent from all adult tissues, including cardia from *G. morsitans*. Nonetheless, it is transcribed at extremely low levels in cardia RNA from adult *G. pallidipes* (Fig. 36A) and *G. palpalis* (Fig. 36B). The homologue of GmPro5, C17826 is expressed in *Drosophila* adults at much lower levels (copy no. mRNA) in comparison to larvae (Chapter 5). Thus, the developmental expression pattern of *GmPro5*, as defined by RT-PCR, is consistent with the expression of *DmPro2* in *Drosophila*. *GmPro7* and *8* were found to be transcribed at lower levels in the larval gut, compared to adult *G. morsitans*. This expression pattern is not supported by that of the homologues P-15 from *C.bezziana* and *DmPro6* from *Drosophila* respectively, since both are highly expressed within the larval stage. A number of tsetse-control strategies focusing on reducing fecundity by disrupting the PM structure have been suggested (Hao and Aksoy, 2002). They essentially suggest that the development of intra-uterine larvae could be severely impaired either indirectly by disrupting the adult PM, thereby reducing the supply of nutrients vital to larval development or directly by using antibodies or PM proteins as vaccines to specifically target the larval PM. The latter strategy has already been shown to have a huge impact on *Lucilia cuprina* larvae, in which the peritrophins P-55 and P-95 have a profound inhibitory effect on larval growth (Casu *et al.*, 1997; Tellam *et al.*, 2001; Tellam *et al.*, 2003). For this reason *GmPro5* may be a suitable candidate for the development of a similar vaccine, targeted specifically to the intra-uterine larvae of tsetse. To this end it would be necessary to firstly determine the distribution of *GmPro5* with the PM, either by immuno-gold or immuno-fluorescence microscopy. If *GmPro5* proves to be localized to the surface of the PM it could potentially be developed into a vaccine that would have the effect of gumming up the porous PM, drastically reducing its permeability to the products of digestion, leading to the impoverished development or death of the larva.

It has been speculated that the transcription of *GmPro1*, a cardia specific gene may be modulated to some extent by trypanosomes, in order to enhance their survival and development within the midgut, and thus transmission (Hao and Aksoy, 2002). To this end, teneral *G. m. morsitans* were infected with the YTAT1.1 strain of *Trypanosoma brucei* in blood containing D(+)-glucosamine. The transcripts were shown to be unresponsive to the presence of the procyclic form of the parasites 21 d post infection. This implies that they may not responsive to infection, or if they are it is post transcriptionally regulated. Alternatively, they may be transcriptionally responsive earlier during the infection process. A hypothesis which is supported by the downregulation of *GmPro1* detected 2-8 h post infection (Hao and Aksoy, 2002).

Previous experiments with tsetse species and strains have reported inter- and intra-specific variation in the innate susceptibility of flies to trypanosome infection (Okolo *et al.*, 1990). It has been postulated that susceptible lines of *G. m. morsitans* produce more lectin binding sites compared to refractory strains. The variations in sequence homology of *GmPro 1* and *2* (Hao and Aksoy, 2002), and *GmPro5*, *6* and *9* (this study) between the homologs in *G. pallidipes* and *G. palpalis* was evaluated in order to ascertain the link between the role of the PM as a mediator of parasite infection and susceptibility. There appears to be a wide range in the extent of mutations of the putative peritrophin homologs sequenced. However, the majority of mutations occurring between homologs result in conserved changes in the amino acid sequence, indicating that it is likely that the secondary structure, and thus function of the mature peptide is not affected in even the most severely affected peritrophin *GmPro5*. Moreover, mutations are entirely limited to hypervariable regions of all sequences, the conserved cysteine and aromatic regions that are characteristic of CBDs remain unaffected. Thus, the homologs should retain their chitin binding function, and so would be unlikely to be defective peritrophins. However, the mutations within the homologs might play a role in the observed variations in susceptibility between species.

The PM of intra-uterine larvae appears to be much weaker structure in comparison to the adult PM (T.E.M. data not shown). The adult PM is a ~320 nm thick trilaminate structure, with distinct electron dense and amorphous electron opaque layers (Lehane *et al.*, 1996). In contrast to larvae of *Drosophila* and *Aedes aegypti*, *Glossina* intra-uterine larvae have a very privileged nutrient source, provided directly by the mother. It is thus likely to be sterile in comparison to the decaying vegetable matter or bacterial suspensions on which Drosophila and *Aedes aegypti* larvae feed. Thus, the PM of intra-uterine larvae may reflect the benign nature of their nutrient source, since the PM of *Drosophila* and *Aedes aegypti* larvae provides a more effective barrier to the bacterial pathogens that are abundant in their diet (Lehane, 1997).

Chapter 7. Overall Discussion, future perspectives and general summary

This chapter conveys an overarching, contemplative discussion and summary of the whole of this work. It is here that I shall reconsider the aims of this investigation, and to what extent they have been achieved. Additionally, a broader view of the results obtained will allow the identification of future perspectives in this area of research.

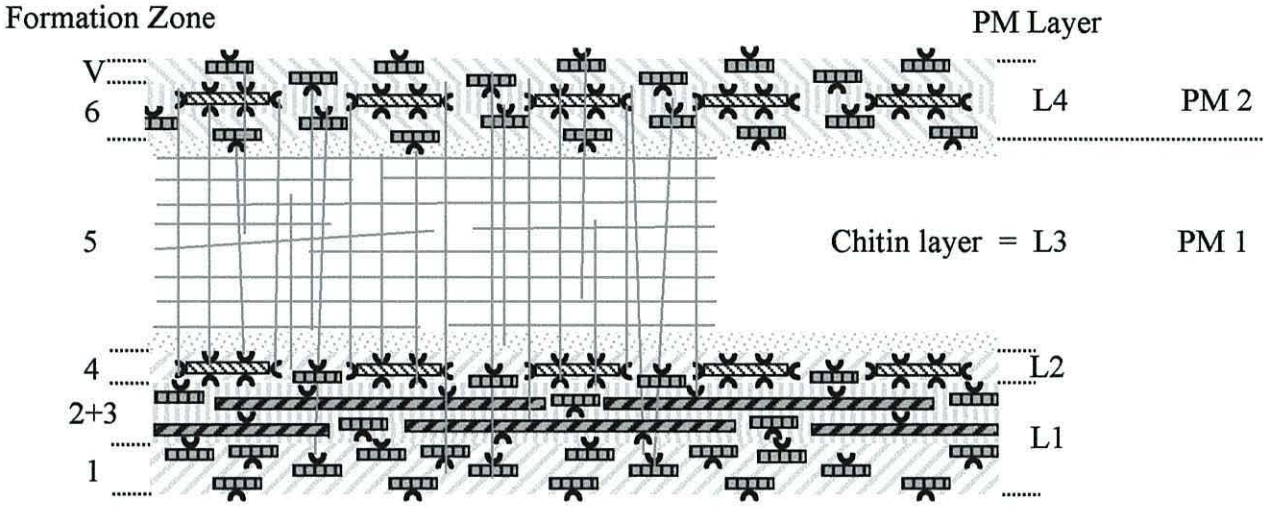
7.1. Concluding Remarks on the type 2 PM in Diptera

In conclusion, our aims were accomplished, although not completely, since a number of key points remain to be resolved. The project provides the first comprehensive study of peritrophins in the type 2 PM of *Drosophila* and *Glossina* and yields evidence in support of the fundamental importance of an intact PM in determining the outcome of *per os* infection in insects. Adult hematophagous Diptera producing type 2 PMs are more refractory to infection, and thus less likely to act as vectors than those possessing a type 1 PM since it is a more effective barrier. The innovative nature of this work is four fold: firstly the isolation, characterisation and *in-situ* detection of novel peritrophin transcripts in *Drosophila*, secondly the use of a deficiency strain (in which there is maximal disruption to PM integrity) to test the susceptibility of PM-compromised larvae to bacterial pathogens, the detection of changes in peritrophin transcription levels in response to age dependent and infection variables, and lastly the identification and characterisation of homologs in *Glossina*.

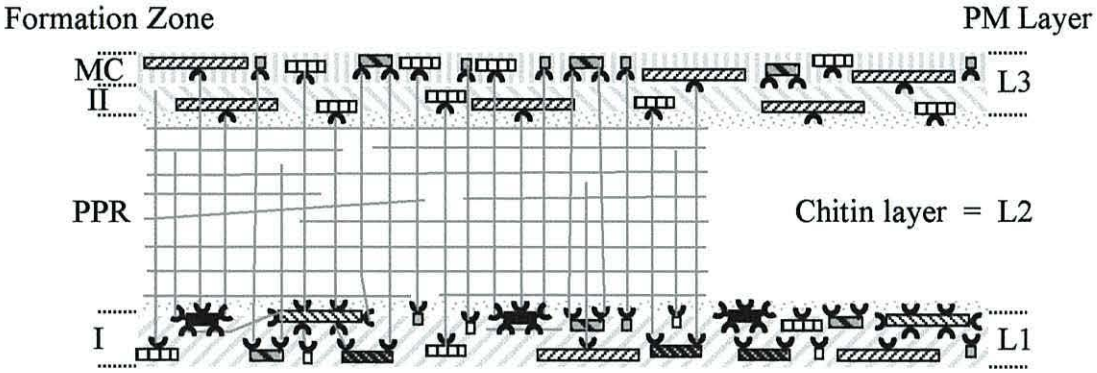
The results demonstrate the potential use of RNAi to knock out the expression of the genes encoding the remaining peritrophins with a view to generating a PM-compromised adult. The traditional classification of PM formation zones within the cardia epithelium (based purely on cell morphology) is out of date and inaccurate in comparison to the (F)ISH study here, which provides a comprehensive set of images of peritrophin transcription zones, including gaps in peritrophin transcription that may be relevant for chitin synthesis. This version of cardia morphology and PM structure may serve as a temporary alternative to that proposed by King (1988) and Rizki (1956) for PM formation in *Drosophila*, prior to conducting further T.E.M. and (F)ISH studies. The PM structure inferred from peritrophin expression profiles within the cardia led to the conclusion that the adult PM is degenerate in comparison to that of larvae, since the adult-specific peritrophin is encoded by the gene *DmPro1*, with the characteristics of a gene becoming defective during the course of evolution. The model suggests that the integrity of the adult PM is mostly retained by a single peritrophin, *DmPro2*, for which there are six CBDs, and therefore the potential for the formation of multiple links to other PM components. The model for PM formation, constructed from (F)ISH data, predicts that chitin and protein components of

Fig. 39. A diagrammatic representation of the newly synthesised *Drosophila* PM in: **A.** adult and **B.** larval as inferred from FISH localisation of transcripts within the cardia (refer to Table 27A and 27B). Note that the chitin rich and peritrophin rich layers are separate; in the mature PM structure the chitin component is likely to infiltrate the peritrophin layers and vice versa through bi-directional diffusion during subsequent maturation of the PM. The thickness of the layers is not proportionate to the actual thickness. Peritrophins are denoted by horizontal bars for which each protrusion equals one CBD. The number in brackets represents the efficiency ratio of the number of CBDs to the length of the predicted amino acid sequence.

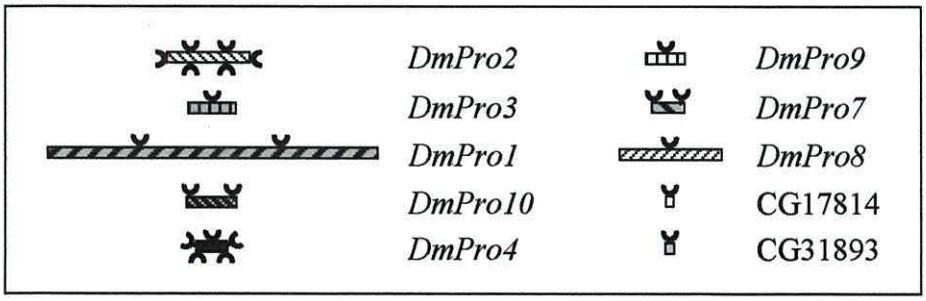
A



B



Key



the PM are more localised than previously thought, and thus are a valuable contribution to our current understanding of type 2 PM structure. Those peritrophins that are more widespread in the PM do not necessarily contribute more to the structural integrity of the PM, since those peritrophins with a single or fewer CBDs are less likely to have an essential structural role. Whilst the loss of *DmPro3* and *DmPro2* function in the deficient strain at the adult stage had no phenotypic change on the adult PM.

There are indications that a degree of redundancy exists between PM proteins in *Drosophila*, which confounded attempts to develop a PM-compromised fly line in which transcription of specific peritrophins were knocked down using RNA interference (RNAi). Thus, peritrophins must play important roles in the physiology of the insect, such as an essential protective or digestion function in order to merit the cellular resources required for the synthesis of multiple peritrophins.

The data suggest that although peritrophin transcription is constitutive overall, it is, at least in part regulated by both physical and chemical stimuli, providing further evidence that some peritrophins have unique roles within the PM. The observed variation in peritrophin transcription during development and in response to infection may be explained in view of the possible role of these molecules in the adaptive modulation of the PM in response to infection. In common with insect cuticle, PMs are composed of chitin microfibrils (embedded in a matrix of proteins), very similar polymers that are organized into a helicoidal and extended pattern in cuticle and PM respectively (Neville, 1975). Moreover, in common with the outer epicuticle (Binnington, 1993), chitin is absent from the outermost, 3-4 nm thick electron-lucent layer of the PM in *Stomoxys calcitrans* (Lehane, 1976). Thus, the PM may be considered as a degenerate cuticle, despite the paradox that there is no evolutionary link between endodermal cells of the cardia, that secrete the chitin containing PM and ectodermal cells, from which the cuticle lined fore- and hindgut originate. However, the cardia may be considered as a composite organ, in which there are cells of ectodermal origin within the endodermal cells of the midgut (annular pad), in addition to those of the foregut invagination (stomodeal valve). Such a perculiar cellular organisation within the cardia may be explained by the presence of an ectopic germlayer of ectodermal cells within the endoderm of the cardia, so that the endodermal cells of the anterior midgut are not the sole progenitors of the midgut, meaning that the foregut/ midgut junction is less distinct than first thought. The migration of proventriculus primordial cells following keyhole formation during cardia morphogenesis may influence the fate decisions that occur during morphogenesis (Skaer, 1993). Alternatively, heterotopic transplantations have been identified by Technau and Gampos-Ortega (1985; 1986), in which foregut cells of ectodermal origin may transmigrate through the midgut epithelium, to reside within the endodermal portion

of the cardia, thereby contributing to the anterior midgut. Therefore, it is realistic that regional localisation of chitin synthesis within the cardia may arise from the specification and differentiation of specialised cells, but definitive evidence is not yet available to distinguish which of the aforementioned two views is correct. Moreover, the intima of the cardia of crickets and cockroaches is made up of cuticular teeth, which serve to disrupt food (Snodgrass, 1935).

7.2. General Summary

As an introduction, **Chapter 1** provides a comprehensive overview of our current knowledge about the origin, occurrence and formation of the PM. In particular, this first chapter focuses on the background that is pertinent to this study, namely, PM proteins and the implications of their degradation on the integrity and thus protective function of the PM.

The objective of this project was to provide a detailed molecular understanding of the type 2 PM of *Drosophila* and *Glossina*. Our first aim was met in the work conducted in **Chapter 2** in which a differential library screening strategy, led to a total of ten previously unknown, cardia-specific clones being isolated from both the adult and larval PM. Reverse Transcription-PCR of putative peritrophins from this initial screen led to the identification of three adult-specific and eight larval specific peritrophins that were further characterised. This approach provided a very sound basis on which to base further characterisation of the peritrophin components of the type 2 PM from *Drosophila* through the isolation of cloned DNAs (cDNAs) that encode cardia-specific genes.

In the first instance, we used a (Fluorescent) *In situ* Hybridisation or (F)ISH approach to accurately determine the site of peritrophin transcription in wholemount cardia (plus adjacent tissue) with confocal microscopy, thereby indirectly determining the distribution of peritrophins within the PM of *D. melanogaster* (**Appendix**). We successfully applied (F)ISH to not only provide decisive proof of the identity of the genes of interest, but also precisely locate their site of transcription within the cardia epithelium. The images obtained using this elegant technique provide evidence of clear-cut zones of expression within the cardia, and thus the localised distribution of peritrophins within the PM. The transcription of adult peritrophins occurs in a number of overlapping regions, with all formation zones synthesising a peritrophin except zone 5: foregut (1-3), 4 and 6. Whilst in larvae, the transcription of peritrophins occurs in only three formation zones: I (PR cells), II (equivalent to zone 6 in the adult cardia) and midgut caecae.

Moreover, the results were used to infer the particular layer of the PM into which these proteins are inserted, and thus predict their distribution within the mature PM.

Therefore, the results of this study provide indirect evidence for a model of the construction of type 2 PM. Furthermore, the number of chitin binding domains (CBDs) on each peritrophin may coincide with suitable structural roles for peritrophins at their predicted location within the adult PM. The expression of larval peritrophins also occurs at distinct formation zones within the larval cardia, separated spatially by a zone of negative-peritrophin expression. It is proposed that, the cells of these peritrophin-minus zones within the adult and larval cardia are responsible for the synthesis of the chitin component of the PM since they actively secrete PM precursors. A new model for the structure of type 2 PMs is proposed, in which rather than being homogeneous, chitin and peritrophins are predominantly localised to the electron lucent and electron dense layers respectively, a degree of fluidity existing between the two. A number of peritrophins have been identified that are most likely to be involved in maintaining structural integrity of the PM. In this way this technique may aid a more thorough understanding of the structure of type 2 PMs. The complex, multilaminar type 2 PM of *Drosophila* and *Glossina* is synthesized by several spatially distinct, readily identifiable formation zones within the cardia epithelium.

During the functional analysis of peritrophins we originally attempted to combine the robustness of deficient fly strains with the specificity of RNA interference (RNAi) in the pursuit of generating a fly strain in which the integrity of the PM is sufficiently compromised to provide direct evidence in support of the protective role of the PM. We attempted to generate fly strains in which individual peritrophins were specifically targeted by RNAi. However, the degree of redundancy between peritrophins in the PM structure made this technique intractable. **Chapter 3** reveals the deficiency *Df(3L)vin3*, in which eight separate larval peritrophins are simultaneously knocked down, causing maximal disruption to the integrity of the PM in larvae. However, the PM-compromised phenotype is characterised by only partial breaks to the PM, in which disruption seems to be limited to those layers of the PM in which the affected peritrophins predominate. The PM-compromised phenotype was manifested by partial thickness breaks in the second and third layers of the PM. Thus, integrity was only moderately weakened in deficient flies, rather than grossly disrupted (such as those phenotypes resulting from experiments in which chitin synthesis is targeted). This suggests that the loss of eight separate integral peritrophins causes sufficient disruption to the PM structure (through the loss of those intrinsic peritrophins required to form invaluable links between PM components) for it to be manifested as localised breaks in the PM. In addition, one of the phenotypes observed in PM-compromised flies is the separation of the larval PM along a plane similar to that of L3 and L4 in

adults. Suggesting that peritrophin(s) with adhesive properties (whose function is knocked out in the deficient stock) may be found at the boundary between L2 and L3 in the larval PM. The findings of the (F)ISH Chapter show that peritrophins may conceivably form links between layers, since *DmPro2* expression pattern predicts that this peritrophin (carrying six chitin binding domains) would be localised to the boundary between the equivalent layers in the adult PM.

Our third aim was to examine the effect of a PM-compromised phenotype on survival. This was carefully explored by a series of bioassays conducted on PM-compromised and PM-normal adults and larvae, involving strains of *S. marcescens* and *S. entomophila*, as described in (**Chapter 4**). Fitness studies yielded tentative evidence that the PM may protect against bacteria and/ their toxins. PM-compromised larvae infected *per os* with pathogenic bacterial strains showed a three to four-fold rise in mortality compared to controls. By contrast deficient adults, that secrete a PM phenotypically equivalent to that of control flies were no more susceptible to infection than control adults.

In order to assess the effects of age, nutritional status and *per os* bacterial infection on peritrophin transcription (**Chapter 5**), adults and larvae were subjected to a series of feeding experiments and the transcription levels of peritrophins assessed using Quantitative Reverse Transcription-PCR (QRT-PCR). Despite starving flies for two days, peritrophin transcription levels in older flies are three times more than those in newly eclosed flies. Transcription of peritrophins was upregulated in response to *per os* bacterial infection in larvae only, whilst adult peritrophins are down regulated. This suggests that the PM may not be purely a mechanical barrier, having the additional capacity to act as a chemical barrier, supported by previous studies in which the expression of other peritrophins from *Drosophila* and *Glossina* are modulated in response to bacterial infection. Perhaps the dynamic changes in transcription levels of the peritrophins encoded by *DmPro4* serve to protect chitin fibrils within the PM from the action of chitinases and other enzymes or toxins in response to infection.

Finally, homologs were identified in *Glossina morsitans morsitans* using a bioinformatics based approach (**Chapter 6**). Eight putative peritrophins were identified from the *G. m. morsitans* midgut expressed sequence tag (EST) database, five of which shared similarities with known PM proteins. Expression analysis indicated that transcripts were preferentially expressed in the cardia of *G. m. morsitans*, *G. pallidipes* and *G. palpalis*. However, their transcription was not modulated in response to trypanosome infection (21 d post ingestion of procyclic trypanosomes). Sequencing analysis of the homologs in G. *pallidipes* and *G. palpalis* showed that sequence variability between homologs was limited to hypervariable regions between the conserved cysteine and aromatic regions that are characteristic of chitin-binding domains

(CBDs). Finally, aspects of each chapter worthy of future investigation are suggested in **Chapter 7.**

7.3. Future Perspectives

Perspectives for future research should include an in depth T.E.M. study of the cardia of *Drosophila* larvae, so as to allow full use to be made of the FISH data, by providing detailed cell morphology information on which to base further distinctions between formation zones and thus their corresponding PM layers. The further use of (F)ISH to localize the remaining PM components will allow us to make more precise models of PM structure. For example, this technique could be used to pinpoint the region(s) of the cardia responsible for chitin synthesis. This study suggests that the zones in which peritrophin transcription is negative are responsible for the secretion of the chitin component of the PM. Zone 5 of the adult cardia and the APR or PPR zones of the larval cardia are presumed to be chitin synthase positive. This would provide direct evidence in support of the hypothesis that chitin is synthesised by specific zones within the cardia. Therefore, chitin may be expected to be predominantly found within the third layer of the adult PM and the second layer of the larval PM, rather than being diffuse throughout all layers.

The precise molecular role of the protein encoded by the gene *DmPro2* within the PM may be elucidated by determining either the 3D structure of the mature peptide or the length and number of CBDs within the mature peptide. The latter could be carried out by a Western blot of PM extract, using a combination of a universal CBD domain and peritrophin specific (artificially synthesised oligopeptide) probe. The fold intensity of the signal from the CBD probe would be expected to reveal the number of CBDs and the protein specific probe the total length of the target. This information could be used in conjunction with the amino acid sequence to determine if the mature peptide is full length or cleaved into a number of shorter peptides. Since these scenarios would have numerous implications in terms of determining the molecular properties and thus function of this, and other key peritrophins within the PM (Wang *et al.*, 2004).

Furthermore, the FISH localisation of peritrophin expression within the cardia of an insect vector, particularly a species for which the larval stage has a different diet from the adult (such as *S. calcitrans*) may prove fruitful in determining the possible relationship between diet and PM structure and thus function.

To date, there have been no attempts to model the tertiary structure of peritrophins. The conformation of peritrophins may be critical to their role within the PM, since steric hindrance may prevent some potential linkages from forming. Whilst the majority of the peritrophin

sequences are too short for it to be possible to perform analysis of the crystal structure (as there would be insufficient constraints to form a meaningful 3D structure) basic tertiary prediction programmes, such as iMolTalk (http://i.moltalk.org) would give some rudimentary structural analysis. In addition, the 3D structure of Tachycitin, an antimicrobial peptide, analogous to peritrophins (GI:10835818, pdb: molecule 1DQC), is available for use as an algorithm in comparative modelling using protein folding prediction programs, such as Swiss Model (http://swissmodel.expasy.org/SWISS-MODEL.html). However, such models are limited in their usefulness, since predicted tertiary structures can often be inaccurate when compared to the real molecule.

This study has also highlighted a number of further questions concerning the modulation of peritrophin transcription in response to physiological and immunological stimulation. The answers to these questions may be sought by conducting similar experiments in which a wider range of variables are taken into account. Firstly, the transcription levels of peritrophins in larvae throughout development would need to be determined, since age appears to be a factor in adults.

Secondly, further analysis of the transcript levels at time points throughout the bacterial treatment period may detect a more significant induction or reduction in gene expression of peritrophins in response to infection. In addition, the flies used in this study had been maintained as a laboratory culture for several years, they may have lost the capacity to react strongly to the presence of pathogens in the gut as shown in previous studies (Boulanger *et al.*, 2001). Flies collected from the field may show relatively strong expression of peritrophins in response to *per os* infection compared to laboratory cultures.

A study in which a range of chemical and biological insults could be utilised to further elucidate the protective role of the PM by determining their effect on transcription of peritrophins. The endotoxin from *Bacillus thuringiensis* (Gill and Pietrantonio, 1996) (Gill and Pietrantonio, 1996) would be used to determine the effect of an abrasive on transcription of peritrophins and thus PM secretion. Whilst the study by Abedi and Brown (1961) may suggest that DDT could be a good candidate, exposure to DDT is unlikely to cause a detectable change in gene expression of peritrophins in wild type flies, since PM hypersecretion appears to be an epiphenomenon, dependant on resistance of the fly strains to DDT, rather than a direct dose-response. This suggests that rather than wild-type flies, DDT resistant fly strains would be necessary for a similar response be detected by QRT-PCR in *Drosophila*. Trypanosomes that infect *Drosophila* may induce peritrophin expression, since *GmPro1* from *Glossina* type 2 PM was found to be modulated by trypanosomes during early infection (Hao and Aksoy, 2002). Furthermore, a study in which peritrophin expression is measured in insects in which the PM is

disrupted by degradation of chitin or PM assembly has been inhibited with Calcofluor may reveal other important aspects of the way in which the PM is modulated in response to such challenges. Thus, it would be beneficial to make some comparison between peritrophin transcription and the expression of other genes involved in PM synthesis, e.g. chitin synthase, in response to development and biological and chemical insults.

The resolution of the remaining questions on *Glossina* peritrophins awaits the analysis of their location within the PM; as such a study may allow the elucidation of their possible role in trypanosome-tsetse interactions. There distribution within the PM may be indirectly determined from wholemount (F)ISH to tsetse cardia and midgut using probes designed to the mRNA, from which the layer within the PM could be inferred. Alternatively they may be directly localized to the PM by immuno-gold or immuno-fluorescence studies.

It has been debated for many years that teneral *Glossina* are particularly susceptible to infection, on the basis of enhanced susceptibility due to a semi-fluid PM. This suggests that the barrier function of the PM is impaired, allowing proportionally more parasites to gain access to the ectoperitrophic space (Willett, 1966). However, since other workers have suggested that flies can be infected at any age, the PM may form as much of an effective barrier to infection in teneral as mature flies (Lehane and Msangi, 1991). Therefore, the use of RNA interference (RNAi) to selectively target key peritrophins in adult tsetse PM and monitoring trypanosome prevalence 20 days post ingestion of an infective blood meal (an indicator of susceptibility to infection) may improve our understanding of the possible role of the PM in infection.

The considerable overlap between the genes encoding *Drosophila* and *Glossina* peritrophins suggests that there may be an evolutionary link between their peritrophin encoding genes. Thus, similar analysis of the nearest *An. gambiae* homologs to these sequences would allow the direct comparison of peritrophins in three Dipterans: *Drosophila*, *Glossina* and *Anopheles*. Which in turn may lead to a more thorough understanding of the composition and structure of the type 2 PM in Diptera.

T. E. M. studies on the PM of intra-uterine larvae would serve to elucidate the role of the larval specific peritrophin *GmPro5* through immuno-gold or immuno-fluorescence localisation, providing useful data on which to base future vector control strategies. Since the already low reproductive rate of tsetse flies could be targeted by reducing the fecundity of tsetse further, using a *GmPro5* vaccine specifically targeted to larvae to inhibit larval growth. This is already being attempted with recombinant *GmPro 1* and *2* (Terry Pearson and Serape Aksoy, pers. communication). It may be productive to conduct similar experiments using the best candidate from my screen, *GmPro5*. Alternatively, since the *GmPro5* transcript is of lowest abundance in

adult *G. pallidipes* and *G. palpalis* it may be possible obtain a null phenotype of reduced fecundity or enhanced infection rate by performing RNAi targeted against this gene.

It is possible that the *GmPro4 - 9*, being putative PM constituents may have a role in tsetse immunity during trypanosome transmission. If they are immune responsive, they may be transcriptionally up- or downregulated earlier during infection, like *GmPro1*. Thus, taking RNA samples from the cardia of flies at earlier time points post ingestion (6-12 d) of the infectious blood meal may yield more meaningful results than those at day 21.

Appendix. Localisation of mRNA transcripts of peritrophins within the *Drosophila* cardia epithelium by *in situ* hybridisation

A.1. Abstract

The purpose of this study was to examine adult and larval *Drosophila* peritrophic matrix (PM) formation *in vivo* using *in situ* hybridisation (ISH) to delineate those regions of the cardia responsible for the secretion of the chitin binding protein (peritrophin) components of the PM. In this way we can indirectly determine the content of each layer of the PM, as we know which layer is produced by each region of the cardia. This coupled with previous studies of descriptive morphology (Rizki, 1956; King, 1988; Dimitriadis, 1985) may lead to a better understanding of PM formation in type 2 PM producing insects. Indeed, a unique insight into the formation of the type 2 PM in *Drosophila* has been gained using this technique to delineate the formation zones of a number of peritrophins.

A.2. Introduction

The anatomy of the cardia has been examined in detail in a number of species, including *D. melanogaster* (King, 1988; Rizki, 1956), *S. calcitrans* (Lehane, 1976a), *M. domestica* (Zhuzhikov, 1963), *Lucilia cuprina* (Eisemann *et al.*, 2001), *Glossina* (Wigglesworth, 1929; Moloo *et al.*, 1970; Lehane *et al.*, 1996;) and *C. erythrocephala* (Becker, 1977). The discovery that this impressive, complex organ is composed of a number of distinct zones and that some Diptera are capable of synthesizing multiple PMs from an elaborate network of cardia epithelia suggests that the secretion of type 2 PMs meets a fundamental physiological requirement in insects and therefore merits further study. Becker (1976) showed that each of the 3 PMs in *C. erythrocephala* is secreted by its own distinct formation zone. Moreover, previous studies of the adult cardia in *D. melanogaster* (King, 1988) suggest that even the four layers of the single PM can be traced back to a number of specific regions within the cardia epithelium, each with its own characteristic cellular specialisation. The report by Rizki (1956) identifies a ring of cells that secretes the PM in *Drosophila* larvae, but this interpretation seems to be more subjective than well supported, and has not been documented since. However, the detailed localization of cells responsible for the secretion of PM chitin binding proteins within the cardia of *Drosophila* is lacking, in spite of the attention that the midgut and this model insect has received. Indeed, it is possible to probe specific PM components since Wijffels (2001) successfully localized P-15 secretion within the cardia of *L. cuprina* larvae by immunogold staining of the protein.

First developed during the 1970's, *in-situ* hybridisation (ISH) is an extremely useful technique that allows the localisation of DNA or RNA *in-situ*. ISH to mRNA provides a valuable insight into the pattern of gene expression, both temporally and spatially of the target,

with the added insight of cellular resolution. Whilst the related techniques of Northern hybridisation, subtractive cDNA library screening and RT-PCR are much more sensitive in comparison, they are better suited to assay transcript levels in relation to various specific tissues, developmental stages and physiological states.

The basic principle of the technique is to fix the tissue so that all the mRNA being transcribed at the time is retained within the cells, including those transcripts of the gene of interest. Tissue is normally fixed using a crosslinking fixative such as paraformaldehyde or formaldehyde. The mRNA is detected *in-situ* by hybridisation with a RNA probe (riboprobe) labelled so that it can be later detected either by ISH or Fluorescence ISH (FISH). The size of the tissue dictates if hybridisation can be carried out on intact (i.e. whole mount) or sectioned material. An understanding of the thickness and fixation of the material is paramount, as both may impede penetration of the probe. Whole mounts have the advantage of simplicity and can give a much better sense of the pattern of gene expression by higher resolution afforded by the maintenance of cell structure. Transcripts can be detected using either DNA or RNA probes. The former being synthesised from cloned DNA in a variety of ways, including polymerase chain reaction (PCR), random priming and nick translation; riboprobes are prepared by in-vitro transcription of either PCR products in which SP6, T3 or T7 polymerase promoters have been incorporated or cloned DNA within a suitable vector such as pBluescript that has the bacteriophage promoters located either side of the multi-cloning site. Riboprobes are usually more sensitive and reliable than DNA probes for the determination of transcript location. Oligoprobes, in comparison are capable of even more precise labelling, provided they can be designed so that they span a known exon-intron boundary. Some of the earliest protocols involved the use of isotopically labelled probes (^{3}H, ^{32}P, ^{33}P, ^{35}S, ^{125}I), detected following hybridisation using autoradiography. However, there were many limitations of this technique, as with any technique involving radioisotopes, mainly that resolution was poor, tissue preparation was more involved and exposure times had to be determined empirically. Derivatized nonisotopically labelled (biotinylated, digoxygenin (DIG) or flourescein) nucleotides, have since replaced radiolabelled nucleotides.

One important consideration when using biotin- or dioxygenin-labelled rNTPs to label probes is that the modified nucleotide must be used at a low concentration in the transcription reaction, so as to ensure that relatively few labelled nucleotides (typically 1 labelled nucleotide per 20-25 unlabeled nucleotides) are incorporated per molecule of probe. If more modified nucleotides are incorporated into the probe than required, the hybridisation efficiency of the probe to the target mRNA or DNA can be affected. In addition the posthybridisation recognition of the labels on the probe by antibodies is most efficient when only a few bases are replaced by

labelled nucleotides. Furthermore, riboprobes exceeding 300 nucleotides long are unsuitable as *in situ* probes for ISH to wholemounts. Small probes (~100 nt) can penetrate tissue that has been partially crosslinked by fixation more efficiently than larger fragments of RNA. If there are penetration problems that are believed to be caused by the length of the probe, it is possible to reduce the length of longer RNA probes by limited alkaline hydrolysis.

Related *in-vivo* techniques to (F)ISH include the incorporation of PCR into the reaction and staining with an antibody to label the nascent protein. InSitu PCR and RT-PCR techniques were specifically developed for the detection of rare transcripts that (F)ISH cannot easily detect, combining the cellular localization of ISH with the sensitivity of PCR (Nuovo, 1994; Nuovo, 2001). Bauer (2002) used immunostaining to localize the secreted proteins encoded by the genes *fork head* (Fkh) and *defective proventriculus* (*Dve*) in the cardia of *Drosophila* embryos. Similarly, Kolmer (1994) detected diazepam-binding inhibitor (DBI) immunoreactivity in the cardia of adult flies treated with and Anti-DBI antibody. Furthermore, Meola *et al.* (1998) characterised the location of three tachykinin-related peptides using whole mount immunocytochemistry in the mosquito *Culex salinarius*, the cardia being one of the immunopositive organs.

A.3. Materials and Methods

The ISH procedures detailed in protocol 2 and 3 chart the development of a series of modifications made to protocol 1, originally described by Tautz and Pfeifle (1989) that were required to successfully label transcripts of chitin binding proteins expressed within the epithelium of wholemount cardia. The modifications made by the Carroll Lab to the original protocol included an increase in the number, and the duration of the hybridization washes with hybridization buffer. The background signal was dramatically reduced as a result, probably because the hybridization buffer was more stringent than PBT, used during washes in the original protocol. The Carroll Lab protocol is available at: http://www.molbio.wisc.edu/carroll/methods/insitus/DigInSitu.html.

All the following steps were carried out in Eppendorf tubes with the simple replacement of fluids for at each step. Each prep was triplicated and comprised of ~ 20 cardia, including a short portion of the adjacent foregut and midgut, plus gastric caecae in larvae. Adult and larval tissues were dissected out in cold phosphate-buffered saline (PBS) (139mM NaCl, 10mM Na_3PO_4, pH 7.2).

Fig. 40. The molecular structures of the fluorophores used in the FISH analysis: A, DAPI; B, FITC; C, Cy3; and D, Cy5.

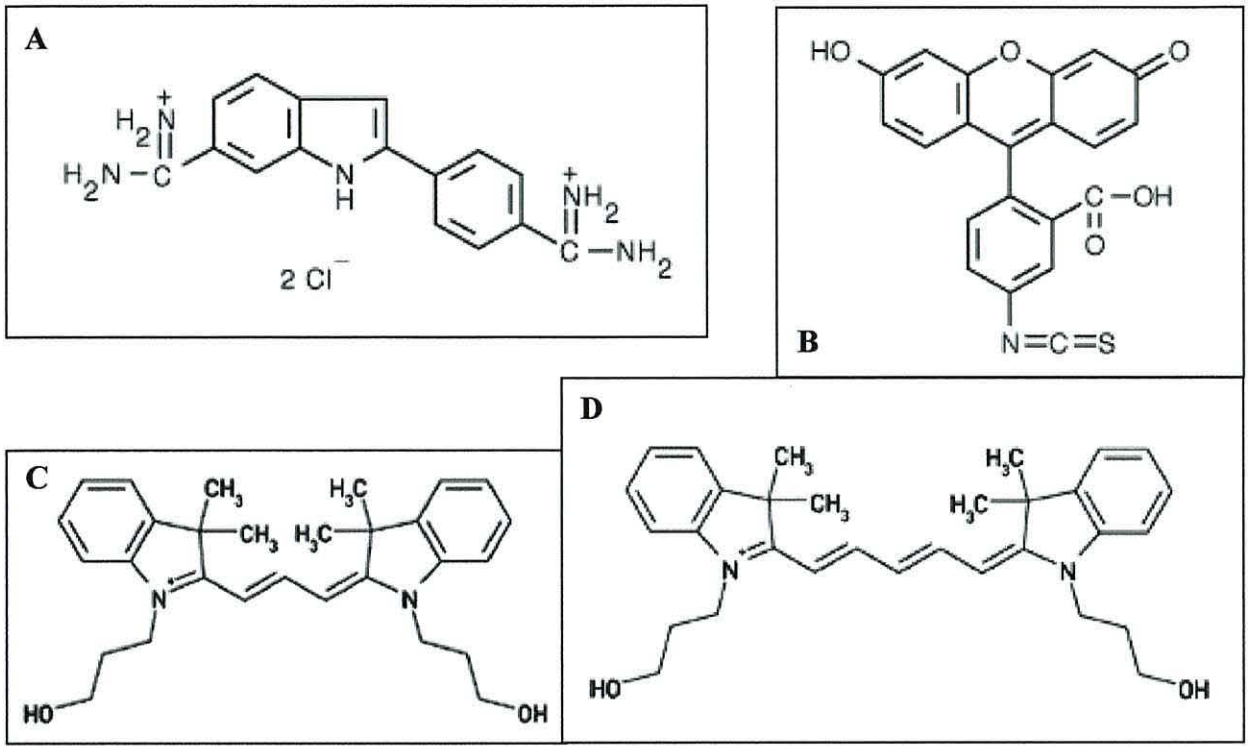


Table 24. Peak wavelengths of the absorption and emission spectra of the above fluorophores, plus DAPI. From Jackson Research Labs Inc. Technical Information.

Fluorophore	Absorption Peak (nm)	Emission Peak (nm)
DAPI	365	420
Fluorescein, FITC	492	520
Cyanine, Cy2	492	510
Indocarbocyanine, Cy3	550	570
Indodicarbocyanine, Cy5	650	670

Note: Approximate values are given for the purpose of comparison between fluorophores only.

A.3.1. Protocol 1. Carroll Lab Protocol for ISH of Whole Mounts with RNA probes

A.3.1.1. Tissue Preparation

Dissected tissue was placed in 4 % Paraformaldehyde fixative for 2 hours at R.T. or O/N at 4°C. Material was washed 3 X in 1 X PBS, 5 min each and treated with 200 µl Proteinase K (5 ug/ml working from 500 ug ml^{-1} stock soln) for 10-30 min at 37 °C, before being washed 2 X 10 min in DEPC SDW at 4 °C. The tissue was post-fix treated in 4% Paraformaldehyde for 10 min at R.T. or 20 min at 4 °C and washed 3 X in PBS containing 1 % hydroxylammonium chloride, 5 min each. The tissue was then rinsed in PBS for 5 min, before pre-incubating in HS for 30 min to 2 hr at 50 °C (RNA target-RNA probe).

A.3.1.2. Probe Synthesis

Short (approx 100nt) digoxigenin labelled riboprobes were synthesized using the T7 Mega-shortscript Transcription Kit (Ambion) and digoxigenin-11-UTP (Roche). The labelled RNA was recovered by ethanol precipitation and resuspended in nuclease-free water at a final concentration of 17 ng/µl. The precise concentration of purified resuspended RNA probe was determined using the Picogreen RNA quantification protocol (Molecular Probes) on the Wallac

Table 25. Details of the primer sequences and the length of the resulting ISH probes. Note: the length includes the T7 or T3 promoter on the 5' end of the primer in each case, the sequences of which are T3, 5'-ATTAACCCTCACTAAAGGGAGA-3' and T7, 5'-TAATACGACTCACTATAGGGAGA-3'.

Name	Sequence (5'-3')	Probe length (nt)
DmPro1 (T3 S)	GACACCGAATCGACTCCTG	102
DmPro1 (T7 AS)	AACCTGCTGCACGACTTTG	
DmPro3 (T3 S)	TGGTCTGGATGTTGATGTC	115
DmPro3 (T7 AS)	GCTTTGGTGAAGCAGTTG	
DmPro2 (T3 S)	CGCTACGCAGTTCTTCAATAC	111
DmPro2 (T7 AS)	AGTCGGGATCGCAGGTTTTG	
CG14645 (T3 S)	CATCGTATTGGAAGTGTGAGAC	111
CG14645 (T7 AS)	CTTGAGACTATCCATGAATCCTG	
GAPDH-2 (T3 S)	CGGCAACTAAGCTCACAAG	96
GAPDH-2 (T7 AS)	TATGGCCATTTCACCTGCTC	

Victor 2 1420 multilabel counter (Perkin-Elmer, Life Sciences, Turku, Finland) with the aid of Wallac Workout Software Version 1.5. The hybridisation probes are described in Table 25. Probes are transcribed from PCR products synthesised from clones of the genes encoding the genes of interest.

A.3.1.3. Probe Hybridisation and Detection

Each probe was used at a working concentration of 2 – 10 ng μl^{-1} (5X final concentration in formamide), with 6 µl of probe-formaldehyde mixture (1 µl probe, 2 µl SDW, 3 µl formamide) being denatured at 80 °C for 10 min and quenched on ice. To which 24 µl hybridisation buffer was added to create a hybridisation mixture. During hybridisation the tissue was incubated for 16 - 20 hr at 50 °C in a humid chamber. Next, the specimens were washed stringently during a series of posthybridization washes in: 2 X SSC at R.T.for 1 hr; 1 X SSC at R.T.for 1 hr; 0.5 X SSC at R.T. for 30 min, plus an additional 30 min at 37 °C. The tissue was incubated in Anti-DIG FITC for 30-90 min (working solution is ~ 1:500 dilution of stock solution in PBS) at 37°C and washed 3 X PBS, 42°C, for 20 min each. The tissue is finally mounted in glycerol PBS on slides.

A.3.2. Protocol 2. Silviter Lab Protocol for ISH of *Drosophila* Gut Tissue using Riboprobes

There were a number of key modifications made to the protocol so that it was suitable for the detection of mRNA within *Drosophila* gut tissue. The DIG labelled probes from Protocol 1 were used.

A.3.2.1. Tissue Preparation and Fixation

The cardia and a short portion of the midgut adjacent to the cardia were dissected out in PBS and fixed for 40 min in 5% formaldehyde in PBT. The tissue was washed 3x with PBT for 5 min each and incubated for 3 min in 4 µg ml^{-1} proteinase K in PBT (this is optimised for *Drosophila* embryos and gut tissue, other tissues may be require either longer or reduced incubation times, even the elimination of the proteinase K treatment). The protease reaction was stopped by washing 2x with PBT containing 2 mg ml^{-1} glycine for 10 min.

A.3.2.2. Hybridisation and Washing

The tissue was washed 2x with PBT for 10 min and postfixed in 5% formaldehyde in PBT for 20 min. They were then washed 5x with PBT; 1x with 50% PBT: 50% hybridization solution (HS)

(50% deionized formamide, 5x SSC, 200 µg/ml tRNA, 100 µg/ml sonicated, boiled salmon sperm DNA, 0.1% Tween-20) and 1x with 100% HS prior to placing them in 1 ml of HS for 1 hr of prehybridization at 55°C. The majority of the HS was removed, so that only 100µl remained, to which 17 ng of heat-denatured probe was added to each tube containing a separate tissue sample in 100 µl of HS. The probes were hybridized at 55°C for between 24-48 hrs (usually 40h). During posthybridisation washes the tissue was rinsed 5x in 1 ml of 55°C HS, each for 1 hr, with the final wash overnight. Next, the tissue was washed 1x with 50% HS: 50% PBT, and 4x with room temperature (R.T.) PBT before commencing with probe detection.

A.3.2.3. Probe Detection

The tissue samples were incubated in 1 ml of a 1:2000 dilution of a preadsorbed Anti-DIG antibody conjugated to alkaline phosphatase (AP) (Roche, Cat # 1093274) for 2 hrs at R.T. The excess of antibody was washed out with at least 10 changes of PBT over 2 hrs, before equilibrating the tissue with 2 changes of staining buffer [100 mM NaCl, 100 mM TrisHCl (pH 9.5), 0.1% Tween 20, 1x PBS] and developing the stain (for 15 min - 2 hrs) in 1 ml of staining buffer to which 20 µl of NBT/BCIP solution (Roche, Cat # 1681451) had been added. Poorly transcribed messages can be detected by developing the stain overnight at 4°C after 1 or 2 changes of developing solution. The developing reaction was stopped by washing 5x with PBT + 50 mM EDTA, and the tissue cleared overnight at 4°C in 10-90% glycerol, 50 mM EDTA to further reduce background, before mounting the tissue on slides the next day in a drop of gelatin and glycerol mountant heated gently to 40-50 °C (7g gelatin melted gently in 42ml SDW, to which 50 µl glycerol and 50ml Phenol:OH/$CHCl_3$ 1:1 are added, mix well) before sufficient further melting to seal the coverslip. The slides were stored at 4 °C until viewing on a Differential Interference Contrast (DIC) (Olympus, T1R 1X71) microscope, to obtain DIC and brightfield images.

A.3.2.4. Probe Synthesis

i) DNA Template Preparation

PCR synthesis was performed to create cDNAs corresponding to target sequences from a cloned fragment of DNA, with a T7 promoter conjugated to the gene specific portion of the sense primer and a T3 promoter on the antisense primer (see Table 25). So that the PCR product can be used to synthesize both the antisense RNA (positive probe) and sense RNA (negative probe). The PCR products were purified with an equal volume of phenol:chloroform:isoamyl alcohol. From this point on the DNA was handled with RNase-free tips using RNase-free reagents. The DNA was precipitated with ammonium acetate (0.5M) and 2 volumes of EtOH (100%),

incubated 20 min at -80°C, and centrifuged for 20 min at 19200 xG, 4°C (Jouan), the supernatant aspirated and the pellet rinsed with 70% EtOH, air dried and resuspended in 10 µl UPW, before 1 % being utilised for the PicoGreen protocol (Victor) to estimate concentration of the purified template DNA for the transcription reaction, ideally 100-200 ng μl^{-1}.

ii) Transcription

For each transcription reaction, the following components were combined at R.T.: 2 µl reaction buffer; 2 µl of the rNTPs A, G and C, and 1.3 µl UTP; 0.7 µl Dig-11-UTP; 200ng template DNA; 2 µl T7 or T3 Megashortscript enzyme and UPW to a total reaction volume of 20 µl. The reaction was mixed gently by pipetting and incubated at 37 °C for 2-4 h.

iii) DNase I Treatment and Quantification

The DNA template within the transcription reaction was destroyed by firstly treating with 5 µl "DNA-free" sludge (DNA-free™, Ambion) and recovering the supernatant and placing it into a new tube, before being denatured at 95 °C for 3 min, quenched on ice, and adding 2 µl 10x DNase buffer and 2 µl (4U) of RNase-free DNase I to the reaction, and incubated at 37°C for 15 min. The "DNA-free" sludge procedure was repeated, and the RNA ppt at –80 °C for 20 min and purified by washing the pellet 3x in 70 % EtOH. The pellet was dried and resuspended in 20 µl UPW or RNase-free TE. 1 µl of resuspend probe was quantified using the RiboGreen assay (Molecular Probes) and on an RNase-free 2 % agarose gel.

iv) Estimation of Incorporation Efficiency of DIG-labelled RNA

The labelling efficiency of the synthesized probe was measured by dot blot in comparison to digoxigenin-labelled RNA standards (Roche) with Anti-DIG-antibody detection. Aliquots of the probe were prediluted, 1 µl of neat RNA probe (10 ng/µl) dotted onto nylon membrane (Hybond-N) and the same with a series of 10-fold dilutions down to 1pg, including a water control. The membrane was crosslinked by UV in a Stratalinker for 3-5 min, and blocked in 10 ml 1x blocking solution [(Roche, Cat # 1096176) diluted from 10x stock in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl pH 7.5)] for 30 min. The blocking solution was replaced with 10 ml antibody solution [Anti-DIG-alkaline phosphatase (Roche, Cat # 1093274) diluted to a concentration of 1:5000 (150 mU ml^{-1}) in blocking solution] and incubated for 30 min. The membrane was washed 2 x in 10 ml washing buffer [0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3 % (v/v) tween 20] for 15 min each, and equilibrated in 10 ml detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 2-5 min. The detection buffer was replaced by colour subtrate solution [prepared freshly from NBT/BCIP stock solution (Roche, Cat # 1681451), 40 µl in 2 ml detection buffer] and the colour was allowed to develop in the dark until the spots appeared at a sufficient intensity to read, the reaction being stopped by washing with TE buffer for 5 min. The spot intensities of the control and experimental dilutions can be used to estimate the

concentration of the experimental probes accurately. The probes were used in hybridization reactions at a concentration of ~17ng 100 μl^{-1} hybridisation solution and stored at -80°C in small aliquots before use.

A.3.3. Protocol 3. Short Protocol for FISH of *Drosophila* Gut Tissue using Oligoprobes

A.3.3.1. Fixation

Dissected tissue was transferred to formaldehyde PBT fixative and incubated at R.T. for 40 min. The fixative [5% formaldehyde in PBT (PBS, 0.1% Tween 20)] was prepared in sterile nuclease-free water, whereas all other solutions were rendered nuclease-free by treatment with diethyl-pyrocarbonate (DEPC) overnight at room temperature, followed by autoclaving. After fixation, the preparations were washed by quickly replacing the fixative with PBT plus twice more in PBT for 5 mins and permeabilised in 4 $\mu g\ ml^{-1}$ proteinase K for 3 min. Tissues were then incubated in two changes of PBT containing glycine (2mg ml^{-1}) for 5 min to arrest digestion and then washed twice in PBT for 10 min, before finally being postfixed in 5% formaldehyde in PBT for 20 min, followed by washing out the fixative immediately in PBT and then rewashing twice more. Tissues were washed by replacing the solution with HS, plus once more in HS in order to prepare the tissue for hybridisation.

A.3.3.2. Hybridisation and Washing

Tissues were hybridised according to a modification of the method developed for digoxigenin-labelled DNA probes by the Carroll. The hybridisation solution contained no formamide, instead equally stringent conditions were obtained in accordance with the desired conditions of 0.9 M NaCl, 0 % formamide and ~55 °C hybridisation according with the universal melting temperature (Tm) formula of Lathe (1985). The hybridisation solution (HS) consisted of 10mM KPO_4, 5 x SSC (20 x SSC is 3M NaCl, 0.3M sodium citrate, pH 7.0), 140mM NaCl, and 0.1% Tween 20. The tissues were first washed for 10 mins in 1/1 PBT/HS and for 10 min in HS. Pre-hybridisation took place for 60 min at 55°C in a heat block, after which most of the supernatant was removed so that only 100 µl remained, whereupon 1 µl oligoprobes were added in a total of 15 µl HS at a concentration of 1 µM (= ± 5 ng μl^{-1}) per probe. Hybridisation took place at maximum stringency by keeping the closed eppendorfs at 55 °C in a heat block overnight (~ 15 h). Next the tissues were washed four times at 55 °C by firstly replacing the solution and then incubating in fresh HS for periods of 60 min, 180 min and 60 min, once in HS/PBT and finally 2x in 100 % PBT.

A.3.3.3. Mounting and DAPI-counterstaining

The experimental cardia were pipetted out of the preceding PBT washing buffer onto microscope slides and mounted in ~ 25 µl Vectashield mountant (Vector Labs) containing DAPI (4',6-diamidino-2-phenylindole) (1.5 µg/ml) to preserve the fluorescence signal and counterstain tissue (Note: Cy3 will dissipate in the tissue over time). The preparations were covered, sealed and stored in the dark at 4 °C until they could be examined.

A.3.3.4. Confocal Microscopy

Images were created in the form of Z-stacks and reconstructed into 3D-images using a laser scanning confocal microscope (LSM 510 confocal, Zeiss), equipped with a Krypton-Argon laser for use with FITC (excitation filter: 450-490 nm; emission filter: 515-586 32 nm), Cy3 ™ (excitation filter: 540-552 nm; emission filter: 590^{+} nm) and Cy5™ (excitation filter: 610-650 nm; emission filter: 670-720 nm), a multiphoton laser (excitation filter: 357 ± 10 nm; emission filter: > 460 nm) for use with DAPI. A combination of 10× (numerical aperture of 0.3), 20× (numerical aperture of 0.5) and 40× oil immersion (numerical aperture of 1.3) objectives were used. Each fluorochrome was scanned individually to avoid crosstalk between channels (particularly those scanning FITC and Cy3). Dual images were subsequently combined where necessary using Adobe Photoshop 6.0.

A.3.3.5. Probe Design and Synthesis

The probes were designed to be complementary to a region of the transcribed sequence that corresponded to an intron-exon boundary within the genomic DNA so as to serve as a control for non-specific binding of the probe to genomic DNA in addition to the target mRNA. Deoxy-oligoprobes were synthesised in Copenhagen by TAGN Ltd. The oligos were directly conjugated to the following modifications at their 5' end: Cy3 ™ (5-N-N'-diethyltetramethylindocarbocyanine), sulfoindocyanine chromofore Cy5™ (5-N-N'-diethyltetramethylindodicarbocyanine) and FITC (fluorescein isothiocyanate). Aliquotted stock solutions of 10 µM in TE-buffer (10mM Tris-HCl, 1 mM EDTA, pH 7.4) were made; 1 µl thereof was used for each 100 µl final hybridization mixture.

Probes corresponding to the genes of interest were grouped together for double and triple FISH experiments based on their predicted location within the cardia, so as to attempt to prevent overlapping signals in the final images. Probes *DmPro3* and *DmPro2* were used in the first adult preparation, *DmPro3* and *DmPro1* in the second. The first larval tissue preparation included the probes *DmPro2* and *DmPro10*. The second larval FISH experiment was conducted with the

probes *DmPro9*, CG31893 and *DmPro8*. The third larval preparation included the probes corresponding to *DmPro4*, CG17814 and *DmPro7*.

Table 26. Details of fluorescent labels covalently linked to the oligos used in this study, including the sequence of the gene specific portion of each probe.

Probe Name	Sequence (5'-3') including 5' label
DmPro1	Cy3-ACUCUCAUGAGACGACAC
DmPro3	FITC-AUGAAGUUGGUCUGGAUG
DmPro2	Cy3-AAACACUAUGGAGUGGCC
DmPro10	Cy5-AAUGCGAGGACUUCCUAG
DmPro9	Cy3-GAAAGCCGUGAUCUUUGUG
DmPro8	Cy5-ACGCCAUAGUAAUUGCAUG
CG31893	FITC-AUGAAAGCAGCUUUAGUCC
DmPro4	Cy3-GGGGGUCUCUGGACCGUUC
DmPro7	Cy5-CUGUGCAUGAAUGAGAUAG
CG17814	FITC-AAGUCCGCACUACUUUUGA

A.4. Results

PM secretion patterns were examined in the cardia and anterior midgut by (F)ISH. Whole mounts of cardia and adjacent tissue were probed with peritrophin-specific probes. Morphologically, adult cardia appear to be more discrete in comparison to those of larvae, indicated by narrowing of the ventricular lumen at the tip of the stomodeal valve and the absence of gastric caeca (e.g. Fig. 41 and 47). The results of this study suggest that the formation zone of the adult cardia consists of a number of unique sites of expression, tightly integrated with one another, and consequently could be regarded as being more specialised (Fig. 41) than the larval cardia, in which the expression zones are essentially equivalent (Fig. 47).

The cardia is a prominent organ of the alimentary tract that characterises "higher" or muscoid flies, of the order Diptera (King, 1988). The morphology of the adult cardia in *D. melanogaster* is described in detail by King (1988); briefly, it is a conical organ with radial symmetry (longitudinal axis along the oesophageal lumen) that is broadest at the oesophagus, narrowing towards the ventriculus (Fig. 41). The muscoid cardia consists of a foregut invagination, termed the stomodeal valve together with an annular pad (Fig. 42), forming the outer wall, composed of midgut cells. The origin of the tissue within this composite organ can be easily distinguished, since the foregut epithelium is characterised by the secretion of an extracellular cuticle, whilst midgut epithelium is typically lined by a prominent brush border of microvilli along the apical surface of the epithelium (Richards and Richards, 1977).

Six distinct formation zones can be easily recognised within the adult cardia (Fig. 41 and 3). The first three occur within the foregut, and are characterised by cells containing apical concentrations of smooth endoplasmic reticulum (ER) and basal accumulations of glycogen. Zone 1 begins at the apex of the stomodeal valve, and extends anteriorly to zone 2, which is located at the narrowing of the stomodeal valve. A number of specialized ultrastructural features characterise the columnar cells of zones 1 and 2, including a pleated apical plasma membrane, numerous Golgi bodies and smooth ER and large accumulations of glycogen, the highest concentration of which occurs within cells either side of the zone 1-2 boundary. Zone 3 is found at the neck of the stomodeal valve, and is composed of cells that are much shorter and less morphologically specialised, being less rich in smooth ER and glycogen. The foregut/ midgut boundary is delineated by a small number of cuboidal cells at the neck of the cardia and the start of the basement membrane, which lines the midgut epithelium. The midgut folds back to create a zone of columnar cells along the recurrent wall (anterior to the neck of the stomodeal valve), that are characterised by cells rich in rough ER, Golgi, secretary vesicles and long microvilli, indicating that this zone is adapted for a secretary function. The remaining region of the midgut epithelium forms the posterior outer wall, and is composed of cuboidal cells that are less specialised in morphology. The cells within the midgut are subdivided into three zones, zone 4 appears by light microscopy to be the origin of the majority of the PM, and are characterised by cuboidal to short columnar cells with long loosely packed apical microvilli. The only prominent columnar zone occurs at zone 5, which starts at the anterior limit of the cardia lumen, and is characterised by cells with long widely spaced microvilli, rich in ER and Golgi. The boundary between zone 5 and 6 is marked by an abrupt reduction in epithelial cell height and microvilli length. The cuboidal cells of zone 6 have short, tightly packed microvilli and lack dense concentrations of rough ER and Golgi. The cells of this zone gradually take on the characteristic form of ventriculus cells, a transition that is not sharply defined. Cells of the ventriculus often bulge into the midgut lumen due to these cells being much taller than surrounding cells.

The characteristics and exact origin of the four laminae of the adult PM has been described previously in studies conducted by Peters (1976) and King (1988). In summary, the first layer (L1) of the PM, a well defined but thin electron-dense, lumen (ENPS) facing lamina is secreted by cells within zones 1-3 (Fig. 42), adheres to, but is distinct from L2. L2, an electron-lucent layer originates from an amorphous mass of newly secreted PM components at the tips of the microvilli of cells in zone 4, becomes associated with L1 and L3 as it moves distally, past zone 5. L3 appears to be morphologically indistinct from L2 and L4 in light microscopy, and is composed of numerous electron-dense granules that are secreted by cells of zone 5. L4, a delicate epithelium (ECPS) facing lamina, secreted by zone 6, often separates from the L3 as the

PM moves distally into the ventriculus, forming the "second" PM (PM 2) which is spatially distinct from PM 1, composed of the remaining layers.

The PM of *Drosophila* larvae appears to be secreted almost exclusively by a ring of cells, localised to the equivalent of zone 5 in the adult, which Rizki termed the PR (PM secreting ring) zone (1956). This is in stark contrast to the extended formation of the adult PM over at least four separate zones (King, 1988). Morphologically, the cardia epithelium from the PR cells to the ventriculus resembles that of the adult, and thus might be expected to be capable of secreting PM components. However, he observed that amorphous material was only present in the cardia lumen over the PR cells, and that the PM first appears just distal to this ring of cells, indicating that the single larval PM is formed exclusively by this ring of cells. In this way defining function based purely on cellular morphology can be misleading.

The following (F)ISH results are all Z-stack derived images of LS sections through the cardia and gastric caeca (larvae only) from wild-type adults and larvae.

Table 27. Comparison of the number of formation zones in which the peritrophin mRNA was detected in adult and larval cardia. The relative strength of the signal is given as: +++, strong; ++, moderate; +, weak; and -, no signal (negative). Refer to the Figures listed for details:
A. Adult gene expression.
Abbreviations: numbers 1-6 refer to formation zone 1-6 in Fig. 41; V, ventriculus.

		Formation zone							
Name of probe	Number CBDs	1	2	3	4	5	6	V	Figures
DmPro1	2	-	++	++	-	-	-	-	43, 44 and 46
DmPro2	6	-	-	-	++	-	++	-	45
DmPro3	1	+++	+++	+++	-	-	++	++	45

B. Gene expression in larval cardia.
Abbreviations: numbers I and II refer to formation zones in Fig. 47; MC, midgut.

		Formation zone					
Name of probe	Number CBDs	APR	I	PPR	II	MC	Figures
DmPro2	6	-	+++	-	-	-	48
DmPro10	2	-	+++	-	-	-	48
DmPro9	1	-	+++	-	++	++	49 and 50
DmPro8	1	-	+++	-	+++	+++	49 and 50
CG31893	1	-	+++	-	-	+	49 and 50
CG17814	1	-	+++	-	-	-	51 and 52
DmPro4	5	-	+++	-	-	-	51 and 52
DmPro7	2	-	+++	-	-	+	51 and 52

Fig. 41. Basic overview of the morphology of the adult cardia in longitudinal section. Formation zones 1-6 are shown. Adapted from King (1988). Refer to Fig. 42 for detailed representation of the zones that contribute to each layer of the adult PM.

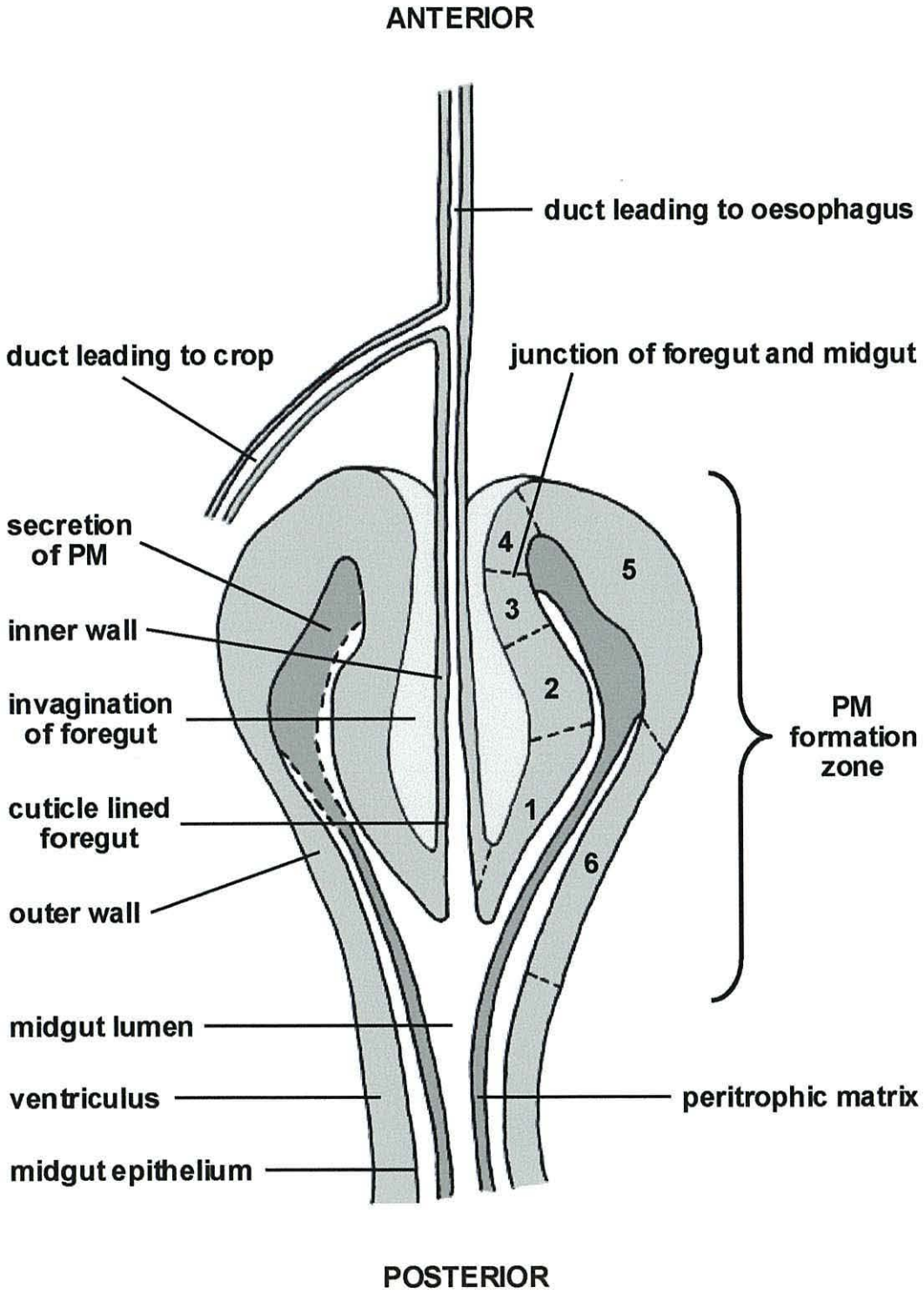


Fig. 42. Detailed representation of the PM formation zone of adult cardia; only the right half of the cardia is shown. The four layers of the PM are clearly depicted with their associated region of the formation zone within the cardia epithelium. From King (1988). Also refer to Fig. 41 for a basic overview of the morphology of the adult cardia. Abbreviations: AP, annular pad; E, oesophagus; F/M, foregut midgut boundary; SV, stomodeal valve; V, ventriculus; PM, peritrophic matrix; cu, cuticle lined foregut; L1, formed by zone 1-3; L2, secreted by zone 4; L3, formed by zone 5; and L4, secreted by zone 6. L1 and L4 face the Endo- and ECPS, respectively.

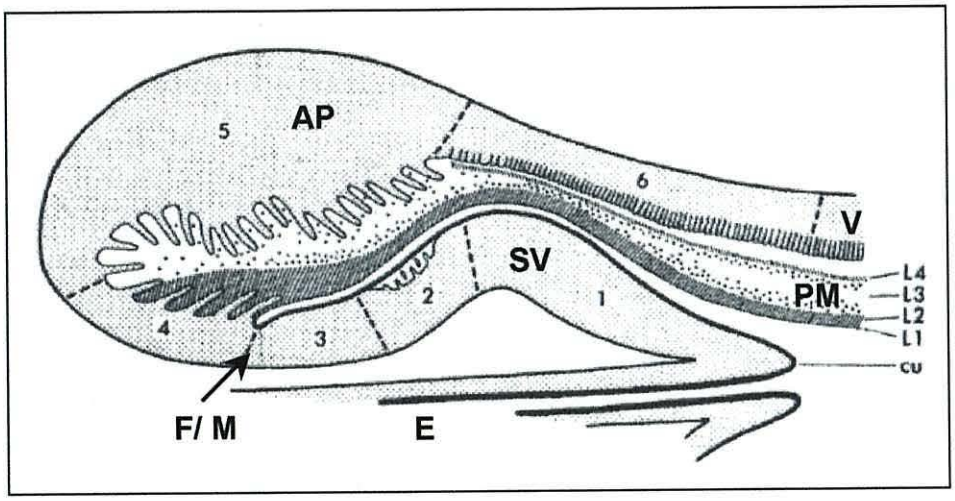


Fig. 43. Specificity of the cRNA probe for *DmPro1* with anti-DIG FITC.
In situ hybridisation of mRNA encoding *DmPro1* with an anti-sense cRNA DIG-labelled probe against *DmPro1* mRNA, detected using anti-DIG FITC. Positive reactions are visible in cells of formation zone 2 and 3. Note: Arrows indicate the corresponding formation zones within the adult cardia (Fig. 42). Abbreviations: V, ventriculus; FG, foregut; and MG, midgut. Scale bar: 50 µm.

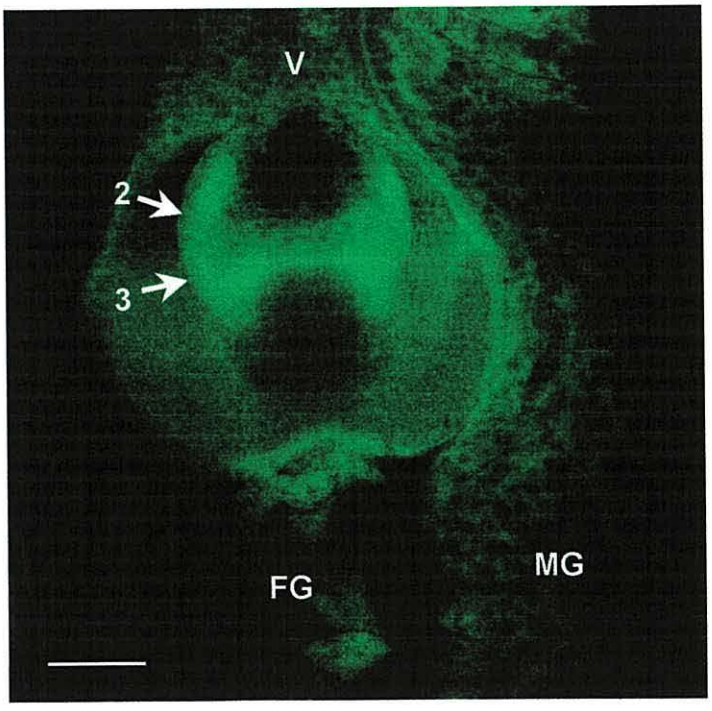
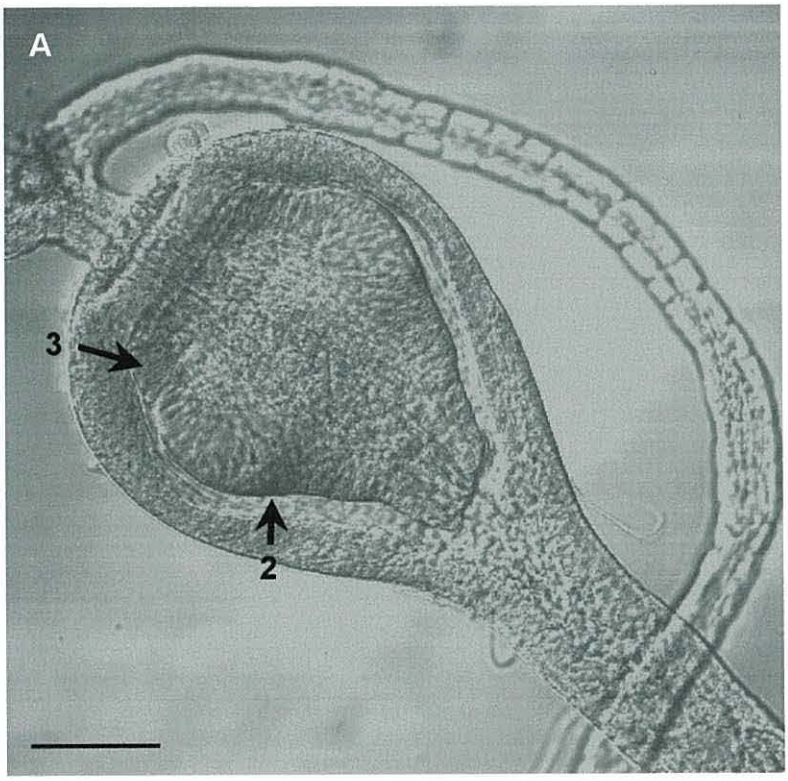


Fig. 44. Specificity of the cRNA probe for *DmPro1* with anti-DIG AP.
Fig. 5A. *In situ* hybridisation of mRNA encoding *DmPro1* with an anti-sense cRNA DIG-labelled probe against *DmPro1* mRNA, detected using anti-DIG AP.
Weak positive signals are found in cells of formation zones 2 and 3.
Fig. 5B. Negative control, demonstrating no hybridisation after using the control *DmPro1* sense cRNA probe. Note: Arrows indicate the corresponding formation zones within the adult cardia (Fig. 42). Abbreviations: FZ, formation zone. Scale bar: 50 µm.



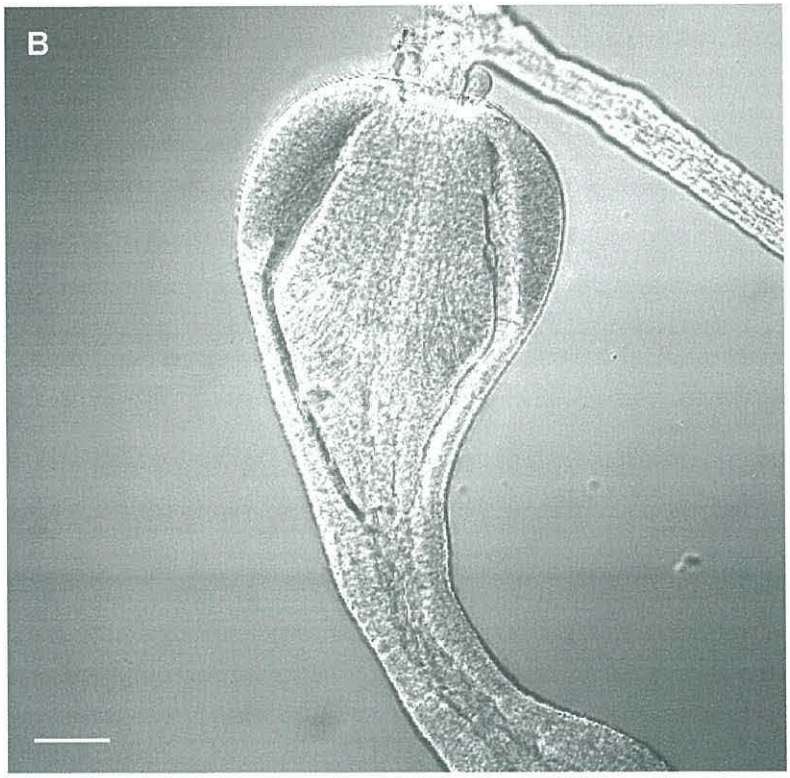


Fig. 45. Co-localization of *DmPro3* and *DmPro2* mRNA within the adult cardia.
A. FISH, *DmPro3* expression is highly enriched in zones 1-3, 6 and the ventriculus, i.e. diffuse compared with *DmPro2* mRNA, which is only present in a small number of cells in zones 4 and 6 of the cardia. All other parts of the cardia and midgut were negative, including the foregut, which does have autofluoresent cuticle (Fig. 53).
B. Corresponding transmission image. Note: asterisks indicate the end of the zone of expression of *DmPro3* in the ventriculus at the very anterior of the midgut.
arrows indicate the cells expressing mRNA encoding *DmPro3* (FITC, in green) and *DmPro2* (Cy3, in red), numbers refer to formation zones shown in Fig. 42. Abbreviations: cu, autofluorescent cuticle; V, ventriculus. Scale bar: 50 µm

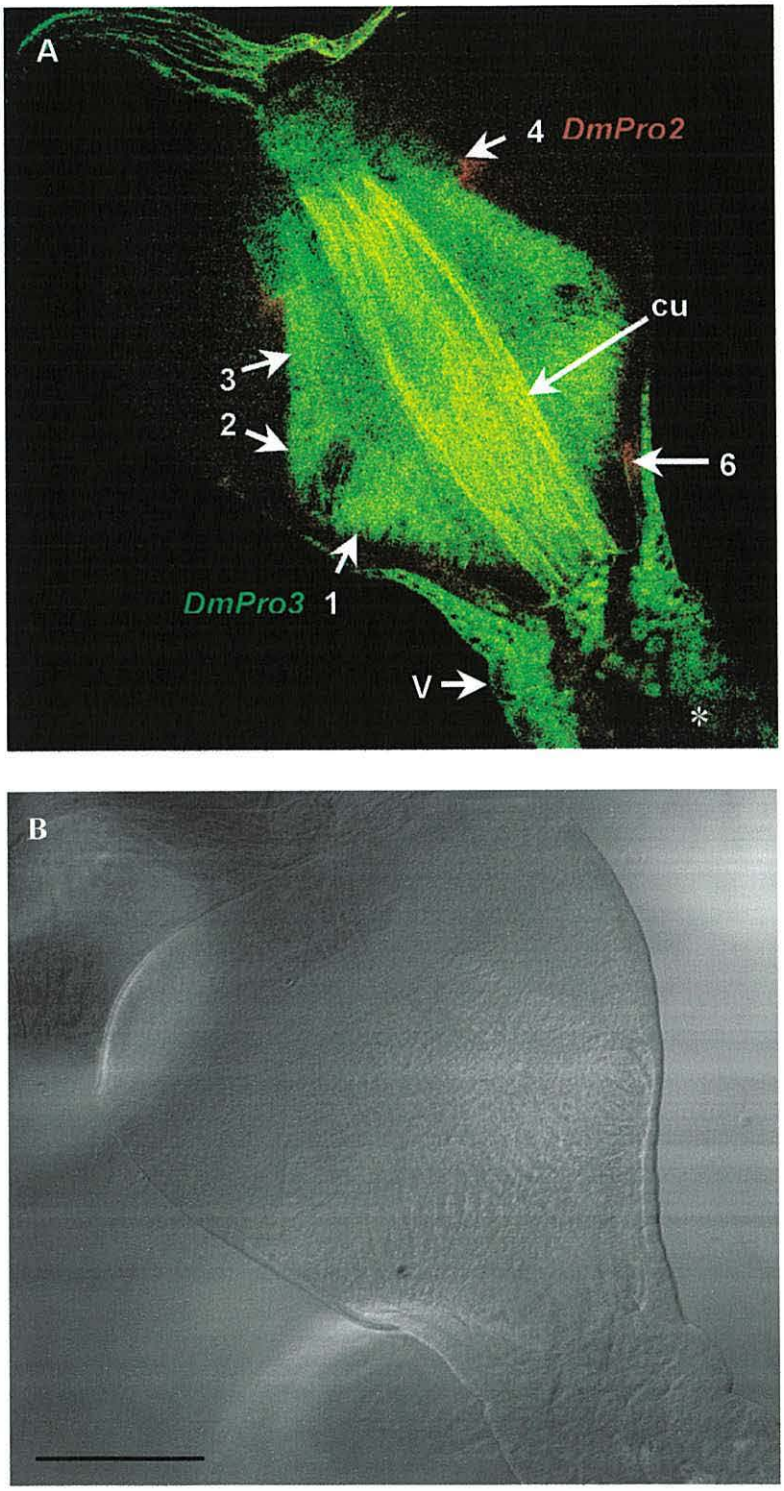


Fig. 46. Localisation of the *DmPro1* oligoprobe within the adult cardia.
A. Positive signal in formation zones 2 and 3, indicating that the cells of this zone express mRNA encoding *DmPro1*.
B. Corresponding transmission image.
Abbreviations: numbers indicate formation zones. Scale bar: 50 µm.

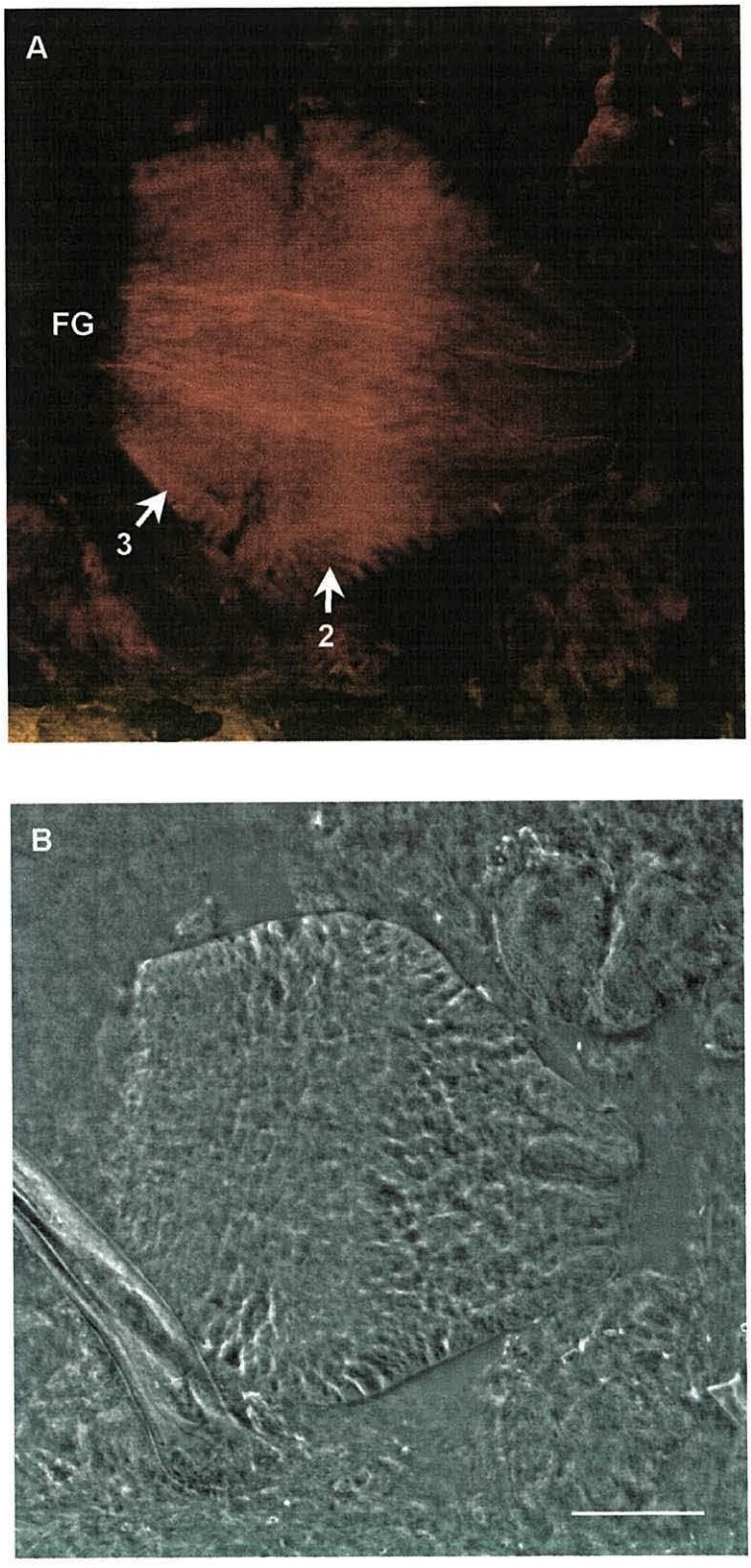


Fig. 47. Overview of the morphology of the cardia from *Drosophila* larvae in longitudinal section. Note: I and (PR cells, cells within the PM forming ring) and II used to denote formation zones 1 and 2 respectively, since retaining the adult terminology could become confusing. Abbreviations: APR, cells anterior to the PR zone (termed zone I here); PPR, cells posterior to the PR zone (zone I). Midgut caecae not shown. Drawn after Rizki (1956).

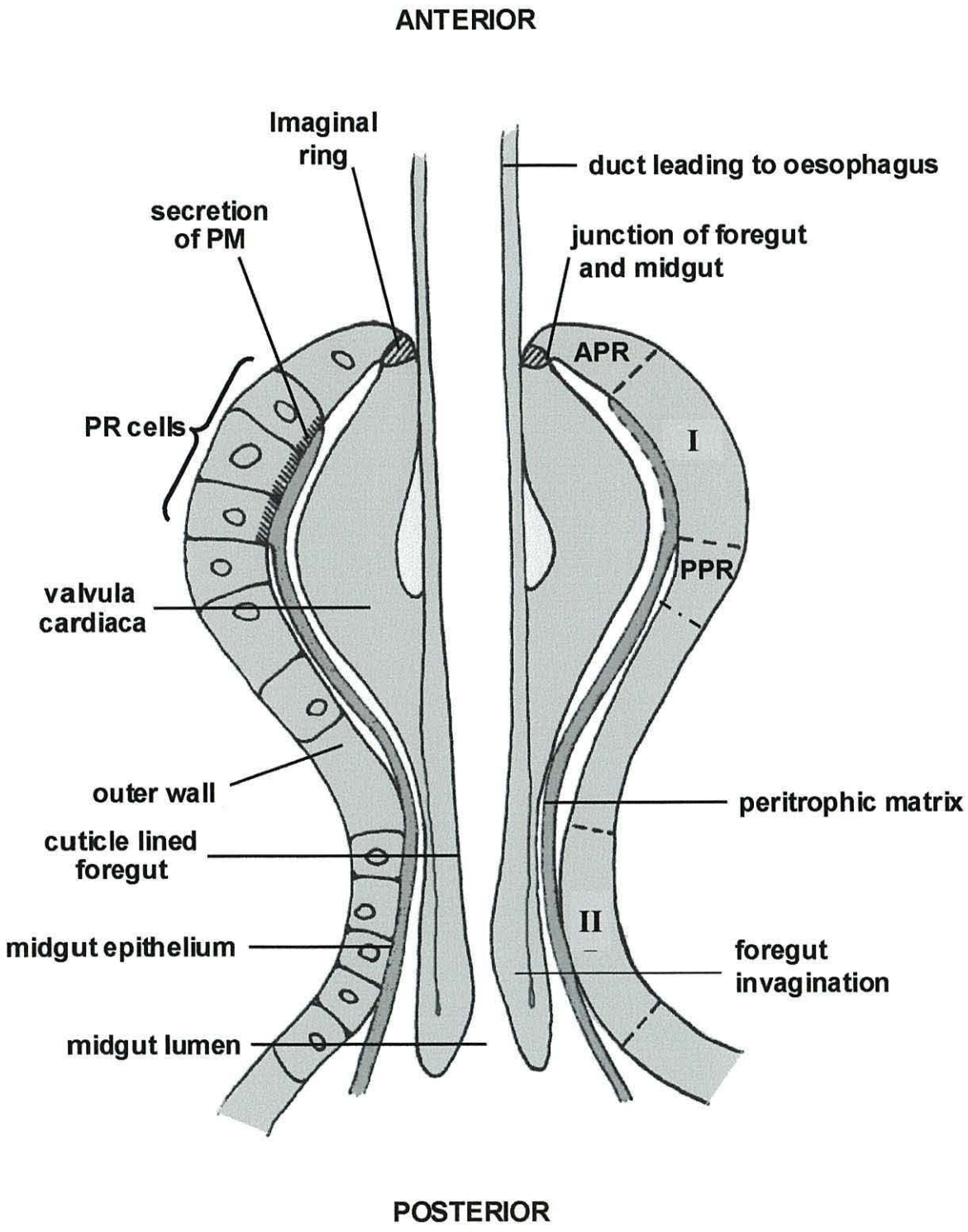


Fig. 48. Co-localisation of *DmPro2* and *DmPro10* to cells within the larval cardia.

C. FISH of mRNA encoding *DmPro2*.

D. FISH of mRNA encoding *DmPro10*.

C. Corresponding transmission image.

Positive signal only present within secretion zone 1, an annulus of expression by a series of three cells within this zone (Fig. 47). Abbreviations: I, formation zone 1. Scale bar: 100 μm.

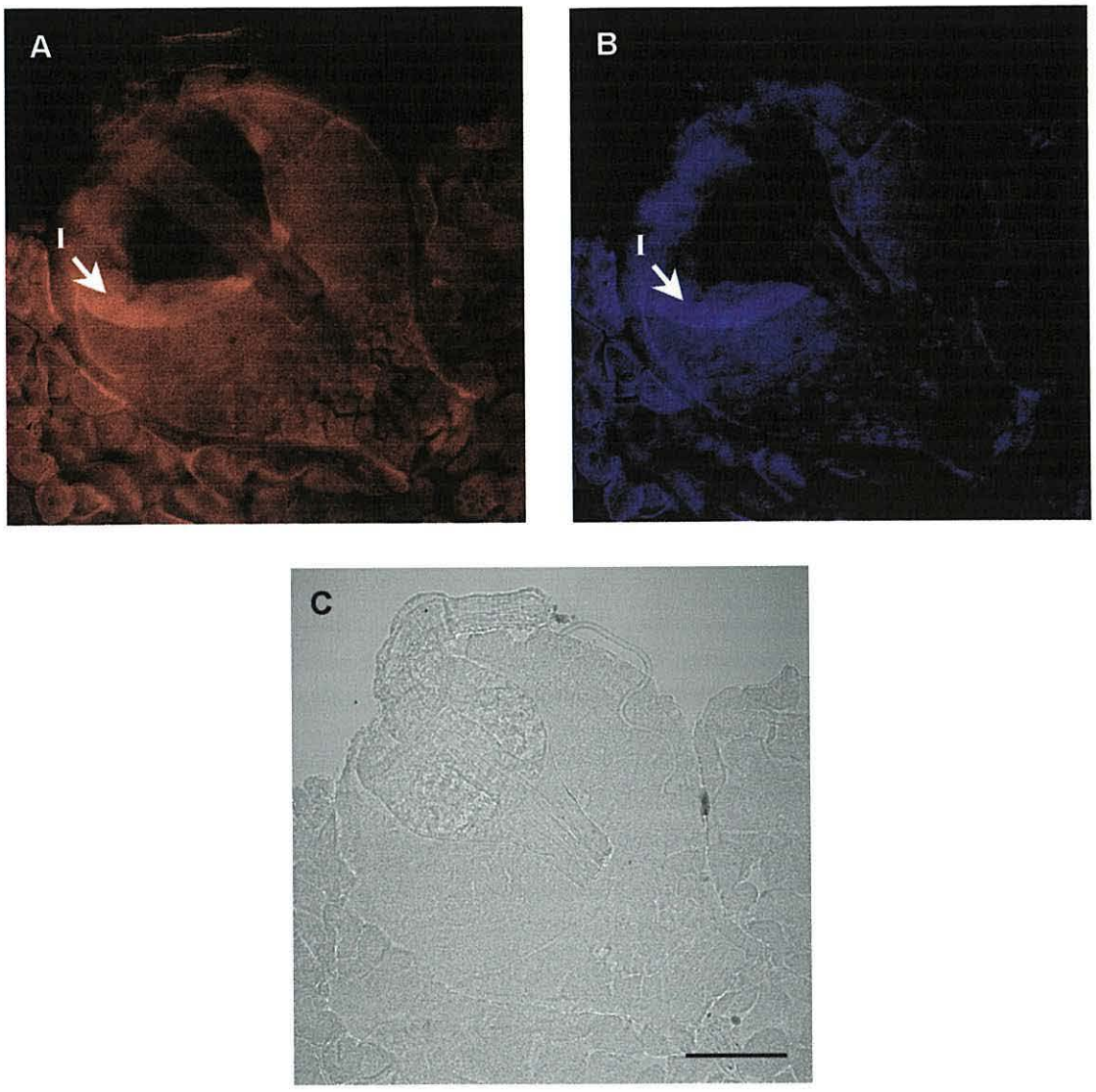


Fig. 49. Co-localisation of CG31893, *DmPro9* and *DmPro8* to cells within the larval cardia. The images all clearly show the accumulation of mRNA in the apical region of the three cell thick zone I (PR cells) that results in a clear annulus of expression (may also have arisen due to superposition of the signal in the cell layers).
A. FISH to mRNA encoding CG31893, showing specific hybridisation to formation zone 1 only (expression in midgut caeca is diffuse in comparison). The signal from the foregut lumen is due to autofluorescence from cuticle lining the foregut.
B. FISH to mRNA encoding *DmPro9*. The positive signal is present in formation zone 1 and 2 and the midgut caeca.
C. FISH to mRNA encoding *DmPro8*. The positive signal is present in formation zone 1 and 2 and the midgut caeca.
D. Corresponding transmission image.
Abbreviations: I and II, formation zone 1 and 2 (Fig. 47); MC, midgut caeca.
Scale bar: 100 μm.

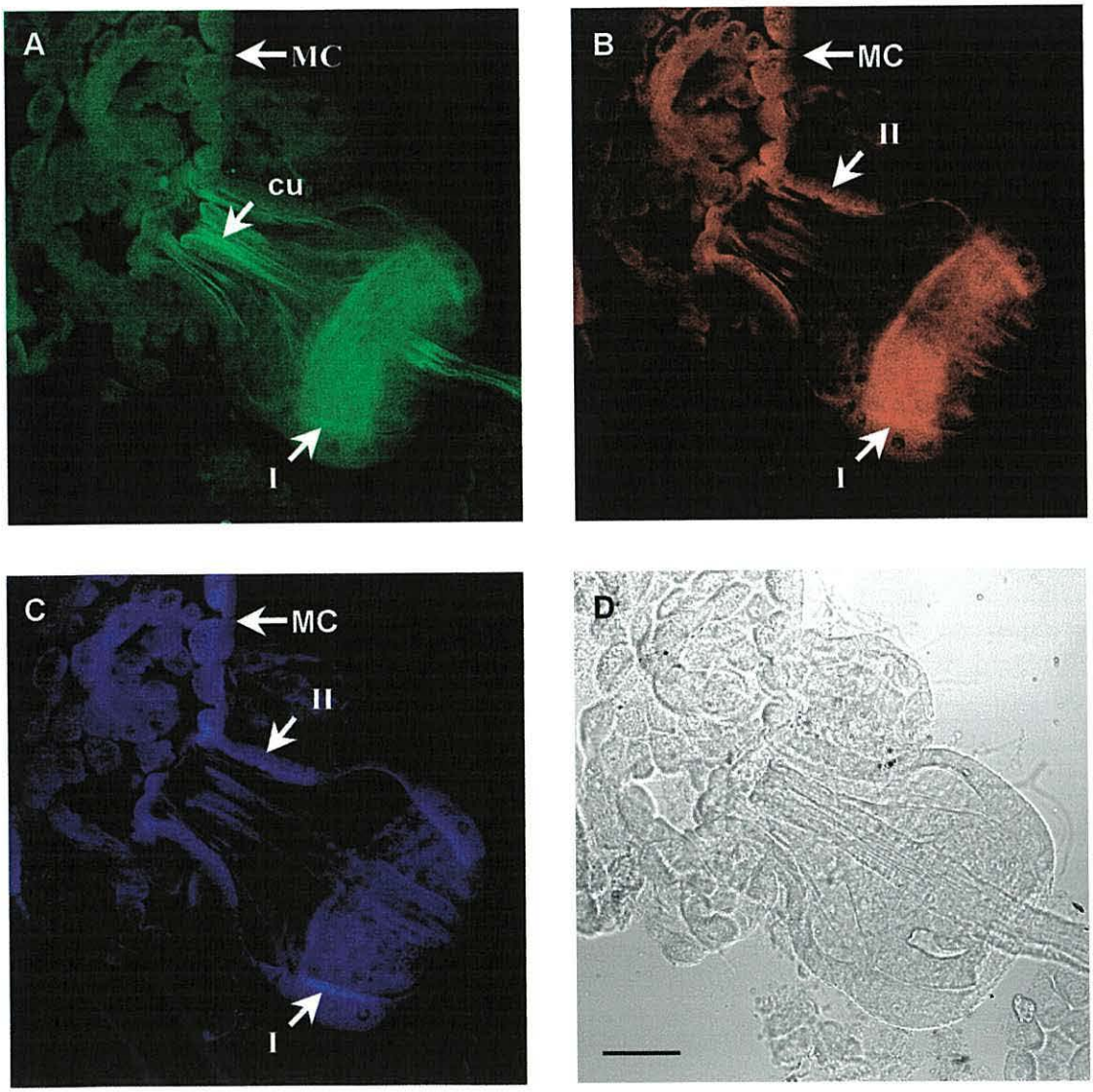


Fig. 50. Co-localisation of CG31893, *DmPro9* and *DmPro8* to cells within the larval cardia. The following images clearly show an annulus of expression in formation zone 1(the cells of zone 2 are not visible) and the midgut caeca (stronger signal than that in Fig. 50B and 11C).
A. FISH of mRNA encoding CG31893. The signal from the foregut lining is due to autofluorescence from cuticle.
B. FISH of mRNA encoding *DmPro9*.
C. FISH of mRNA encoding *DmPro8*.
D. Corresponding transmission image.
Abbreviations: I, formation zone 1 (Fig. 47); MC, midgut caeca. Scale bar: 100 μm.

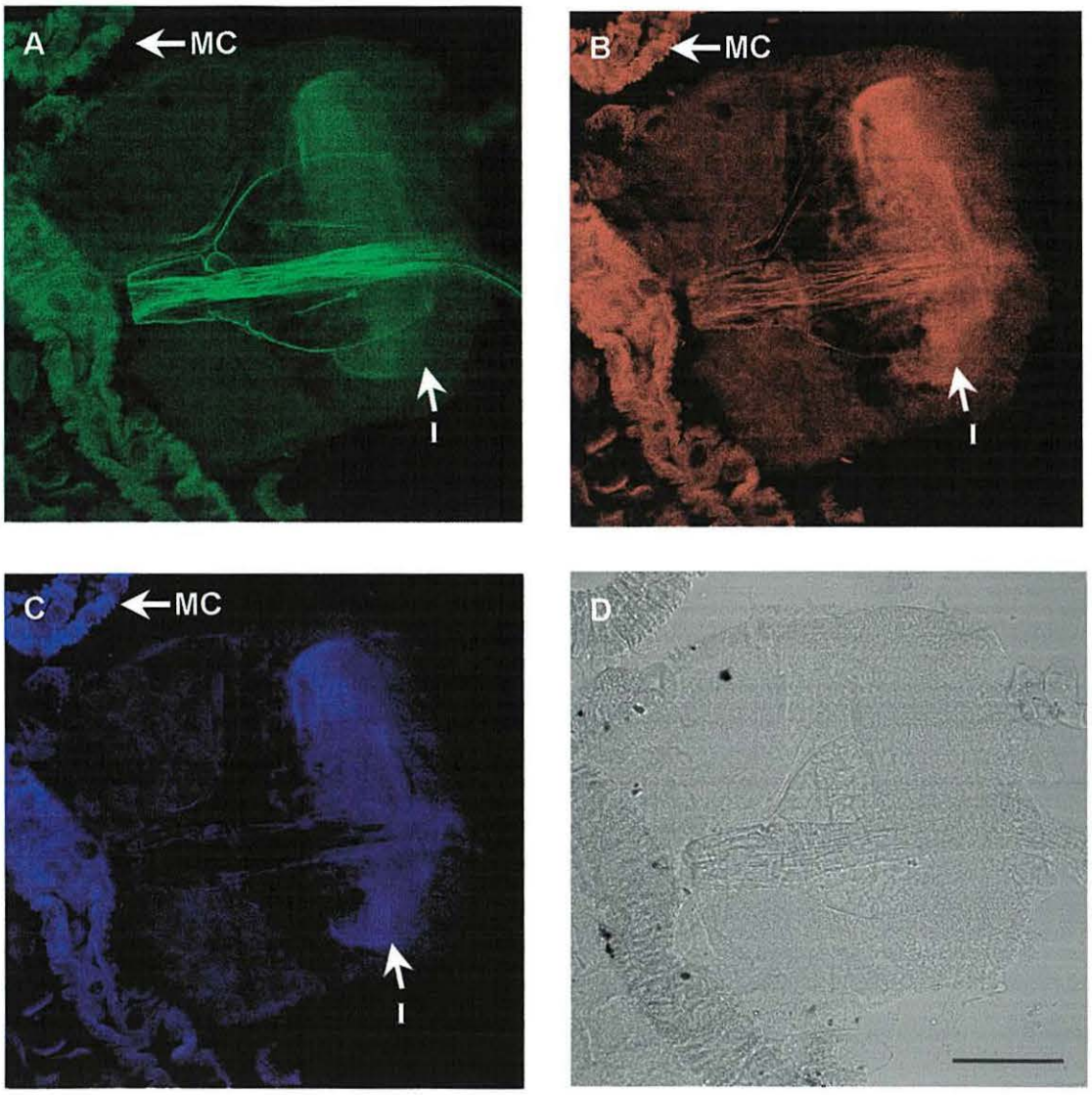


Fig. 51. Co-localisation of *DmPro4*, CG17814 and *DmPro7* within the cardia epithelium of larvae, showing specific hybridisation to formation zone 1 only. The foregut lining is lined with autofluorescent cuticle. The stomodeal valve has become torn from the annular pad at the narrowest cell layer between the foregut-midgut boundary during the preparation, indicated by the strong positive signal in Fig. 51 C.
A. FISH of mRNA encoding *DmPro4*.
B. FISH of mRNA encoding CG17814.
C. FISH of mRNA encoding *DmPro7*.
D. Corresponding transmission image.
Note: double headed arrow indicates the actual and expected position of the stomodeal valve.
Abbreviations: I, formation zone 1; PM, peritrophic matrix; cu, cuticle lined foregut (Fig. 47).
Scale bar: 100 µm.

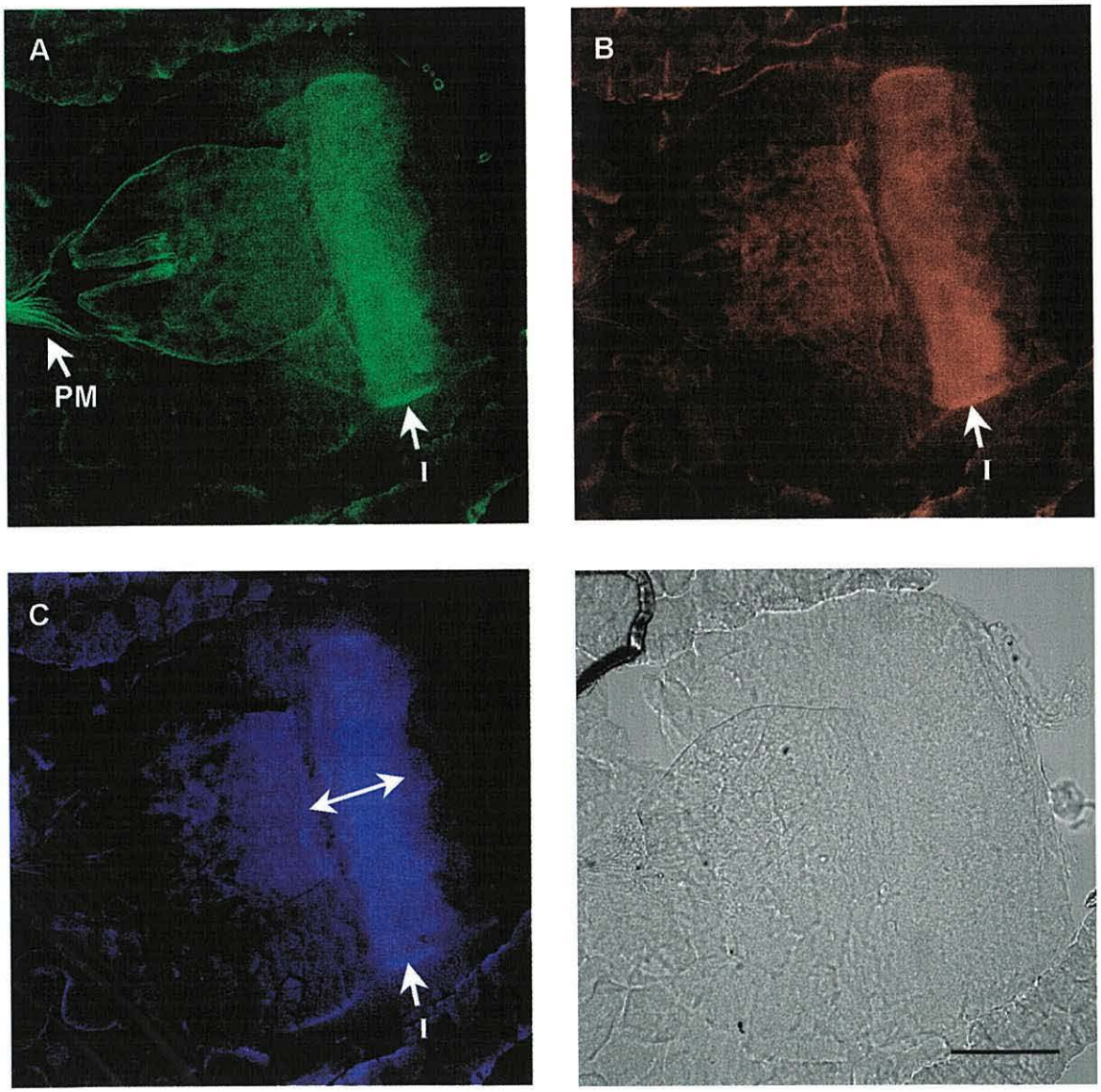


Fig. 52. Co-localisation of *DmPro4*, CG17814 and *DmPro7* within the cardia epithelium of larvae, showing specific hybridisation to formation zone 1 only. The signal from the foregut cuticular lining is due to autofluorescence. Zone II is not visible in this preparation. The lack of expression in cells of the APR and PPR zone is shown clearly by the distinct borders of zone 1 (indicated by asterisks). The stomodeal valve has become torn from the annular pad at the foregut-midgut junction during the preparation; the double headed arrow indicates the present and expected position of the stomodeal valve.
A. FISH of mRNA encoding *DmPro4*.
B. FISH of mRNA encoding CG17814.
C. FISH of mRNA encoding *DmPro7*.
D. Corresponding transmission image.
Abbreviations: I, formation zone 1; cu, cuticle lined foregut (Fig. 47). Scale bar: 100 µm.

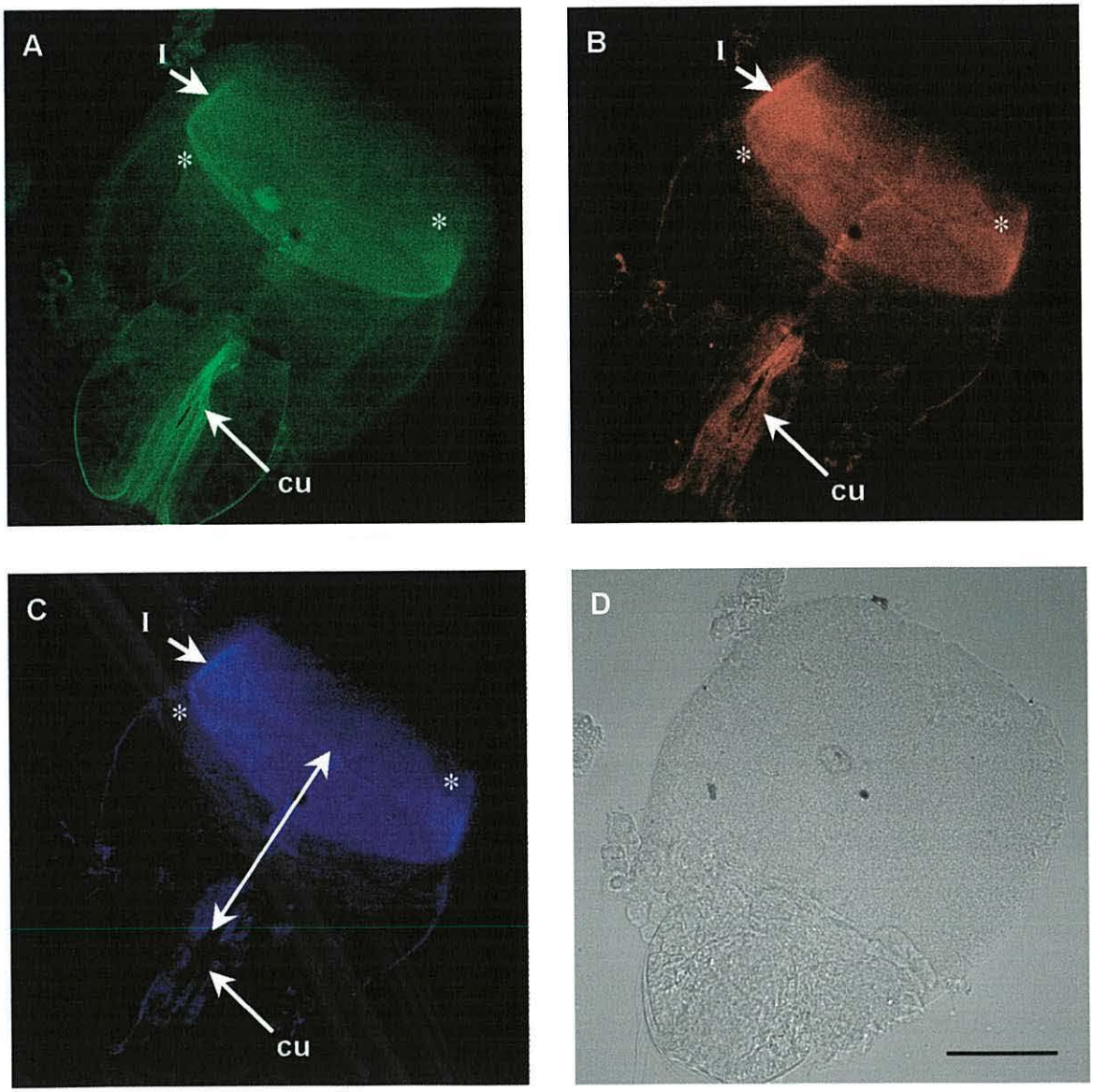
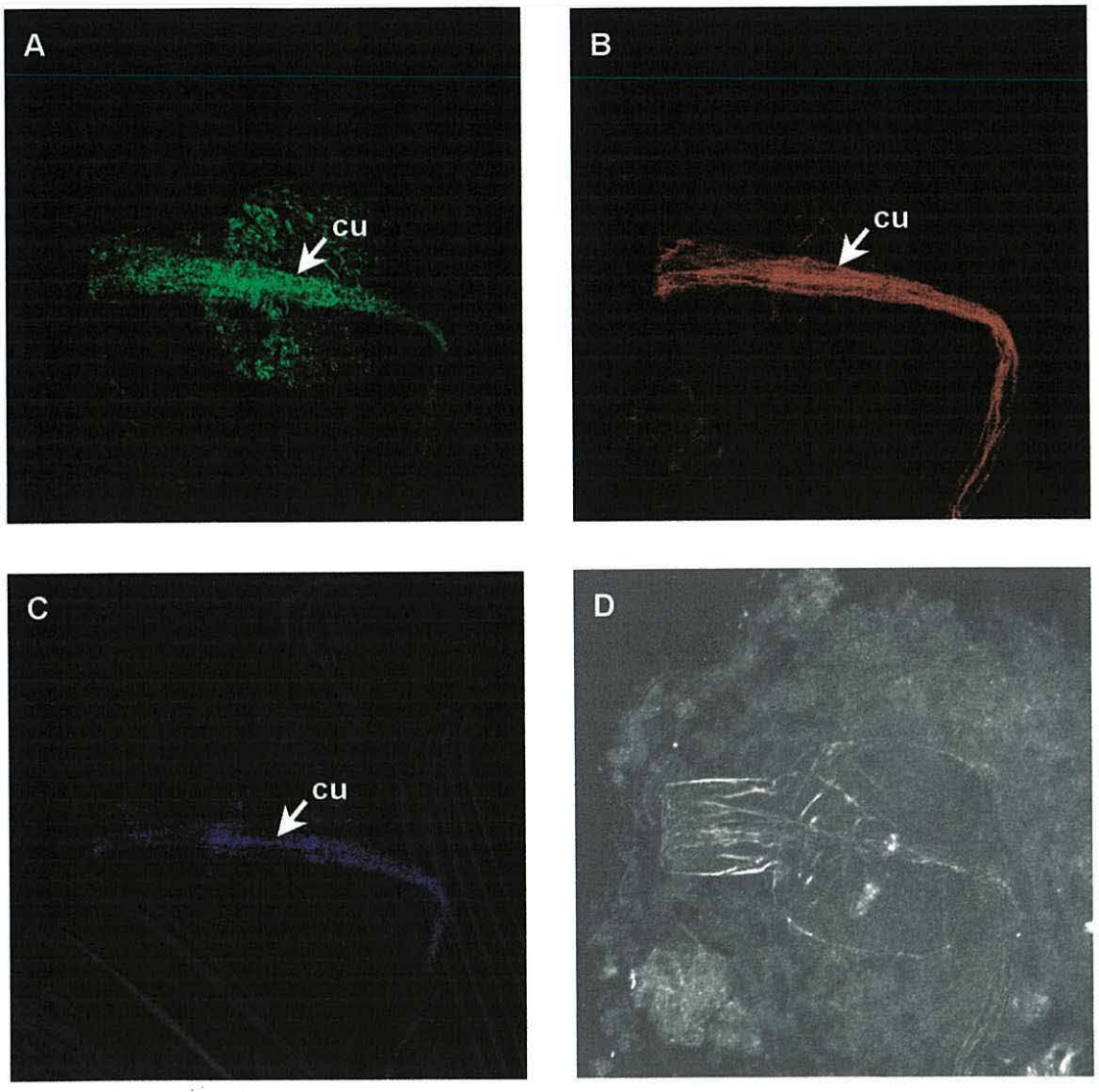
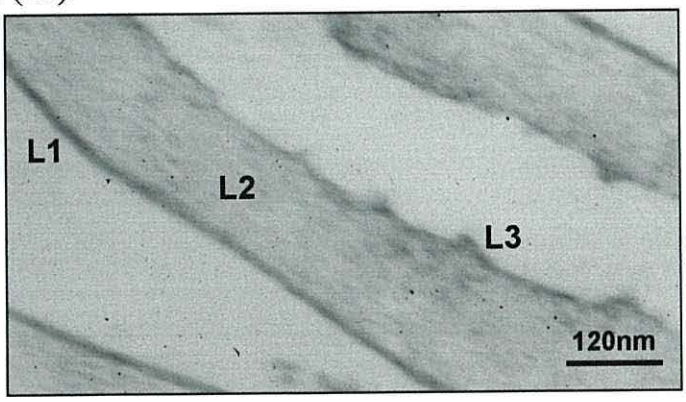


Fig. 53. Negative control, in which the RNA signal was degraded using RNase. Where cells of the cardia show no hybridisation, the positive signal in all images is accounted for by autofluorescence of the foregut cuticular lining.
A. FISH with a FITC labelled oligoprobe.
B. FISH with a Cy3 labelled oligoprobe.
C. FISH with a Cy5 labelled oligoprobe.
D. Corresponding transmission image.
Abbreviations: cu, cuticle lining of the foregut (Fig. 47). Scale bar: 100 µm.



In the most favourable set of serial confocal sections of dual-labelled cardia from adult *Drosophila* it is possible to clearly see the co-localisation of mRNA from two adult peritrophins, in which *DmPro2* mRNA is localised to zones 4 and 6, and *DmPro3* mRNA to zones 1-3, 6 and the ventriculus (Fig. 45) when adopting the terminology of King (1988). The image in Fig. 45A confirms the aforementioned statements about the formation zone of the adult cardia, but also suggests that not all cells within the zone react positively to peritrophin specific-probes. Whilst the oligoprobe for *DmPro3* appears to be constitutively expressed by the majority of cells within the cardia and the ventriculus epithelium (Fig. 45), there is a band of negative signal at the boundary between zones 1 and 2, which may be ascribable to the presence of dense stores of glycogen within this region (King, 1988). The oligoprobe specific for *DmPro1* expression produced an expression pattern (Fig. 46) that confirms that viewed in the DIG-FITC and DIG-AP experiments (Fig. 43 and 44), namely that it is expressed exclusively by cells of zones 2 and 3. Interestingly, none of the peritrophin probes tested labelled zone 5. It is likely that this consistent gap in expression of genes encoding peritrophins results from the synthesis and secretion of another PM component, namely chitin. A distinct function for the cells in this zone is suggested by the observations of King (1988), in that the boundaries of this zone are marked by an abrupt transition in cell morphology, to cells that appear to be highly secretary. Moreover, he observed that the vesicles at the tips of the cytoplasmic prominences (projections at the apical end) of the cells comprising this zone secrete a distinct "flocculent" secretion product, which gives rise to the electron-dense granules that are characteristic of the third layer of the PM. Thus, the formation zones of the adult cardia may be such that synthesis of the chitin component, rather than being diffuse throughout the entire cardia epithelium, is localised to zone 5, thereby forming a PM composed of a central layer of chitin sandwiched between the peritrophins (peritrophins and mucins), which in their turn are secreted by the cells of zones 1-4, and 6. This study provides the first indirect evidence that chitin may be localised to a single layer within the PM rather than being present in all layers, at least in the nascent (newly synthesised) PM. Nonetheless, the results are not conclusive as it remains to be seen if this apparent gap in expression of peritrophins can be accounted for by the cells within this zone synthesising a yet unknown adult peritrophin rather than the presence

Fig. 54. The PM in *Drosophila* larvae, showing an electron dense layer of uniform thickness (L1), thick electron lucent layer (L2) and much thinner second electron dense layer of irregular thickness (L3).



of chitin synthase and/or other enzymes involved in chitin synthesis (Zimoch and Merzendorfer, 2002). The four layered adult PM initially secreted by the cardia, splits into two distinct matrices by the time it has moved distally into the ventriculus (King, 1988). This cleavage in the mature PM may be accounted for by the third layer being predominantly composed of chitin, sandwiched between two peritrophin rich layers (Fig. 39A). It may be inferred from the expression pattern of peritrophins within the adult cardia that the protein encoded by *DmPro3*, originates from zones 1 to 3 and 6 to the ventriculus, suggesting that it is an abundant component of the first and fourth layer (refer to Fig. 42). In comparison, the putative peritrophin encoded by *DmPro2* is specifically localised to zone 4 and the posterior portion of zone 6, hence it is likely to be present in the second and fourth layers of the PM. The peritrophin encoded by *DmPro1* is specifically expressed within the second and third zones, implying that it may be present within the first layer, at the boundary with the second. The significance of the distribution of these PM components in terms of the putative roles of the peritrophins within the PM is discussed later.

Images from the larval cardia reveal a comparatively less complex formation zone since there are no substantial differences in peritrophin expression within the anterior region, peritrophins appear to be consistently transcribed from zone I, any variation in expression pattern being restricted to zone II and the midgut caeca. All specimens clearly showed strong expression in a ring of cells in the anterior region of the cardia, termed formation zone I. In addition, weaker expression can be seen for *DmPro7* mRNA in the cells of the anterior midgut that surround the terminal portion of the conical foregut invagination, denoted formation zone II (Fig. 51C), and for *DmPro9* and *DmPro8* in the midgut caeca (Fig. 49B, 50B and 49C, 50C respectively). Zones I and II are located within the larval cardia structure in the equivalent of zones 4/5 and 6 respectively of the adult cardia. Although neither of the additional zones (zone II and midgut caeca) have been previously documented to participate in the secretion of the PM in *Drosophila* larvae (Rizki, 1956), studies in *Trichoplusia ni* and *Heliothis virescens* larvae

indicate that this is indeed the case in Lepidoptera (Harper and Granados, 1999; Ryerse *et al.*, 1992). Thus, it is possible that the nascent larval PM initially secreted by zones I, PPR and II within the cardia, is reinforced by the addition of additional material, supplied by the midgut caeca.

The T.E.M. studies conducted by King (1988) suggest that the PM of adult *D. melanogaster* is composed of four layers, formed by six formation zones as described previously. Conversely, Peters (1976) observed that a single trilaminate PM is secreted by the cardia of third instar *D. melanogaster* larvae. T.E.M. examination of wild type third instar *D. melanogaster* larvae in this thesis reveals a 110-130 nm thick PM composed of a thin electron dense layer on the lumen side and a much thinner electron dense layer on the epithelial side, separated by a thick layer of amorphous, electron lucent material, in support of Peters (Fig. 54). The exact location within the PM of the peritrophins can again be deduced from their expression patterns within the cardia (Fig. 39B). The apparent absence of transcription by genes encoding peritrophins (in this study) in the APR and PPR zones and the observation by Peters (1976) that PM precursors are secreted by both zones implies that the cells of these zones are either secreting chitin or are not actively synthesising the peritrophins targeted in this study. However, in my opinion, the secretion of chitin by the cells of the APR zone is questionable, since this would imply that the first layer of the resulting PM would be chitin rich.

King (1989) suggests that, in contrast to his earlier observations (King, 1988) the adult cardia is composed of only four formation zones: foregut, zones 4, 5 and 6. This study confirms that chitin and peritrophin components of the PM are expressed by a total of four formation zones (Table 27A and Fig. 39A). Similarly, this study indicates that a trilaminate PM is produced by three formation zones in *D. melanogaster* larvae (Table 27B and Fig. 39B). Consequently, it may be concluded that there is a direct correlation between the number of lamellae within the PM and the number of formation zones that secreted it. Furthermore, the three formation zones within the cardia of tsetse flies (*Glossina*) give rise to a bilaminate PM, composed of a 310 nm thick amorphous, electron lucent layer and a 20 nm thick electron dense layer (Moloo *et al.*, 1970). Moreover, the PM of *Stomoxys calcitrans* is reported to be secreted by four formation zones, forming five distinct layers according to Lehane (1976a), which Peters (1992) suggests are separated into two distinct PMs.

A.5. Discussion

Despite the wealth of information on the morphology of the cardia and the PM in type 2 PM producing insects (Peters, 1992), it appears that we are still at an early stage in fully understanding the implications of our observations in terms of the as yet unresolved question: the function of the PM. The aim of this study was to use a (F)ISH approach to precisely locate the zone within the cardia in which each peritrophin is transcribed in order to predict the layer of the PM in which each peritrophin is incorporated. Allowing the implications of the composition of each layer on the structure and physiology of the PM to be determined, and thus gain valuable information about the putative role of the PM in *D. melanogaster* (Rizki, 1956; King, 1988). This set of mutually confirming (F)ISH data has provided evidence in the favour this hypothesis.

The summarized PM structures (Fig. 39) suggest that the PM is diffuse in construction, in which the boundary between peritrophin and chitin rich layers is fluid, due to the dynamics between spatially separate layers. The position of peritrophins in relation to chitin fibrils is of paramount importance to maintaining the structural integrity of the PM. The chitin fibrils lying in the plane of the PM are stationary, whilst those that are perpendicular to this plane are free to diffuse through the pores in the PM and form links with peritophins within the peritrophin rich layers prior to polymerisation. *DmPro3* may encode a peritrophin that acts as a capping protein that protects the free ends of the chitin fibrils against exo-chitinases within the endoperitrophic space. In addition, *DmPro2* may act as a coronator (chelating agent) with chitin fibrils, thereby directly protecting them from the action of endo-chitinases, whilst still having four CBDs available to form links between components of the PM matrix. Moreover, the presence of six CBDs may compensate for the much lower amounts of the *DmPro2* transcript (since only two small zones of cells are involved in its synthesis). The synthesis of *DmPro2* might be expected to consume less cellular resources in comparison to the production of the equivalent number of CBDs within peritrophins with fewer CBDs per molecule. Which may support the hypothesis that *DmPro2* is replacing *DmPro3* in L1 and L4. Since this peritrophin, even as a cleaved peptide (Wang *et al.*, 2004) is capable of acting as both a capping and coronating protein, since the CBDs are separated by at least 40 aa, providing the necessary flexibility for both functions. Furthermore, the expression of *DmPro1* is limited to zones 2 and 3 only of the foregut, which may indicate that this peritrophin is becoming evolutionary redundant, also being replaced by *DmPro2*. The presence of only two CBDs and highly repetitive sequences within the nucleotide sequence of *DmPro1* provides evidence in support of this hypothesis, since this could result from the protein becoming redundant as a result of *DmPro2* becoming the dominant peritrophin. A higher mutation rate without any deleterious effect on function (Mullers ratchet) is a

consequence of less evolutionary pressure on the gene. Therefore, there is a congruence between PM structure and gene expression of peritrophins, as the adult PM appears to be degenerate in comparison to the larval PM.

From the (F)ISH images of adult cardia it can be demonstrated that the adult specific peritrophin *DmPro3* mRNA is the most ubiquitous in its distribution (Fig. 45), being localised to zones 1-3 (foregut) and 6 of the PM formation zone in addition to the ventriculus. Evidently, this peritrophin must be present within the first and fourth layers of the PM, since these layers originate from the aforementioned zones. This indicates that *DmPro3* encodes a peritrophin that may perform a unique role in the PM, since it is abundant in the luminal and epithelial facing PM layers (Fig. 39A). The significance of its distribution within the PM is heightened by the protein possessing only one chitin binding domain (CBD). This suggests that this peritrophin may play a similar role to that of P-15 in *L. cuprina*, believed to act as a short capping protein that protects the ends of the chitin microfibrils from degradation by exochitinases present in the midgut (Wijffels *et al.*, 2001). Alternatively, *DmPro3* may encode an immune responsive peritrophin, since the anti-microbial peptide tachycitin (from Horseshoe crab's) possesses a single CBD (Kawabata *et al.*, 1996). Both roles would be advantageous for a peritrophin localised to the luminal and epithelial face of the PM, since this would serve to protect the PM structure against degradation or microbial invasion. Wijffels (2001) predicted that the *Drosophila* homologs of P-15 from *L. cuprina*, P-15a (CG17814) and P-15b (CG31893) would have a similar role. Conversely, this has not been the case in cardia from larvae (Fig. 49A, 50A and 51A, 52A) which further suggests that larval PM synthesis within the larval cardia is distinct from that in the adult organ.

Transcription of *DmPro1* is more localized than *DmPro3*, being expressed soley by the second and third zones of the cardia epithelium (Fig.39, 43, 44, and 46). Moreover, *DmPro2* has the most localised expression pattern of all the peritrophin genes studied, as it appears to be transcribed only by cells of zone 4 and a small portion of zone 6. This is the region of the cardia in which the second and forth layer of the PM originates, implying that *DmPro2* encodes a peritrophin that is principally involved in maintaining the structure of the PM through mediating adhesion between the chitin rich third layer and adjacent peritrophin rich layers. This role is supported by the presence of six CBDs within the predicted protein, since this would allow the protein to form numerous links between the chitin and peritrophin rich layers. In this way, the comparatively less specialised expression pattern of *DmPro1*, which encodes a peritrophin with only two CBDs may simply reflect the number of CBDs available to form cross-links with other PM components. The localization of *DmPro1* to the second and third formation zones of the adult cardia indicates that it is found within the first layer, between *DmPro3* and *DmPro2*,

suggesting that it may form linkages between peritrophins (Fig. 39A). Moreover, the mature adult PM seperates into two separate PMs, termed PM 1 and PM 2 by Peters (1976). This supports the adhesive role of *DmPro2*, since the split occurs between the third and forth layers. As unlike the boundary between the second and third layer, where *DmPro2* is predicted on the boundary itself (Fig. 39A), it is present within the middle of the forth layer rather than where it adjoins the third. Thus, it is not in direct contact with the chitin rich layer, which suggests that it is unable to form linkages with the chitin fibrils (Fig. 39A). Since *DmPro3* encodes only a single CBD, it is unable to form the necessary links with chitin, leading to a weakness in the structure at the boundary between the third and forth layers, which could account for them becoming dissociated once the PM moves distally into the ventriculus.

All the larval specific peritrophins studied, without exception, are expressed within an annulus of cells, termed formation zone I of the larval cardia (Table 27A and Fig. 47), spatially equivalent to zone 5 if adult cardia terminology were adopted. This is in accordance with Rizki (1956), whose light microscopic study reported that the PM in *Drosophila* larvae is secreted by a ring of cells within the PR (PM secreting ring) zone, characterised by heavy staining with periodic acid-Schiff (PAS) reagent. He also identified fewer apparent PM formation zones in larvae in comparison to the adult, although this may be accounted for by the inherent higher resolution of TEM since Peters (1976) suggests that the cells adjacent to this zone, termed zones APR and PPR by Rizki (1956), also contribute to PM secretion. The images presented here indicate that the cells adjacent to zone I are devoid of expression (particularly distinct in Fig. 52), although they may still be involved in PM formation, through the synthesis of peritrophins other than those within this study, or alternatively a non peritrophin component of the PM, i.e. chitin. The former is possible, but unlikely since this study conducted FISH on 8 of the genes encoding putative peritrophins in *D. melanogaster* larvae. The latter explanation suggests that the larval PM (like that of the adult) may be a composite structure in which the chitin component is sandwiched between two peritrophin rich layers, since Peters (1976) reported that the material secreted by the APR cells had already coalesced into a PM prior to reaching zone I. However, this would imply that the larval PM is composed of four or more layers, which is in conflict with the image in Fig. 54. Alternatively, this would suggest that within the zones from APR through to PPR, the nascent PM is still relatively fluid, allowing the chitin component to become embedded within the peritrophin rich matrix, which is secreted by zone I (PR). I propose that as the nascent PM moves distally towards the posterior cardia it may be supplemented by the addition of a number of peritrophins by zone II and the midgut caeca. The secretion of peritrophins would also account for the electron dense layer on the epithelial side of the PM. Moreover, the initial secretion of the PM in a zone which extends from the APR-PPR zone, a

distance of only 75-100 μm, may account for the more compact nature of the larval PM (110-130 nm thick) in comparison to the adult PM (200 nm thick), since this may not be fully attributed to the former having an additional layer.

The most intense signal for all larval peritrophin probes was found in the apical region of the cells in formation zone I, in agreement with the supposed PM initiation site (Rizki, 1956). Nonetheless, there is some minor discrepancy in terms of the exact size of the secretion zone: this study indicates that it is composed of three cells (most clearly seen in Fig. 49), as opposed to Rizki (1956), who rather dubiously (after bad fixation resulting in significant cell shrinkage) counted four cells.

The expression of *DmPro9*, *DmPro7*, *DmPro8* and CG31893 by zone II and the gastric caeca suggests that the cells of these zones may secrete further peritrophins, required to reinforce the structural integrity of the PM. However, the subsequent incorporation of the majority of the additional peritrophins (*DmPro9*, *DmPro8* and CG31893) would provide capping proteins to protect the free ends of the chitin fibrils (lying perpendicular to the PM face) rather than additional cross-links between chitin fibrils and peritrophin components since *DmPro7* is the only peritrophin having two CBDs that is located in these layers. Indeed, invertebrate intestinal mucin (IIM) is secreted by cells throughout the length of the mesenteron of *T. ni* and *H. virescens* caterpillars in addition to the anterior mesenteron (homologous to the PR zone of *Drosophila* larvae), where the PM is initially formed (Ryerse *et al.*, 1992; Harper and Granados, 1999). This role of the midgut caeca in *Drosophila* larvae may be similar to that suggested in some grasshoppers, in which the caeca form a caecal membrane that is believed to supplement the PM (Lee, 1968; Baines, 1978; Bernays, 1981). Conversely, the caecal membrane formed in mosquito larvae (*Culex pipiens*, *Aedes aegypti* and *Anopheles stephensi*) has been shown not to contribute to the PM secreted by the midgut epithelium (Volkmann and Peters, 1989a and 1989b).

Whether the constrained larval PM formation zone has more conspicuous (than the ones mentioned above) effects on PM structure and/or function remains to be seen. Future work should thus include probing adult-specific peritrophins in the larval cardia and vice versa. In addition, other genes involved in PM secretion may be probed, such as chitin synthase in order to gain an even broader picture of PM formation that encompasses chitin synthesis. Furthermore, this technique could be used to provide a useful tool to directly determine the effect of various physiological conditions on the rate of PM production, including the response to starvation or infection, since modulation of the amount of mRNA will cause a corresponding induction or reduction in the specific signal.

T.E.M. studies conducted by King (1988) and Peters (1976) report a multilaminar, ~245 nm thick adult PM (PM1 of 200 nm, including a 23 nm electron dense layer, and a 45 nm thick PM2). By contrast, the larval PM shows less complexity, whilst being more compact (110-130 nm, including an electron-dense layer of ~12 nm), which implies that fewer formation zones are required to secrete it. Indeed, the PM of larvae appears to consist of a single integrated layer composed of a thin electron dense layer and amorphous electron lucent material (Fig. 54). This suggests that the larval cardia secretes a more compact PM from formation zone 1, this nascent PM being supplemented with material formed by zone 2 and the midgut caeca as it moves distally. By contrast, the lamellae of the adult PM are formed by the differential expression of it components from the extensive formation zone of the adult cardia. Indeed, these (F)ISH studies suggest that adult peritrophins are secreted by a number of staggered secretion zones (see Table 27 for summary), initially forming separate layers that converge after their formation (King, 1988). The split of the adult PM post maturation into PM1 and PM2 along the boundary of layers 3 and 4, indicates that the individual lamellae are not tightly bound, which may also account for the much thicker adult PM. Indeed, one of the phenotypes of the larval PM deficient in all eight of the larval peritrophins involved in this study resembles that of the split adult PM. This may suggest that peritrophins are indeed critical to the structural integrity of the PM, both in adults and larvae, in which the PM components are integrated to form a nascent PM in a staggered (adult) or more uniform (larvae) fashion.

The elegant functional specialisation of adult cardia epithelial cells (Fig. 41) may be correlated with a comparatively more differentiated PM morphologically (King, 1988) (Fig. 54A). This suggests that the evolution of the cardia is linked with the adaptive transformation of the PM to suit the food source. In nature, larvae feed on the natal substrate, particularly yeast which commonly occurs on the fermenting fruit and the eukaryotic decomposition products of their conspecifics; adults ingest primarily fungi and yeast, in addition to prokaryotic decomposition products as liquid food via a protrusive sucking proboscis. Thus, the larval diet is much more particulate, and therefore abrasive in comparison to that of the adult. However, there is no indication of a link between an abrasive diet and a PM structure that is less impaired by abrasive damage in *Drosophila* larvae, whilst there appears to be a link between the ingestion of the abrasive *Bacillus thuringiensis* endotoxin and PM integrity in *Manduca sexta* (Rupp and Spence, 1985).

From the (F)ISH results it may be concluded that the majority of epithelial cells within the larval cardia do not have the potential to express peritrophins. This is reflected by the localisation of peritrophin mRNA to only two formation zones within the cardia itself, and the midgut caeca in some cases. Conversely, within the adult cardia virtually all the epithelial cells

are capable of expressing peritrophins, with the apparent exception of zone 5 (Fig. 45). The absence of expression of peritrophins within this zone could be ascribable to either the cells within this zone having subsequently lost their ability to express peritrophins or their activity is involved with the secretion of other molecules, including enzymes such as aminopeptidases that are believed to be integrated into the PM structure (Peters and Kalnins, 1985). The former theory is supported by the specialised expression pattern of *DmPro2* in comparison to the more generalised expression pattern of *DmPro3*. Thus, it can be concluded that *DmPro2* is the only adult gene that is both specifically and locally expressed. The remaining two adult genes are more generalised in expression, as are all the larval peritrophins. This is in contrast to the traditional view conveyed in many morphological studies that link structure and function in often too rigid a fashion by over-categorising cells.

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