The Molecular Mechanism of Immune Evasion by the Eggs and Larvae of the Endoparasitoid *Venturia canescens* in its Host, *Ephestia kühniella.*

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Venturia canescens ovipositing in host *Ephestia kühniella*.
(Photo, courtesy of Rüdiger Buhl, Pressestelle der Albert- Ludwigs Universität, Freiburg).

TABLE OF CONTENTS

Summary

Venturia canescens (Gravenhorst) is unique among hymenopteran endoparasitoids. Instead of using wasp secretions to actively suppress the host immune defence as seen in other systems analysed so far, the egg and larva are protected against the host defence of *Ephestia kühniella* (Zeller) by a passive mechanism, leaving the host defence system intact. The protective feature is a layer consisting of several components found on the parasitoid egg and larval surface which preclude immune recognition and/or prevent encapsulation by host hemocytes. In this study, the molecular composition of parasitoid surface components were analysed using serological methods and specific sugar-binding lectins as diagnostic tools. These data revealed that the protective layer consists of at least two parts:

i) A mucin-like glycoprotein, produced by the adult female wasp during oogenesis covers the egg surface. After oviposition, the developing embryo secretes the glycoprotein which covers the larval cuticle during late embryogenesis.

ii) In addition to the mucin-like glycoprotein layer, additional components from the wasp calyx fluid and the host hemolymph are added to the egg and larval surface.

Using a lectin against specific GalNac sugar, which was previously shown to recognise hemomucin, a glycoprotein implied to be involved in immune response in *Drosophila melanogaster*, a similar compound was identified in *V. canescens*. In *V. canescens*, like in *D. melanogaster*, the glycoprotein is found on the surface of sclerotised structures and adjacent tissues. The protein is found on the chorion layer which is produced by the follicle cells, and on the peritrophic membrane produced by the cardia and gut cells. This protein may serve to physically separate and lubricate these sclerotised structures from cells and tissues. In addition, the glycoprotein is found on the surface of hemocytes, where it has an immune function.

Previously a 42 kDa host protein was identified by antigenic similarities to VLP-proteins of 52 and 60 kDa. This component was shown to be present in the host hemolymph and on the surface of hemocytes. A VLP-protein of 40 kDa was cloned and shown to contain a protein domain with structural similarities to vertebrate phospholipid-associated glutathione peroxidase (PHGPX). Antibodies against bacterial fusion proteins of the PHGPX-

like domain revealed a host protein which was mainly expressed in the silk glands of the host caterpillars.

The identification of three different components with structural similarities to host-like components on the egg surface suggests that the VLP-proteins and the mucin-like glycoprotein may resemble insect structures that are conserved between the two species. This study suggests that the wasp larval cuticle is protected in a similar fashion as the egg chorion, except that the calyx-specific VLPs are probably replaced on the larval cuticle by host hemolymph proteins. Thus the protective layer is a complex mixture of several components, where some may be produced by the wasp embryo (mucin-like glycoprotein), and some may be derived from the host hemolymph (42 kDa protein) which become attached to the cuticle while the late embryo is still inside the egg shell.

These findings suggest that the mechanism of passive immune evasion may have emerged during the evolution of the wasp-host interactions using conserved insect components that are found both in the parasitoid species and in the potential host. The implication is that structurally conserved components may have similar functions in the parasitic and non-parasitic species and therefore could constitute a useful pre-adaptations for an endoparasitoid lifestyle. Thus one of the possible functions of the symbiotic virus in *V. canescens* could be to modify the structure and expression of wasp genes to allow its products to be secreted in the calyx gland, from where it is transported into the host during oviposition.

Declaration

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

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signed :

Wanja M. Kinuthia, M.Sc

Dated this twenty fifth day of June, 1996.

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List of Abbreviations

BCIP	5-bromo-4-chloro-3'-indolyphosphate (p-toluidine salt)
bp	base pair
BPB	bromophenol blue
ßme	ß-mercaptoethanol
BSA	bovine serum albumin
cDNA	complimentary deoxyribonucleic acid
cm	centimetre
DAB	3,3'-diaminobenzidine
DBO	1,4-diazo-bicyclo-[2,2,2] octane
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylendiaminetetra-acetic acid di-sodium salt
EtOH	ethanol
FITC	fluorescein isothiocyanate
g	gram(s)
Gal-NAc	N-acetyl-D-galactosamine
H_2O_2	hydrogen peroxide
H. p.	*Helix pomatia* (the Roman or edible snail)
hr	hour(s)
l	litre(s)
kb	kilo bases
kDa	kiloDalton
m	molar
mA	milliampere
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimole
MW	molecular weight

NBT	nitro blue tetrazolium
NP-40	nonylphenoxy polyethoxy ethanol
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
SDS	sodium dodecyl sulphate
s	second(s)
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SNDW	sterile nanopole distilled water
TEMED	N,N,N',N'-Tramethylethylenediamine
Tween-20	polyoxyethylenesorbitan monolaurate
µg	microgram(s)
µl	microlitre(s)
U.V.	ultra violet
V	volt(s)
v/v	volume per volume
VLPs	Virus-like particles
w/v	weight/volume

Chapter One

General Introduction

Review of Literature

1.1 Introduction

Invertebrates constitute approximately 96% of all animal species. The capability of occupying almost every ecological niche, may stem from the ability to overcome the potential threat of pathogenic micro-organisms which these creatures encounter in their respective ecosystems. However, the molecular knowledge on how these creatures recognise foreign objects and distinguish "self" from "non-self" is lagging relative to higher vertebrates. In addition to micro-organisms, insects are exposed to multicellular parasitoids, where some have managed to live inside the hemocoel of the insect. In general, study of the survival mechanisms of endoparasitic invertebrates inside living host insects is still in its infancy. Nevertheless, there is some progress in the case of vector arthropod species which carry organisms responsible for human diseases like malaria (Aikawa *et al.*, 1981) and schistosomiasis (Schmidt, 1995).

Insects, like vertebrates, have evolved successful strategies to reduce their potential of serving as hosts for the entomophagous and entomopathogenic organisms encountered in their natural habitats. Godfray (1994) described several behavioural and physiological mechanisms such as avoidance, escape, mimicry and repellency to overcome some of these environmental hazards. Avoidance is the mechanism of concealing self by feeding inside the host plant, *eg.* the leaf mining insects whereas escape is the violent wriggling of the host when parasitation is attempted. Two forms of mimicry have been demonstrated; chemical and morphological mimicry of the host by the parasitoid as exemplified by Eucharitidae ant larval and pupal parasitoid and *Echthrodape africana*, the ectoparasitoid of bee larvae, respectively. Many ichneumonids and braconids produce a repugnant odour when disturbed (Godfray, 1994). On-the-other-hand, the exocuticle acts as an external barrier to keep potential intruders at bay and the peritrophic membrane of the gut excludes micro-organisms from the hemocoel.

An important factor in the successful establishment of insects has been the development of immune defence reactions that are both strong enough to protect against infection and flexible enough to allow adjustment to a diverse array of ecological niches (Klein, 1989). The internal immune systems (cellular and humoral) constitute the final

line of defence to eliminate foreign objects from within the hemocoel (Ratcliffe and Rowley, 1979; Götz and Boman, 1985; Dunn, 1991). This is achieved by utilising certain molecules involved in recognition, for example lectins, which interact with glyco-conjugates on hemocytes, several anti-bacterial peptides and the prophenoloxidase system, thus facilitating phagocytosis or encapsulation of foreign matter (Bayne, 1990).

Some of these protective strategies, known to be effective in insects for defence against pathogenic and parasitic invaders are reviewed below.

1.2 Physical barriers

External barriers are not only important for preventing mechanical damage to the internal organs, but also in the exclusion of potential pathogens and parasitoids from the hemocoel. The external barrier varies within groups of invertebrates for example, the hard crustacean cuticle has a high calcium content which is lacking in the sclerotised insect cuticle. However, the two types of cuticle have similar proteins, like chitin and certain amino acids, though at very different concentrations (Welinder, 1974; Kumari *et al.*, 1995).

The external milieu, from an immunological point of view, includes the lumina of the digestive and respiratory systems as possible entry sites for pathogens. In insects it comprises the exoskeleton, and the cuticular lining of the foregut, hindgut, and the tracheae as well as the peritrophic membrane of the midgut. Nevertheless, micro-organisms and parasitoids may still enter through the spiracles, mouth parts and even via the ovipositor during probing and oviposition. In other invertebrates additional features are involved in protection, for example, the cuticle of the crayfish, *Astacus astacus*, contains protease inhibitors which may prevent parasitic invasion (Hall and Söderhäll, 1982).

The mucous layer, which covers the soft bodies of coelenterates, annelids, mollusc and protochordates may have a defence function (Casteels *et al.*, 1990). Its sticky, gel-like properties trap bacteria and other micro-organisms, thus preventing them from reaching the underlying tissue. Molecules such as lectins, protease inhibitors and antibacterial factors secreted into the mucus kill or render the trapped micro-organism

inactive (Magarinos *et al.*, 1995). On-the-other-hand, the water dwelling amphibians use anti-microbial proteins from their skin as an additional protection against infection (Bevins and Zasloff, 1980; Zasloff, 1987).

In general, arthropods appear to be well protected even at their most vulnerable moulting period. During this time, additional protective substances like anti-microbial factors (such as fungitoxic quinones) are produced (Söderhäll and Ajaxon, 1982).

The internal immune systems will be discussed under two categories, cellular and humoral reactions (Dunn, 1986; Boman and Hultmark, 1987; Vinson, 1990a). However, this classification is arbitrary.

1.3 Cellular immune response

In general, the cellular defence reaction of invertebrates is based on hemocytes that are present in the hemolymph. Invertebrate cellular defence reactions include wound repair, cell-mediated coagulation, phagocytosis, nodule formation and encapsulation (Ratcliffe and Rowley, 1979; Ratner and Vinson, 1983a; 1983b; Ratcliffe and Götz, 1990). Each of these reactions is evoked under specific circumstances, sometimes depending on the size of the intruding object (Ratcliffe, 1993). Morphology, and thus the classification of hemocytes, is different within groups of insect species and therefore not well defined (Brehelin and Zachary, 1986; Ratcliffe *et al.*, 1986). The two main groups of hemocytes, involved in defence in the case of arthropods are plasmatocytes, responsible for phagocytosis of small organisms and wound healing, and granulocytes for encapsulation of larger objects in conjunction with plasmatocytes (Ratcliffe and Götz, 1990; Gupta, 1991a). The two cell types can be found to cooperate during immune responses. An example is the enhancement of the process of phagocytosis of *Bacillus cereus* by *Galleria mellonella* plasmatocytes, caused by the addition of granulocytes (Anggraeni and Ratcliffe, 1991). This indicates that secreted components of granulocytes are important in phagocytosis (Ratcliffe and Götz, 1990). However, other hemocytes like oenocytoids, spherulocytes and adipohemocytes may also be involved.

Phagocytosis is the ingestion of foreign matter and its encasement in a cytoplasmic vacuole. This mechanism involves recognition, attachment and internalisation of small

foreign objects, such as bacteria, viruses, protozoan, fungi, *etc.* Vinson, (1990a). Gupta, (1991b), described the process as an unidirectional chemotactic movement of hemocytes towards the foreign body where recognition is achieved by surface receptors through opsonins added onto the object from the hemolymph and the surface carbohydrates of the phagocytes (Sharon, 1984). The phagocytes then engulf the particles by formation of vesicles transferring them into lysosomes, where organic structures are broken down by enzymes and other anti-microbial molecules. Breakdown of the bacterial cell wall releases lipopolysaccharides (LPS) or peptidoglycans, which are strong elicitors of antibacterial proteins in insects (Kanost *et al.*, 1988; Dunn, 1991; Weisner and Götz, 1993). The cellular defence reactions collaborate effectively with the humoral immune components discussed below.

1.4 Humoral immune response

The humoral response involves the production of a variety of hemolymph proteins that are specifically induced or increased in relative amounts, in response to wounding and infection (Boman and Hultmark, 1987; Boman *et al.*, 1991). Some of these humoral response proteins are antibacterial (Faye and Wyatt, 1980; Boman, 1991; Kimbrell, 1991; Faye and Hultmark, 1993), either through lytic or bacteriostatic effect and, together with phagocytosis, constitute a potent defence against bacteria. In addition, hemostatic mechanisms are activated to repair wounds and prevent further entry of bacteria (Rizki and Rizki, 1984; Ratcliffe *et al.*, 1985; Dunn, 1986; Boman and Hultmark, 1987). In addition to the classical antibacterial and bacteriostatic substances, other components (recognition molecules, adhesion proteins, *etc.*), are produced. However, their function is not well defined in the cascade of events leading to the inactivation of micro-organisms.

1.4.1 Antibacterial proteins

Insect anti-bacterial proteins have recently been categorised on the basis of their protein sequence homology as belonging to one of five families (Hetru *et al.*, 1994):

1. Peptides forming amphipathic α-helices *eg.* cecropins (Hultmark *et al.*, 1980;

Hultmark *et al.*, 1982).

2. Peptides with intramolecular disulfide bridges *eg.* "defensins" (Matsuyama and Natori, 1988).

3. The glycine-rich polypeptides *eg.* attacins and diptericins, identified in *Phormia terranovae* (Dimarcq *et al.*, 1990).

4. The proline-rich peptides *eg.* drosocin from *Drosophila melanogaster* (Bulet *et al.*, 1993) and

5. Other antibacterial peptides and polypeptides, *eg.* lactoferrins and lactoferricins, iron binding proteins present in many biological fluids of mammals (Arnold *et al.*, 1977; Ellison *et al.*, 1990).

Of all antibacterial proteins characterised in invertebrates, cecropins and defensins are the only ones shown to have counterparts in vertebrates (Hetru *et al.*, 1994). The human homologues appear to have a similar mode of action as those in invertebrates.

1.4.1.1 Cecropins

Cecropins from *Hyalophora cecropia* were the first antibacterial peptides isolated from insects (Hultmark *et al.*, 1980) and characterised (Steiner *et al.*, 1981). The mode of action of these amphipathic molecules has been investigated (von Hofsten *et al.*, 1985; Christensen *et al.*, 1988; Boman *et al.*, 1991; Faye and Hultmark, 1993; Gazit *et al.*, 1994). The interaction of cecropins with the lipid bilayer of bacterial cell membranes form voltage-dependent ion channels which block the generation of ATP, essential for oxidative phosphorylation (Okada and Natori, 1985; Hultmark, 1993). This reaction is thought to be prevented by cholesterol, a compound found in eukaryotic cytoplasmic membranes, which renders them resistant to lysis by cecropin (Nakajima *et al.*, 1987; Natori, 1988).

Cecropins in conjunction with lysozymes have been found to be induced during the degradation of larval tissue during pupariation, this is required for protection against gut bacteria (Kylsten *et al.*, 1990). These facts point to the crucial role cecropins play in preserving the integrity of both higher and lower organisms.

1.4.1.2 Insect defensins

The insect defensins are positively charged, small peptides of 40 amino acids (Dimarcq *et al.*, 1990). They have been isolated from an embryonic cell line of *Sarcophaga peregrina* (Matsuyama and Natori, 1988) and from the royal jelly of *Apis mellifera*, where they are termed sapecins and royalisin, respectively (Fujiwara *et al.*, 1990). At the protein level, insect and mammalian defensins have common or similar disulfide bridges but vary in that the bridges are formed between alternative cysteine and therefore have different structures (Lambert *et al.*, 1989). Defensins are the major factors that act against gram-positive bacteria in many insects (Hultmark, 1993), but have little activity against either gram-negative bacteria or eukaryotic cells (Hetru *et al.*, 1994).

1.4.1.3 Attacins

Attacins are antibacterial proteins produced in insects in response to bacterial infection (Götz and Boman, 1985) and are found in basic or acidic forms. Attacins are synthesised as pro-proteins and undergo post-translational modifications during maturation and secretion (Hultmark *et al.*, 1983; Engström *et al.*, 1984a). They were first characterised by Pye and Boman (1977) in *H. cecropia* and were called P5 and are of 20-30 kDa in size. They are distantly related to a heterogenous group of antibacterial insect proteins which include sarcotoxin II, diptericin and coleoptericin (Wicker *et al.*, 1990; Hultmark, 1993). The attacins are bacteriostatic and may act by increasing the permeability of the outer membrane of bacterial cells, thus increasing their sensitivity to enzymes like lysozymes (Engström *et al.*, 1984b). They are also reported to affect growing gram-negative bacteria including *Escherichia coli*, causing them to grow in long chains (Hultmark *et al.*, 1983; Ishikawa *et al.*, 1992). The mode of action of attacins on *E. coli*, though not well understood, is believed to be via blocking the synthesis of the major outer membrane proteins (Carlsson *et al.*, 1991).

1.4.1.4 Lysozymes

Lysozyme is a universal and probably multi-functional enzyme found in microbes, plants, invertebrates and vertebrates. The main role of the enzyme is suggested to be the digestion of polysaccharides by hydrolysing the glycosidic linkage between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan layer of the bacterial cell wall (Boman and Hultmark, 1981). In addition to its role in the immune system, it is a digestive enzyme in ruminants (Stewart *et al.*, 1987) and insects (Boman and Hultmark, 1987). It was the first antibacterial protein to be isolated from insects, initially in *G. mellonella* and *Bombyx mori* (Powning and Davidson, 1973), and later in *D. melanogaster* (Kylsten, 1991). It has since been isolated from many other insects (Powning and Davidson, 1973; Croizier and Croizier, 1978; Hultmark *et al.*, 1980). The lysozyme from *G. mellonella* has been characterised with regard to enzymatic properties (Powning and Davidson, 1976), and is similar in many respects to chicken lysozyme (Jolles *et al.*, 1979).

1.4.2 Recognition molecules

Insects utilise recognition molecules to identify and possibly opsonise foreign objects which are then phagocytosed or encapsulated. These molecules are present in the open circulatory system but their exact role remains to be established.

1.4.2.1 Lectins/hemagglutinins

Lectins are large protein complexes (70-1500 kDa) composed of sub-units of 30-40 kDa in size (Kanost *et al.*, 1990) and belong to a family of carbohydrate binding proteins found in both plants and animals (Barondes, 1981). They have been identified in the hemolymph of several insects (Yeaton, 1981; Castro *et al.*, 1987) including the orders Lepidoptera (Bernheimer, 1952) and Diptera (Natori, 1988). Lectins have been shown to be involved in immune responses as opsonins (Komano and Natori, 1985; Pendland *et al.*, 1988). In *S. peregrina* larvae, lectin titre increases during injury, infection or moulting as well as during agglutinating activity of sheep erythrocytes (Komano *et al.*, 1980). Many of the purified insect lectins have galactose specificity

(Lackie and Vasta, 1988; Pendland *et al.*, 1988), though Kubo and Natori (1987) described a 1500 kDa lectin with specificity for N-acetylgalactosamine from *Periplaneta americana*. Lectins of other sugar specificity have been reported in other invertebrates (Renwrantz, 1983; Renwrantz, 1986).

Lectins interact with surface receptors (Nicholson, 1974; Daimon and Sano-Martins, 1989), where they specifically bind to carbohydrates on the surfaces of blood cells or extracellular matrix. At the same time lectins are able to bind to surface components of micro-organisms (Götz and Boman, 1985; Van der Knaap and Loker, 1990). For example, a lectin isolated from cockroach binds to the polysaccharide side-chains in LPS (Jomori *et al.*, 1990). Soluble lectins, are often functionally described as hemagglutinins. They act as bridges to bind carbohydrates on cellular surfaces and micro-organism leading to agglutination. *In vivo* they are involved in the uptake and destruction of cells as well as lymphocyte migration (Sharon and Lis, 1972; Sharon, 1984; Sharon and Lis, 1989). Indeed, Alberts *et al.* (1989) suggested that lectin molecules present on the cell surface recognise oligosaccharides on the surface of other cells and thus initiate cell interaction. This is a prerequisite for the two main cellular immune reactions, phagocytosis and encapsulation, against foreign antigens.

In the case of *Aedes aegypti*, lectins increased five-fold following infection with *Dirofilaria immitus* microspores thus enhancing the binding properties of the hemocytes (Nappi and Christensen, 1986). The bi-functional property of lectins have been established with M13, a lectin of *Manduca sexta*. This was achieved by treatment of M13 with trypsin which eliminated the hemocyte coagulation activity but not the erythrocyte agglutinating ability (Minnick and Spence, 1988; Van der Knaap and Loker, 1990).

1.4.2.2 Hemolin

Hemolin is a 48 kDa immune protein, found in low concentrations in un-induced *H. cecropia* and *M. sexta* hemolymph. It is strongly induced in *H. cecropia* pupae by bacteria (Rasmuson and Boman, 1979; Andersson and Steiner, 1987), although it has no direct antibacterial activity (Andersson and Steiner, 1987). Sun *et al.* (1990) characterised hemolin and showed that it belongs to the immunoglobulin superfamily.

Hemolin is closely related to the mouse neural cell adhesion molecule L1 (Moss *et al.*, 1988), and the insect cell adhesion molecules of extracellular extensions neuroglian and amalgam which are involved in neural cell-cell interactions (Seeger *et al.*, 1988).

Hemolin binds to the bacterial surface by formation of a complex with another protein in the hemolymph (Sun *et al.*, 1990; Schmidt *et al.*, 1993). The complex formation is dependent on the presence of sugar residues in LPS molecules of the bacterial cell wall, as demonstrated in experiments with LPS-mutant *E. coli* (Schmidt *et al.*, 1993). The full significance of this finding is not clear, although Ladendorff and Kanost, (1991) demonstrated that hemolin prevents aggregation of *M. sexta* hemocytes after binding to the cell surface, and may therefore be involved in both recognition of "non-self" and, at the same time, signalling "self".

1.4.2.3 Prophenoloxidase activating system

The presence of phenol oxidases (PO) has been demonstrated in hemocytes, serum and integument (Ashida and Yamazaki, 1990; Charalambidis *et al.*, 1994). POs are naturally occurring and widely distributed enzymes which generate melanin by oxidising phenolic substances. Two distinct types of POs have been reported in insects:

1) Monophenol mono-oxygenases, such as tyrosinase found in hemocytes, serum and integument, and

2) Laccase-type oxygenase, with a preference for *p*-diphenol found only in the integument (Ashida and Yamazaki, 1990).

Both PO types occur as inert pro-phenoloxidase (pPO), in the hemolymph of most insects, *eg. B. mori* (Ashida, 1990), *G. mellonella* (Pye, 1974), *D. melanogaster* (Seybold *et al.*, 1975), *H. cecropia* (Andersson *et al.*, 1989), *Locusta migratoria* (Brehelin *et. al.*, 1989), and *A. aegypti* (Ashida, 1990).

Tyrosinase is involved in malanization, defence reactions, and wound healing, while the main role of laccase is in sclerotisation reactions (Ashida and Yamazaki, 1990; Sugumaran, 1991; Hopkins and Kramer, 1992; Charalambidis *et al.*, 1994). Most of the biochemical studies of steps leading to malanization and sclerotisation were carried out by

Nappi (1973) and Ashida and Yamazaki (1990) and have been reviewed by Sugumaran (1991).

The presence of the pro-phenol oxidase (pPO) in the hemolymph suggests a cascade of enzyme activities (Söderhäll *et al.*, 1979; Ashida *et al.*, 1982; Yoshida and Ashida, 1986), initially demonstrated by Seybold *et al.* (1975). In the pathway leading to malanization, tyrosinase catalyses the initial steps of melanin formation, while in sclerotisation an enzyme is involved in the generation of reactive tyrosine derivatives, leading to protein cross-linking (Sugumaran, 1991). In *Drosophila algonquin*, the PO activating cascade reaction was initiated by a hormonal imbalance following parasitation by *Pseudeucoila bochei* (Nappi, 1973). In other arthropods, pPO is activated by microbial cell wall components such as LPS, ß-1,3 glucan and peptidoglycans (Ashida and Yamazaki, 1990; Söderhäll *et al.*, 1990). However, in the case of non-organic objects, the initiating signal events are yet to be determined.

The PO activating system has been linked to immune responses in arthropods (Ashida *et al.*, 1982; Söderhäll, 1982) and has been suggested to be involved in "self" and "non-self" discrimination (Ashida *et al.*, 1982; Söderhäll, 1982; Ratcliffe and Götz, 1990; Söderhäll *et al.*, 1990). In the case of cellular immune responses, it has been shown that encapsulation and nodule formation are normally accompanied by malanization, due to a pPO activating cascade (Ratcliffe *et al.*, 1984). While phagocytosis of bacteria is stimulated under conditions that trigger the pPO cascade, encapsulation is normal in a PO-deficient mutant of *D. melanogaster* (Rizki and Rizki, 1990a).

Although the exact role of PO in the recognition process is not clear, the pPO-activating cascade constitutes a link between recognition and cellular response (Boman and Hultmark, 1987; Hultmark, 1993; Charalambidis *et al.*, 1994). Work done by Charalambidis *et al.* (1994) on *Ceratitis capitata* larvae suggests that the recognition of foreignness is achieved by certain hemocytes or integumental proteins. However, the entrapment of bacteria by hemocytes, or their immobilisation at the integument, is achieved by a specific tyrosinase (Marmaras and Charalambidis, 1992; Marmaras *et al.*, 1993; Marmaras *et al.*, 1994). The functional differences of the various tyrosinases were demonstrated by Sugumaran (1991) and Charalambidis *et al.* (1994), which

indicated that hemocyte tyrosinase is responsible only for defence, serum tyrosinase for malanization, and the integumental enzyme form(s) for both defence and malanization. Further work on *C. capitata* by Charalambidis *et al.* (1994) found that only glycosylation and adhesiveness distinguish between larval tyrosinases, and suggested that the two enzyme forms could be similar, but not identical and are encoded by different genes.

1.5 Immune suppression by hymenopteran endoparasitoids

Despite the presence of fully functional immune system in insects (see above), some species have adopted an endoparasitic life style and managed to circumvent the host defence system. The means utilised by endoparasitic insects for this process are many and have been categorised as: avoidance, evasion, destruction, suppression and subversion (Vinson, 1990a). This includes laying of eggs inside a host egg which is devoid of any immune response, or in early larval stages that have not yet developed an efficient immune system. In some cases eggs are oviposited in tissues like the muscles which are not directly exposed to the hemolymph (Salt, 1968).

Active suppression and passive evasion of the host's immune responses are the two main strategies employed by endoparasitoid wasps. Active suppression is achieved by utilisation of ovarian secretions of the parasitoid including certain symbiotic virus entities, whereas passive evasion of host immune recognition is achieved by a protective layer around the egg and larval surface.

1.5.1 Active suppression by polydnaviruses

Endoparasitoid wasps are uniquely adopted to living within other insects. In some hymenopteran species, the egg is deposited into the host together with calyx gland secretions (Stoltz and Vinson, 1979; Stoltz, 1986), containing polydnaviruses (Krell, 1991; Fleming, 1992; Stoltz and Whitfield, 1992; Stoltz, 1993) or virus-like particles (Rotheram, 1967; Bedwin, 1979b; Feddersen *et al.*, 1986). In some parasitoid-host systems these secretions are known to protect the developing wasp against the host's defence reactions (Edson *et al.*, 1981; Vinson, 1990a) and/or alter physiological conditions inside the host (Beckage and Thomas, 1986; Vinson, 1990b;

Lavine and Beckage, 1995), allowing the maturation of the wasp. These wasp-induced changes obliterate the immune defence system (Dunn, 1986), and the host becomes vulnerable to normally non-pathogenic organisms (Stoltz and Guzo, 1986) or bacteria (Ross and Dunn, 1989).

The focus of studies on the immune suppression has been on symbiotic polydnaviruses (PDV) associated with some members of the hymenopteran families, Braconidae and Ichneumonidae (Krell *et al.*, 1982; Brown, 1986). Polydnaviruses possess segmented genomes composed of double stranded circular DNAs (Fleming and Krell, 1993). These viruses replicate in specific cells lining the calyx gland of the female wasps, and are released into the oviduct and co-injected with the egg during oviposition (Krell, 1987; Stoltz, 1990; Fleming and Summers, 1991). The PDVs become transcriptionally active shortly after infecting the host cells (Blissard *et al.*, 1989; Fleming and Summers, 1991; Asgari *et al.*, 1996). Expression of the PDV genes cause profound alterations in host physiology, including abrogation of the cellular immune responses, modification of the host's normal developmental progression (Stoltz, 1986; Strand and Noda, 1991; Fleming, 1992; Dushay and Beckage, 1993; Beckage *et al.*, 1994; Soller and Lanzrein, 1996) as well as change of hormonal titre (Zitman *et al.*, 1995). Some of these changes are essential for the survival of the endoparasitoid (Edson *et al.*, 1981; Stoltz, 1993)

The suppression of the immune system by the PDVs is sometimes enhanced by venom proteins produced in the accessory glands of the reproductive organs (Kitano, 1986; Tanaka, 1987; Stoltz *et al.*, 1988; Soller and Lanzrein, 1996). However, other non-viral proteins called ovarian proteins (OP) are also synthesised by calyx cells or specialised oviduct epithelial cells, and possibly other regions of the reproductive system (Webb and Dahlman, 1985; Webb and Luckhart, 1994). Although PDV-expression is required for protection, virus transcripts are usually not detected before four to six hours after deposition. The corollary of this is that, OP and venom proteins may be involved in egg protection during the window between oviposition and PDV gene expression, and may therefore be essential for the initial protection of the parasitoid (Asgari and Schmidt, 1994; Webb and Luckhart, 1994).

In the case of *Heliothis virescens*, hemocytes undergo rapid morphological changes after parasitation by *Campoletis sonorensis* and by ninety minutes post-parasitation, the spreading ability of plasmatocytes is greatly impaired while the actin (a globular protein involved in cellular movement), in both plasmatocytes and granulocytes, is virtually disrupted (Webb and Luckhart, 1994). In their earlier studies, Webb and Summers, (1990), showed immunological cross-reactivity between the OP and viral structural proteins in *C. sonorensis*. However, no immunological cross-reactivity or sequence homology was found between OP and the cysteine-rich immuno-suppressive polydnavirus gene (Li and Webb, 1994). They found that *C. sonorensis* viral proteins and OPs are independently produced, causing similar morphological effects on *H. virescens* hemocytes at different times.

A unique virus type was described by Rizki and Rizki (1984, 1990b) in the cynipid, *Leptopilina heterotoma*. Unlike other PDVs, this virus is produced in an accessory gland which is separate from the oviduct and opens directly into the genital valves, from where the virus is co-injected with the eggs into *D. melanogaster* host larvae. The virus specifically infects and destroys the lamellocytes, thus preventing encapsulation. Whether the viruses in this system originated from the same group as other PDV-like viruses, or whether each virus type evolved separately according to host requirements, is not known (Whitfield, 1992). Possibly, these endosymbiotic viruses in hymenopteran parasitoids constitute genetic devices that allow the female wasp to provide its egg with protective maternal secretions (Stoltz and Vinson, 1979; Stoltz and Whitfield, 1992; Schmidt *et al.*, 1993). An alternative hypothesis would be that these viruses might be part of the genetic properties of the parasitoid wasp genome that assist it in adapting to the environment in different hosts (Schmidt and Theopold, 1993; Summers and Dib-Hajj, 1995).

1.5.2 Passive protection

Several endoparasitoids are known to evade the host immune response by molecular mimicry, utilising host-like molecules to cover their surfaces. Although little is known about the recognition of foreignness in invertebrates and how foreign objects are

distinguished from own cells and tissues in the open circulatory system, it is assumed that molecules that are similar to certain host proteins may not be recognised as foreign (Salt, 1970). This phenomenon of passive protection may be common in both insect endoparasitoids and protozoan parasites of arthropods (Gupta, 1991a).

1.5.2.1 Surface protection

The active immunosuppression mediated by PDVs requires at least two hours for gene expression in the host, however, the encapsulation process by host hemocytes is known to be initiated immediately. In this situations, endoparasitoids have evolved a mechanism to utilise ovarian secretions which cover the egg surface, precluding encapsulation by the host defence system.

The idea of ovarian secretions covering and protecting the egg from host defence reactions goes back to studies by Salt (1973) on the protection of *Venturia canescens* eggs in habitual and non-habitual hosts (Salt, 1964, 1973). He tested the possibility of a passive protection mechanism due to egg surface properties being similar to host components. Since transplantation experiments, involving exchange of tissues between two permissive host caterpillar species, resulted in encapsulation reactions, he concluded that the sole basis of protection could not have been the structural similarity between the egg surface and the basement membrane of the open circulatory system (Salt, 1964; 1973).

In the pupal endoparasitoid *Pimpla turionella*, secretions from various glands have been implicated in the protection of the egg inside the host pupa (Kilincer, 1975; Osman, 1978; Osman and Fuhrer, 1979). Although some of the gland secretions appear to actively suppress hemocyte adhesion (Osman, 1978), other gland components are non-soluble in host hemolymph forming a layer around the eggs which is active only in close proximity to the egg surface, but leaving the host defence more or less intact (Kilincer, 1975; Osman, 1978). One of the secretions from the gland consists of mucopolysaccharides (Osman, 1974), whereas another consists mainly of lecithins (the phosphatidyl cholines), an important component of biological membranes and

cholesterolesters, similar to venom secretions of other hymenopterans (Neuman and Habermann, 1956).

Some larval endoparasitoid utilise special egg surface properties to elude encapsulation. In the case of *Cardiochiles nigriceps*, a fibrous layer on the egg surface was attributed to immune evasion in the host *H. virescens* (Davies and Vinson, 1986). This passive protection by the fibrous layer allows the egg to endure immune response of the host until active suppression is established by the *C. nigriceps* virus.

In braconid endoparasitoids, a mixture of components from various glands are responsible for effective protection against host defence reactions. In *Cotesia glomeratus* ovaries, a mucopolysaccharide secretion in the oviduct is probably required but not sufficient for the protection inside *Pieris rapae* caterpillars (Kitano, 1969). Additional secretions from other wasp glands, particularly the venom glands, are necessary to provide protection to the parasitoid (Kitano and Nakatsuji, 1978; Kitano, 1982; Kitano, 1986). However, the respective role in active suppression or evasion of the host defence system is not clear.

A passive protection mechanism in *P. rapae* caterpillars have been established for *Cotesia rubecula* eggs. It is required during the small window before the *C. rubecula* polydnavirus (CrV) genes are expressed (Asgari and Schmidt, 1994). This study showed that a 35 and 65 kDa calyx fluid proteins are antigenically related to a CrV component and that the two proteins are present on the parasitoid egg surface and the CrV envelope (Asgari and Schmidt, 1994; Wellby, 1994). The corollary from these results is, the proteins are involved in immune protection of both the eggs and the virus. This is the first case reported where active disruption of the host's immune system mediated by a PDV has been shown to require collaboration of passive protective components for the survival of both the endoparasitoid and the virus.

1.5.2.2 Virus-like particles

For an endoparasitoid larva to successfully complete its development, it must overcome the host immune responses of the host. However, the integrity of the host should be maintained to protect the caterpillar and the parasitoid against infection or

hyperparasitism. Among the parasitic Hymenoptera, the *Venturia canescens-Ephestia kühniella* system is probably unique in that, the virus-like particle proteins (VLPs) produced in the calyx gland passively protect the endoparasitoid but does not suppress the immune system (Feddersen, 1986; Feddersen *et al.*, 1986).

The VLPs are similar to PDVs in terms of morphology, mode of assembly in the nucleus of calyx gland cells and ability to bud into the calyx lumen. However, the VLPs have no detectable nucleic acids and are obviously not involved in host cell infection (Rotheram, 1973a; Bedwin, 1979b; Feddersen *et al.*, 1986; Schmidt and Feddersen, 1989). The VLPs do not appear to impair the host's immune system, though little is known about their molecular protective properties (Feddersen *et al.*, 1986; Theopold *et al.*, 1994; Hellers *et al.*, 1996).

The basic subunit of VLPs is a complex (Bedwin, 1979a) of four major protein components of 35, 40, 52 and 60 kDa as well as minor proteins at 80 kDa, when analysed on a Western blot (Feddersen *et al.*, 1986). Interestingly, the 52 and 60 kDa proteins are antigenically related to a 42 kDa host protein synthesised by the hemocytes and the fat bodies (Feddersen *et al.*, 1986).

The 40 kDa protein of the VLPs has been cloned (Theopold *et al.*, 1994; Hellers *et al.*, 1996) and named p40. The C-terminal region show similarity to the phospholipid hydroxyperoxide glutathione peroxidase (PHGPX), a vertebrate enzyme (Hellers *et al.*, 1996). The sequence showed a lipophilic region which could possibly serve as a membrane anchor and the overall sequence suggest that the protein is involved in binding phospholipids. Whether the *V. canescens* p40 is a peroxidase remains to be established.

In earlier studies, Salt (1965) reported a translucent layer around the chorion when the eggs were examined by an ordinary optical microscope and Rotheram (1973b) described the layer to be present in both pre- and post-calyx eggs, but the coating was found to be thicker in the latter. This layer was shown in electron microscopy studies to consist of particles 130 nm in diameter, (later called virus-like particles) produced by the calyx cells (Rotheram, 1973a; Rotheram, 1973b). The layer appears to impart immune protection in habitual hosts, which constitute twenty three species (Salt, 1976). Although eggs are not encapsulated in a number of other lepidopteran hosts, embryonic

development is arrested in non-*Ephestia* species (Salt, 1955).

In the *V. canescens-E. kühniella* system, previous studies have shown that antigenic similarities exist between structural components of the wasp egg surface and the caterpillar (Schmidt *et al.*, 1993). Although the concept of structural similarities between host and wasp-related proteins as a basis of immune evasion remains to be demonstrated on a molecular basis, the question of why these components are antigenically related in the endoparasitoid and in the host species can possibly be explained in two ways:

a) Gene transfer between species might be the basis for structural similarities between the respective proteins. In the case of lepidopteran hosts and their endoparasitoids, it is conceivable that an ancestral virus of the caterpillar could have been involved in a transfer of genes between the two species. In this context, the genome of the two main groups of symbiotic viruses in hymenopteran endoparasitoids, the Bracoviridae (Stoltz and Vinson, 1979; Theilmann and Summers, 1987; Fleming, 1992) and the Ichnoviridae (Stoltz and Vinson, 1979; Stoltz *et al.*, 1984; Krell, 1991; Fleming, 1992), have been found to be incorporated into the parasitoids' chromosomes (Stoltz and Xu, 1990). These polydnaviral genes are chromosomally transmitted as an integral part of the wasps' genome (Stoltz, 1990). An ancestral lepidopteran gene, picked up by a recombination event involving virus and lepidopteran DNA, would therefore constitute a wasp gene after virus integration into wasp chromosomes.

b) Structural similarity might be based on evolutionary conserved sequences. If "self" related components exist in insects and are conserved between a parasitoid and its habitual host, such components could be used by an endoparasitoid to protect against immune recognition by the host. In the absence of a clear understanding of "self" and "non-self" recognition in invertebrates, the question is, what constitutes a component that is not recognised as foreign by an insect? It is known that some highly conserved molecules exist on the surface of blood cells and the extracellular matrix of tissues that may not elicit a response if exposed to the recognition system of another insect species. However, other components may also exist at the same locations which are diverse enough to initiate an immune response. The question is whether one insect would be protected inside another species if it were covered with components that are conserved between

parasitoid and host. This is the basis for the concept of pre-adaptation in the development of parasitoid-host interactions.

Given the possible selective advantage of an intact host defence, the *V. canescens-E. kühniella* system may in fact be an evolutionary advanced interaction as compared to the cases utilising PDVs for active immune suppression. However, the question of how the emerging larva is protected remains, and the absence of an active suppression of host defence, implies that the larva has an alternative mechanism of immune protection. The mechanisms utilised by *V. canescens* eggs and larvae are therefore explored in this study under the above assumptions of host immune evasion.

1.6 Mucins

Mucins are serine and threonine-rich proteins that are heavily O-glycosylated and have an extended protein backbone structure (Strous and Dekker, 1992; Khatri *et al.*, 1993). Most mucins have a molecular weight of more than 100 kDa. They are hydrophilic (Hilkens *et al.*, 1992) and form a confluent film on the surface of the intestinal or tracheal tracts of vertebrates, protecting them from bacterial and helminth attack (McNabb and Tomasi, 1981). Most mammalian epithelial surfaces, *eg.* the gastrointestinal, tracheo-bronchial and urogenital tracts, and the oral cavity, have specialised mucosal cells, secreting mucus components which function as lubricants and protect against sudden changes in osmotic pressure and digestion by proteolytic enzymes (Devine and McKenzie, 1992). For example, (Gibbons *et al.*, 1976) demonstrated that salivary glycoproteins inhibit adherence of bacteria, and that gastric mucus reduces the binding of cholera toxin to its cell receptors on gut epithelial cells (Strömbeck and Harrold, 1974). The protective properties of mucus in invertebrates is enhanced by secretion of anti-microbial peptides (Zasloff, 1992).

In addition to the "mucus-mucins", animal cells have been shown to posses "cell membrane" or "cell surface" mucins (Hilkens *et al.*, 1992). The cell surface mucins have elongated protein structures with many proline residues and other helix-breaking amino acids. Such a configuration results in mucins with long, extended protein structures and many ß-turns which are made rigid by the addition of O-linked glycans

(Hilkens *et al.*, 1992; Jentoft, 1990). Their structure, and the fact that they are negatively charged, give mucins the property of shielding the cells. Furthermore, mucins have carbohydrate determinants which are specifically recognised by lectins, thus giving the additional property of adhesion to foreign organisms during an infection (Karlsson *et al.*, 1992; Shimizu and Shaw, 1993). The glycoproteins on tissue surfaces serve the function of blood cell trafficking in the circulatory system (Diamond *et al.*, 1991; Springer, 1994), and in inflammatory responses (Shimizu and Shaw, 1993; Springer, 1994; Lasky, 1995) as well as cell adhesion of germ cells during fertilisation (Gahmberg *et al.*, 1992). However, the molecules which are involved in the initial recognition and induction of immune responses and the regulation of hemocyte adhesion in invertebrates are not known.

In vertebrates, Axelsson *et al.* (1978) described one of the first blood cell mucins. The leucocyte mucin "CD43" (leukosialin, sialoglycoprotein and sialophorin) has many glycoforms which are specifically recognised by the snail *Helix pomatia* lectin (*H. p.* lectin). This mucin was suggested to play an important role in immune responses since patients deficient in CD43, ("Wiskott-Aldrich syndrome"), have an impaired immunity (Parkman *et al.*, 1981; Remold-O'-Donnell *et al.*, 1984).

Since insects lack the immunoglobulin cell surface receptors that characterise the immune-competent cells of vertebrates, it is of interest to investigate how they manifest efficient cell-mediated responses against a diversity of non-self components. Rizki and Rizki (1983) and Nappi and Silvers (1984) demonstrated that *D. melanogaster* hemocytes involved in encapsulation acquired altered surface properties which facilitated their recognition and adhesion responses. Similar results were demonstrated in *A. aegypti* by Nappi and Christensen (1986) using a wheat germ agglutinin. Recently, S. Asgari (unpublished results) demonstrated that polydnavirus-infected hemocytes from *P. rapae* lose their *H. p.* lectin binding property and, possibly in conjunction, their adhesion ability as well as immune recognition capacity. This would indicate that the glycoproteins on insect hemocytes are involved in immune responses.

The evidence of a mucin's possible involvement in immune reactions has been reported by Theopold *et al.* (1996). In brief, a *D. melanogaster* glycoprotein named

'hemomucin" found on hemocytes appears to be involved in the regulation of adhesion. This may be achieved by clustering of mucin-like molecules on the membrane surface and exposing pre-existing cell adhesion molecules. The glycoprotein is also found on the surface of the egg chorion and the peritrophic membrane, where it is apparently involved in the separation (protection) of sclerotised structures from neighbouring cells and membranes. Hemomucin is probably one of a number of immune recognition molecules that are presently being characterised in insects.

From the information on mucin-like glycoproteins from vertebrates and *D. melanogaster*, it appears that a corresponding component in *V. canescens* may play a role in the passive protection of the parasitoid inside the host caterpillar. Given the presence of the mucin-like glycoprotein on the surface of hemocytes and eggs in *D. melanogaster*, the question is whether similar components are present in *V. canescens*.

The experimental demonstration of the presence of mucin-like glycoproteins on the surface of an endoparasitic egg could indicate that these components play a role in the parasitoid-host interaction, constituting an adaptive feature where a glycoprotein of the insect egg surface is used to protect the parasitoid egg against defence reactions of the host. It is possible that the coating of the egg surface developed during oogenesis was an important prerequisite for the evolutionary adaptation to a parasitic lifestyle in these insects. To analyse a possible function of these glycoproteins in an endoparasitoid-host relationship, we must first investigate whether structural similarities exist between components found on the parasitoid surface and within the host. If similar components are found in the host, this analysis should include determination of their normal function and tissue-specific localisation.

To analyse structural similarities between different species, serological approaches as well as DNA and protein sequence analysis are possible. This study utilised some of these methods to investigate the protective components of *V. canescens* in its host, *E. kühniella*.

Anti-VLP antisera has been shown to cross-react with a host-like protein of 42 kDa (Feddersen *et al.*, 1986; Schmidt *et al.*, 1993). To further analyse this relationship, this study used anti-VLP antiserum on Western blots, tissue sections and

hemocyte preparations to identify any cross-reacting components in the host and in the parasitoid.

1.7 Aim and scope of study

V. canescens is a unique endoparasitoid as it protects its eggs and the emerging larva by a passive protection against the defence of its habitual host. In contrast to most other hymenopteran endoparasitoids, *V. canescens* parasitation does not lead to an active immune suppressive activity and the host defence system remains virtually intact during the wasp's embryonic and larval development.

In this study the possibility of passive protection due to structural similarities between host-like components and the endoparasitoid is explored further using molecular approaches. In addition to the previously discovered structural relationship between the two major VLP proteins of 52 and 60 kDa in size, and a host-like protein of 42 kDa in size, other structurally similar components were discovered between the wasp and the lepidopteran host. Using antibodies against a bacterial fusion protein coding for a peroxidase-like domain in the 40 kDa VLP-protein, a similar component was identified in the hemolymph but also in the silk glands of the wasp and the caterpillar. In addition to antigenic similarity to the VLP-proteins, both egg chorion and larval cuticle structures revealed a layer containing a mucin-like glycoprotein, which was identified by specific binding properties of *H. p.* lectin.

From these observations three important questions for V. canescens-E. kühniella endoparasitoid-host system follow and are the focus of this study,

1) What is the nature of the *V. canescens* calyx gland secretion, presently called virus-like particles? Are these particles remnants of previous viruses or nuclear secretions?

2) If these particles protect the egg surface, what is the protective coating on the emerging larval cuticle?

3) If there are structural similarities between egg and larval surface components of the wasp, what are the possible functions of the host-like components that prevent recognition as foreign?

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Insect material

The parasitoid wasp *V. canescens* and *E. kühniella* strains used in this study were obtained from a culture established in 1965 by G. Salt at the University of Cambridge, United Kingdom, and revived in 1982 by I. Feddersen at the University of Freiburg, Germany and at the University of Adelaide in 1992. Host caterpillars were reared on rolled oats. Fourth instar caterpillars were parasitised by exposing them for 24 h to adult wasps reared on 30% honey. The insects were maintained at 25°C and a photo-period of 14 h light: 10 h dark.

2.1.2 Chemicals

Acrylamide	Sigma Chemical Co., St. Louis, Missouri, USA.
Agarose, SeaKem	FMC® BioProducts, Rockland, Maine, USA.
Ammonium persulphate	ICN Biomedicals Inc., Aurora, Ohio, USA.
Bovine serum albumin, Fraction V	Sigma Chemical Co.
5-Bromo-4-chloro-3-indolylphosphate (p-Toluidine salt) (BCIP)	Sigma Chemical Co.
Bromophenol blue	BDH Laboratory Supplies, Poole, Dorset, England.
Chromic potassium sulphate	BDH Laboratory Supplies, Australia
Coomassie Brilliant Blue R250	Sigma Chemical Co.
3, 3'-Diaminobenzidine (DAB)	Sigma Chemical Co.
Dithiothreitol (DTT)	Sigma Chemical Co.
Ethidium bromide	Sigma Chemical Co.
Ethylenediaminetetra-acetic acid (EDTA)	BDH Laboratory Supplies
Formamide	Sigma Chemical Co.
Formaldehyde	Sigma Chemical Co.
Gelatin, Type B	Sigma Chemical Co.
Glycerol	BDH Laboratory Supplies

Glycine (aminoacetic acid)	BDH Laboratory Supplies
Hydrogen peroxide	BDH Laboratory Supplies
ß-Mercapto-ethanol	Riedel-de Haën
NNN'N'-tetramethylethene-diamine (TEMED)	Bio-Rad® Laboratories, Hercules, California, USA.
Needles-injection	Terumo®, Crown Sceintific, Australia
Nitro-blue tetrazolium chloride (NBT)	Sigma Chemical Co.
Nonylphenoxy polyethoxy ethanol (NP-40)	Sigma Chemical Co.
Paraformaldehyde (PFA)	Sigma Chemical Co.
Phenol	BDH Laboratory Supplies
Polyoxyethylenesorbitan monolaurate (Tween-20)	Sigma Chemical Co.
Ponceau S	Sigma Chemical Co.
SDS	BDH Laboratory Supplies
Triethanolamine	ICN Biomedicals Inc.
Trizma® Base [Tris(hydroxymethyl) amino-methane]	Sigma Chemical Co.
Triton X-100	Sigma Chemical Co.

Radiochemicals

[α-^{32}P]dCTP, 3000 Ci/m mole	Amersham, Buckinghamshire, England.

Lectin materials

Lectin from *Helix pomatia*	Sigma Chemical Co.
Lectin from *Helix pomatia*, FITC-conjugated	Sigma Chemical Co.
Lectin from *Helix pomatia*, peroxidase-conjugated	Sigma Chemical Co.
N - Acetyl - D - Galactosamine	Sigma Chemical Co.

2.1.3 Affinity column material

Aprotinin	Sigma Chemical Co
Cyanogen bromide activated Sepharose 4B	Sigma Chemical Co
e-Amino-n-caproic acid	Sigma Chemical Co
Poly-Prep® Chromatography columns	Bio-Rad®

2.1.4 Miscellaneous materials

3MM chromatography paper	Whatman®International Ltd., Maidstone, England.
Cryomold®, disposable vinyl moulds (10 mm x 10 mm x 5 mm)	Tissue-Tek®, Miles Inc., Elkhart, Indiana, USA.
Ectachrome 400X Film	
Kodak Colour Slide Film	Eastman Kodak Co., Rochester, New York, USA.
Polaroid land film 667	Polaroid (UK) Ltd., Hertofdshire, England.
X-ray film (Kodak Diagnostic Film, X-Omat)	Eastman Kodak Co.
Nitrocellulose, 0.2µm pore size	Shleicher and Schuell, Dassel, Germany.
OCT embedding compound 4583	Tissue-Tek®, Miles Inc., Elkhart, Indiana, USA.

2.1.5 Buffers and solutions

Antibody Elution buffer	0.5 M glycine, 1 M NaCl, 100µl Tween 20 and 200µg BSA) at pH 3.5
Antifade solution	9 parts glycerol: 1 part 1M Tris-HCl, pH 7.5: 2% 1,4-diaza-bicyclo-(2,2,2)-octane
Calcium-Tris buffer	75 mM Tris-HCl, pH 7.0, 10 mM $CaCl_2$, 166 mM NaCl
Calcium free buffer	75 mM Tris-HCl, pH 7.0, 166 mM NaCl
Cecropia buffer	100 mM Tricine-HCl, pH 6.8 100 mM NaCl, 40 mM KCl, 15 $MgCl_2$, 4 mM $CaCl_2$

Mucin purification buffers

Lysis buffer	0.1 M Na-phosphate, pH 7.5, 0.5% NP-40, 0.06 trypsin inhibiting units/ml (TIU/ml) aprotinin, 1% ε–amino caproic acid
Washing buffer 1	0.1 M Na-phosphate, pH 7.5, 0.5% NP-40, 1% ε–amino caproic acid
Washing buffer 2	0.1 M $NaHPO_4$ pH 7.5, 0.5% NP-40
Elution buffer	0.1 M $NaHPO_4$ pH 7.5, 0.5% NP-40, 100 mM Gal-Nac, 0.02% BPB (tracing dye)
PBS	138 mM NaCl; 2.7 mM KCl; 1.47 mM KH_2PO_4; 7.3 mM NaH_2PO_4; pH 7.6
20x SSC	3 M NaCl; 0.3 M Na_3 Citrate.$2H_20$, pH 7.0
50x Denhardt's	5 g Ficoll (Type 400); 5 g polyvinylpyrrolidone; 5 g BSA; SNDW to 500 ml
TAE	0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0
TBST	10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.05% Tween 20
Subbing Solution	0.1% (w/v) gelatine SNDW dissolved by boiling 5 min, cool to 65°C, 0.1% (w/v) $CrK(SO_4)2H_2O$. Store solution at 4°C.

Southern blot hybridisation solution (low strigency)

SNDW	9.0 ml
Formamide	25.8 ml
50 x Dehardt's solution	6.0 ml
20 x SSC	15.0 ml
10% SDS	3.0 ml
Herring sperm DNA	1.2 ml
	60.0 ml

30% Acrylamide Stock Solution

Dissolve 29.2 g acrylamide and 0.8 g N'N'-methylenebisacrylamide in SNDW to a final volume of 100ml. Store at 4°C in the dark.

Separating Gel (10%) (volumes for 2x 0.75 mm gels)

SNDW	2.95 ml
30% acrylamide	2.5 ml
1.5 M Tris-HCl, pH 8.8	1.9 ml
10% SDS	750 µl
10% APS	750 µl
TEMED	3 µl

Stacking Gel (5%) (volumes for 2x 0.75 mm gels)

SNDW	1.7 ml
30% acrylamide	415 µl
1.0 M Tris-HCl, pH 6.8	315 µl
10% SDS	25 µl
10% APS	25 µl
TEMED	2.5 µl

SDS reducing buffer

50 mM Tris-HCl. pH 6.8; 5% (v/v) ß-mercaptoethanol; 10% (v/v) glycerol; 2% (w/v) SDS; 0.00125% (w/v) BPB

2.1.6 Molecular weight markers

DNA MW marker VI — Boehringer Mannheim, Mannheim, Germany.

(pBR328-DNA/Bgl I + Hinf I

- 2176, 1766, 1230, 1033, 653,

517, 453, 394, 298, 298, 234,

234, 220, 154, 154 base pairs).

DNA MW marker pGEM

(2645, 1605, 1198, 676,517, 460, 396, 350, 222, 179, 126, 75, 65, 51, 36 base pairs). — Promega Corporation, North America

Protein MW markers:

i) Kaleidoscope pre-stained marker (Myosin (blue), 202; b-galactosidase (magenta), 133; BSA (green), 71; Carbonic acid (violet), 41.8, Soybean trypsin inhibitor (orange), 30.6; Lysozyme (red), 17.8; Aprotinin (blue), 6.9 kDa). — Bio-Rad® Laboratories.

ii) SeeBlue pre-stained standards (Myosin, 250; BSA, 98; Glutamic Dehydrogenase, 64; Alcohol Dehydrogenase, 50; Carbonic Anhydrase, 36; Myoglobin, 30; Lysozyme, 16; Aprotinin, 6; Insulin, B Chain, 4 kDa). — Novex Experimental Technology

iii) SDS-PAGE Broad Range Standards (Myosin, 200; b-galactosidase, 116.25; Phosphorylase B, 97.4; Serum albumin, 66.2; Ovalbumin, 45; Carbonic anhydrase, 31; Trypsin inhibitor, 21.5; Lysozyme, 14.4 Aprotinin, 6.5 kDa). — Bio-Rad® Laboratories.

2.1.7 Antisera adjuvants and enzymes

Anti-rabbit IgG FITC conjugate	Sigma Chemical Co.
Freund's adjuvant (Complete)	Sigma Chemical Co.
Freund's adjuvant (Incomplete)	Sigma Chemical Co.
Proteinase K (IUB 3.4.21.14)	Worthington Biochemical Corporation, Freehold, New Jersey, USA
Ribonuclease A (EC 3.1.27.5)	Sigma Chemical Co.
Taq DNA polymerase	Promega, Madison, Wisconsin, USA.

Antisera against Virus-like particles (VLPs) and PHGPX-like domain were from I. Feddersen, University of Freiburg, Germany and M. Beck, Waite Campus, University of Adelaide, S. Australia.

2.1.8 Polymerase chain reaction (PCR)

PCR conditions

x10 PCR buffer	1.9 µl
25 mM $MgCl_2$	0.8 µl
15 mM dNTP	0.8 µl
2 µM Forward primer	0.5 µl
2 µM Reverse Primer	0.5 µl
SNDW	9.5 µl
DNA	5.0 µl
Tag Polymerase (0.5U μl^{-1})	1.0 µl
	20.0 µl

PCR run, 30 cycles

95°C heating

95°C for 5 min

75°C and add Tag polymerase

95°C for 1 min

53 °C for 1 min

72°C for 2.5 min

72°C for 15 min extension

repeat 29 times

4°C end of run

2.1.9 Equipment

PCR machine

Model, PTC-100, Programmable Thermal Controller, Peltier-Effect Cycling from M. J. Research Inc.

Microtome

Refrigerated microtome model, Cryocut 1800, Reichert-Jung GmbH, Germany.

Microscope

Zeiss, Large Research Microscope, Oberkochen, Germany.

2.2 Methods

2.2.1 Preparation of *E. kühniella* hemolymph antisera

Immobilised caterpillars were bled by cutting two of the prolegs and immersing the wound in an Eppendorf tube containing ice cold calcium-free Tris-buffer (75 mM Tris-HCl, pH 7.0, 166 mM NaCl) saturated with phenylthiourea (PTU). The hemolymph was prepared for antisera production as follows.

Antisera against *E. kühniella* hemolymph components were raised in rabbits by

subcutaneous injection of 500 µl unfractionated hemolymph from 50 caterpillars suspended 1:1 in 500 µl complete Freund adjuvant for the first injection. The rest of the immunisation procedure consisted of two additional injections with the same amounts of unfractionated hemolymph in incomplete Freund adjuvant, repeated twice at two week intervals before taking blood for serum preparation. Adjuvants non-specifically stimulate the immune response in mammals. They are known to:

1. Prevent rapid catabolism of antigen by depositing protective components.
2. Raise the level of many soluble lymphokines.
3. Stimulate the activity of antigen-processing cells directly by causing a local inflammatory reaction at the site of injection (Harlow and Lane, 1988). Freund's adjuvant is an emulsion of water in non-metabolizable oil, containing *Mycobacterium tuberculosis* (Harlow and Lane, 1988).

A 25 G (0.50 x 25.0 mm) needle was used for the immunisation, one booster four weeks after the first injection and subsequent boosters fortnightly. The blood was collected before immunisation as preserum and every two weeks before each boost. The posterior side of the rabbit's ear was shaved and sterilised by an alcohol swab. A table lamp with a 100 W bulb was brought close to the shaved area to warm the ear, dilate the vein and prevent coagulation. An 18 G (1.20 x 38.0 mm) needle was inserted into the vein close to the ear edge with gentle rotation and the blood collected into a sterile beaker. The blood was then allowed to coagulate for 12 h at RT and serum transferred to a 10 ml tube. The plasma was centrifuged at 720 g for 10 min to pellet the remaining blood cells. The clean serum was then mixed 1:1 with glycerol containing 0.02 % sodium azide and stored at -20°C. An Ouchterlony test (Ouchterlony, 1969) and dot blot test (Harlow and Lane, 1988) were performed to determine the strength of the antisera.

2.2.2 Dissection of *V. canescens* eggs and larva

V. canescens eggs were dissected from the oviduct of adult wasps. Adult wasps were kept in fresh containers without host caterpillars and were fed on honey diluted with sterile nanopole distilled water (SNDW) at a ratio of 1:3 for three days. They were then immobilised by cooling for one hour at 4°C. The wasps were then dissected in PBS (see

2.1.5 above) under a dissecting microscope. The ovaries were teased from the body by pulling the ovipositor using watch makers fine forceps No. 4. The ovarioles were severed by micro-scissors and the oviduct placed on cold PBS on ice. The eggs were teased from the oviduct and transferred by pipette to a fresh PBS container on ice. Similarly, parasitised host caterpillars were dissected in cold PBS and the wasp eggs and larvae placed in fresh PBS. The samples were then fixed overnight in paraformaldehyde (PFA) and dried in an ethanol gradient (30%, 50%, 70%, 90%, 100% and 100%) for 5 min in each wash. Final dehydration was carried out in an Emscope CPD 750 thermoelectric control dryer and the specimen scanned by an electron microscopy.

2.2.3 Staining of *V. canescens* eggs and larvae with antibodies

V. canescens eggs and larva dissected as described above, were blocked by a 30 min incubation in 0.5% BSA, washed three times in PBS and incubated at RT for 2 h in anti-VLPs, anti-p40 and anti-hemolymph antisera developed in rabbit. They were then washed three times in PBS, incubated at RT for a further 2 h in anti-rabbit IgG FITC conjugate developed in goat. A similar set of specimens were treated with *H. p.* lectin conjugated with FITC for 30 min. The specimens were then washed three times in PBS and mounted on microscope glass slides in antifade solution (9 parts glycerol: 1 part 1M Tris-HCl, pH 7.5: 2% DBO). The specimens were then photographed under normal and indirect UV-light under a Zeiss compound microscope.

2.2.4 Tissue sections

V. canescens and *E. kühniella* larvae and pupae were collected from the rearing chambers and kept overnight on tissue paper to remove food particles. They were then washed and surface sterilised in water and alcohol, respectively. The ovaries were dissected from *V. canescens* wasps and eggs from parasitised host caterpillars. The frozen specimens were sectioned at 10 µm at -15°C using a Reichert-Jung, cryocut 1800 microtome. Specimen sections were placed on subbed slides (washed with 5% gelatine containing 0.1% $CrK(SO_4)_2$) and allowed to dry. The sections were then fixed in 4% PFA in PBS for 20 min, washed three times in PBS, 5 min each, and dehydrated by incubating

in ethanol gradient as in 2.2.2 (above). 1% BSA in PBS was used as a blocking agent for30 min at RT. The tissue sections were then treated with anti-VLP, anti-p40 (Hellers *et al.*, 1996), anti-hemolymph antisera, preserum and *H. p.* lectin, respectively. They were then examined under a Zeiss compound microscope using UV and normal light.

2.2.5 Staining hemocytes with antibodies

V. canescens larvae and adults and *E. kühniella* caterpillars were each bled in a drop of PTU saturated PBS. The hemocytes were allowed to attach to glass slides for 20 min. Following attachment to the glass slide the buffer was replaced with 1% PFA in PBS for 20 min at RT, washed three times in PBS and blocked in 1% BSA for 10 min. The hemocytes were incubated for 2 h at RT with anti-VLP, anti-p40 (Hellers *et al.*, 1996), anti-hemolymph antisera and preserum, respectively. The hemocytes were also incubated in *H. p.* lectin for 20 min. They were washed three times with PBS, and incubated for a further 1 h in FITC-conjugated secondary antibodies (goat anti-rabbit IgG, Sigma). The slides were washed several times in PBS and mounted in antifade solution and examined under indirect UV-light.

2.3.1 Elution of specific antiserum

Specific antibodies against hemolymph proteins were obtained from antiserum treated Western blots (see below). The corresponding protein bands were cut out from the filter and antibodies were eluted according to the method described by (Smith and Fisher, 1984). In brief, the bands were eluted in 30 s washes in an extraction buffer (0.5 M glycine, 1 M NaCl, 100 µl Tween 20 and 200 µg BSA) at pH 3.5. These eluates were immediately neutralised by addition of Na_2PO_4 to a final concentration of 50 mM. The eluted antiserum was used as required.

2.3.2 In vivo labelling of E. kühniella

E. kühniella caterpillars were injected with about 1 µl of lepidopteran buffer containing diluted serum, preserum (1:50) and alternatively with 1 µl of a 1 mg/ml BSA solution in lepidopteran buffer (see appendix II, A.8) as the control. The caterpillars

were left at RT for 12 h in a petri dish, and then washed and processed for sectioning (see below). The fixed sections were treated with anti-rabbit antibodies FITC conjugate made in goat and analysed under indirect UV-light under a Zeiss compound microscope.

2.3.3 In vitro bacteria recognition assay

Hemolymph proteins that attach to bacterial surface components were isolated as described previously (Sun *et al.*, 1990) with slight modifications. *E. coli* (strain C600) were grown in nutrient broth (Difco) at 37°C for 16 h and heat killed at 120°C for 20 min. The bacteria were washed several times in Calcium-Tris buffer and adjusted to $1.2x10^{10}.ml^{-1}$ in calcium-Tris buffer (see appendix II, A.8). $1.2x10^{9}$ bacteria were carefully mixed with 1 ml of cell-free hemolymph and after incubation pelleted by centrifugation. The pellet was washed twice with 50 µl SNDW and then suspended in 30 µl of 0.5 M ammonium formate solution. After removing the bacteria by centrifugation, the supernatant was mixed with loading buffer and separated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and analysed on Western blots (Maniatis *et al.*, 1982).

2.4.1 SDS-polyacrylamide gel electrophoresis of proteins

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to polypeptide sizes. The proteins were mixed and heated in SDS-reducing buffer (50 mM Tris-HCl, pH 6.8, 5% (v/v) ß-mercaptoethanol, 10% (v/v) glycerol; 2% (w/V) SDS; 0.00125% (w/v) Bromophenol blue (BPB)). The proteins were denatured by SDS, a strong anionic detergent and reduced by ß-mercaptoethanol in conjunction with heat. (BPB) dye was used in the sample loading buffer as a marker for the gel front during the run. The denatured polypeptides bind SDS, masking the surface charge of the native proteins so that they encur a net negative charge which facilitate their migration through the gel matrix due to the electric current passing between the electrodes. A discontinuous polyacrylamide gel system (Laemmli, 1970) was used and consists of an upper stacking gel and a lower separating gel. The stacking gel concentrate the polypeptides to a uniform starting point while the separating gel separate the peptides according to size.

A 30% prepared solution of acrylamide: bis-acrylamide (19.2:0.8) was used to prepare 10% (v/v) horizontal slab gels (Appendix 2-7) to separate proteins by the discontinuous method as above. Protein samples were dissolved in SDS-reducing buffer and heated to 70°C for 10 min prior to loading. A Bio-Rad® Mini Protein II dual Slab Cell was used for protein separation. The gels were run for 45 min at 10 mA and then the current increased to 20 mA until the BPB dye was at the bottom of the gel. Novex SeeBlue™ molecular weight markers (4, 6, 16, 30, 36, 50, 64, 98 and 250 kDa) were used to determine the size of the separated peptides.

2.4.2 Staining of SDS-PAGE gels with coomassie brilliant blue

The gels were removed from the glass slabs and immersed in the staining solution (40% (w/v) Coomassie brilliant blue R250, 40% (v/v) methanol, 10% (v/v) glacial acetic acid) with gentle agitation for 2 h or overnight. The gels were then destained in several changes of a destaining solution (10% (v/v) glacial acetic acid and 30% (v/v) methanol) with gentle agitation. A piece of tissue was placed in each destaining tray to absorb excess Coomassie stain. The gels were then dried at 80°C on a Slab Dryer (Bio-Rad®) onto a Whatman 3MM chromatography paper for 2 h.

2.4.3 Western blot analysis with antibodies

Protein extracts were separated on SDS-polyacrylamide gels (Laemmli, 1970) as described in section 2.4.1 (above) and transferred onto nitrocellulose filter membrane (Schleicher and Schüll) using a transblot apparatus (Bio-Rad®). The filter was washed in PBS for 5 min, incubated at RT in blocking solution 1 (8% w/v non-fat milk powder, 0.02% sodium azide, in PBS), including specific antibodies or a 1:5000 dilution of anti-hemolymph antiserum for 2 h with gentle agitation. The filter was washed three times in PBS and in blocking solution 2 (5% w/v non-fat milk powder, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) for 10 min and then incubated in blocking solution 2 including a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) for 2 h at RT with gentle agitation. The filter was washed four times in buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) for 10 min. The bound antibodies were visualised by incubating

the filter in 10 ml alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5) containing 50 µl Nitroblue Tetrazolium Chloride (NBT) (0.5 g NBT in 10ml 70% dimethyl formamide) and 50 µl 5-Bromo-4-Chloro-3-Indolyl-phosphate (BCIP) (0.5 g BCIP disodium salt in 100% dimethyl formamide). To stop the enzymatic reaction of the phosphatase buffer, the filter was incubated in 200 µl (0.5 M EDTA, 50 ml PBS) for 5 min and air-dried.

2.4.4 Western blot analysis with H. p. lectin

Proteins were extracted from *V. canescens* larvae dissected from parasitised *E. kühniella* host caterpillars, ovaries and calyx glands from adult wasps. The samples were heated in loading buffer (50 mM Tris-HCl. pH 6.8; 5% (v/v) ß-mercaptoethanol; 10% (v/v) glycerol; 2% (w/v) SDS; 0.00125% (w/v) BPB) at 60°C for 10 min. About 5-8 µg of protein from each sample was subjected to SDS-polyacryamide gel electrophoresis and electroblotted as described by Laemmli (1970). The transfer of the separated proteins onto nitrocellulose filter was according to Towbin *et al.* (1979). The filter was then washed twice in TBST (10 mM Tris-HCl, pH 8.0; 150 mM NaCl, 0.05% Tween 20) and incubated overnight in buffer [TBST, 1:20,000 (v/v) dilution of peroxidase-conjugated *H. p.* Lectin (1 mg/ml)] with gentle agitation. The filter was then washed 4 times in TBST, and incubated in freshly prepared colour development solution (0.06% w/v DAB; 50 mM Tris, pH 7.5; 0.03 % H_2O_2) until brown bands were detected. The colour development was stopped by washing in PBS, and the filter was then air dried.

2.5.1 Preparation of Sepharose affinity chromatography column

H. p. lectin was covalently linked to cyanogen bromide (CNBr) activated Sepharose 4B by the basic coupling procedure. For every 1 ml final volume in the column, 0.28 g of CNBr activated Sepharose 4B was allowed to swell in 100 ml, 1 mM HCl for 15 min, then washed over a sintered glass filter funnel with a 100 ml, 1 mM HCl. The mixture was then transferred to a poly-Prep® chromatography column (Bio-Rad®) and Incubated with 5 mg *H. p.* lectin dissolved in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and with gentle end-to-end mixing on an orbital mixer (Extech Equipment PTY, USA) for

2 h. The un-incorparated lectin was removed by filtration and the remaining reactive groups on the gel were blocked by addition of 100 mM Tris-HCl, pH 8.0 with gentle end-to-end mixing on an orbital mixer for 2 h. The column was then used for glycoprotein purification, section 2.5.2 (below).

2.5.2 Purification of mucin-like proteins from V. canescens ovaries

Ovaries were dissected from 300 wasps and homogenised in lysis buffer (0.1 M NaHPO4 pH 7.5, 0.5% NP-40, 0.06 trypsin inhibiting units/ml, (TIU/ml) aprotinin, 1% ε–amino caproic acid) for 30 min with gentle end-to-end mixing on an orbital mixer. The homogenate was centrifuged at 18,849 g for 8 min at 4°C. The supernatant was added to the *H. p.* lectin linked Sepharose column in 2.5.1 (above) and incubated at 4°C for 30 min with gentle end-to-end mixing on an orbital mixer. Unbound solution was collected for comparison. This allowed the ovary proteins to bind to the *H. p.* lectin ligands linked to the column. The column was then washed with 40 ml washing buffer 1 (0.1 M NaHPO4. pH 7.5, 0.5% (v/v) NP-40, 1.0% (w/v) e-amino caproic acid) followed by 200 ml wash with washing buffer 2 (0.1 M $NaHPO_4$, pH 7.5, 0.5% (v/v) NP-40). The bound proteins were eluted in 1 ml elution buffer (0.1 M $NaHPO_4$, pH 7.5, 0.5% (v/v) NP-40, 100mM GalNac + 0.002% (v/v) BPB). The eluant was collected from the column into a 1.5 ml Eppendorf tube as soon as the BPB dye emerged and stored at -20° C until use. The column was then washed twice in 0.1% NaN_3 and stored at 4° C. The eluant was analysed on a 10% SDS-PAGE gel.

2.6.1 Extraction of genomic DNA from V. canescens and D. melanogaster

Three *D. melanogaster* larvae and the abdomen from three *V. canescens* wasps, respectively were homogenised in freshly prepared homogenisation buffer (0.4 mM Tris-HCl, pH 8.0, 0.4 mM EDTA, pH 8.0, 1% SDS), in a Eppendorf tube with a pestle on ice. The homogenate was incubated at 56°C for 1 h, 5 M KOAc was added after cooling to RT, mixed and centrifuged. The supernatant was transferred to a fresh Eppendorf tube and 5 µl 20 µg/ml proteinase K solution added. The tube was then incubated at 40°C overnight,

then 0.8 ml 10 µg/ml RNase A was added and incubated at 37°C for 30 min. RT equilibrated 400 µl phenol was added to each preparation and mixed gently by inversion followed by incubation at RT for 5 min. The tube was then centrifuged for 5 min at 12,063 g. The supernatant was transferred to a fresh Eppendorf tube. 400 µl $CHCl_3$ per tube was added and mixed by inversion, and incubated at RT for 5 min. The tubes were centrifuged for 5 min at 12,063 g, 350 µl supernatant transferred to a fresh tube and 730 µl ethanol, 15 µl 5 M NaCl added, and mixed several times by inversion. The tubes were then cooled on ice for 20 min, centrifuged at 18,849 g for 20 min, the supernatant was discarded and the pelleted DNA was air-dried for 10 min. The pellet was then resuspended in 50 µl 1x TE, depending on the size of the pellet. The DNA concentration and purity were determined (at 260 nm and 280 nm) using a Varian DMS 100/D5 15 spectrophotometer with a 1 cm-path quartz cuvettes. 5 µl of the sample was used for determining the concentration and the rest was stored at 4°C until use.

2.6.2 Double digestion of genomic DNA

G. mellonella and *V. canescens* genomic DNA were prepared as in section 2.6.1 (above). 20 µg from each sample were digested overnight at 37°C with *Apa*1 + *Sac*I, *Bam*HI + *Hin*dIII and *Eco*RI + *Pst*I, respectively in a total volume of 20 µl. 1 U of enzyme per µg DNA was used as recommended by the manufacturers (Promega). 5 µl from each reaction was then run on a 0.8% agarose gel as described in section (2.5.3) below. The gel was stained with 0.5 mg/ml ethidium bromide to allow viewing of the DNA under UV light.

2.6.3 Agarose gel electrophoresis

Electrophoresis of genomic DNA digest and PCR products was carried out in tanks for horizontal submerged gel electrophoresis. Samples were prepared in 1x DNA loading buffer (0.05% BPB; 30% glycerol in SNDW) and electrophoresed in 0.8% and 2% (w/v) agarose gels for genomic DNA and PCR products, respectively. DNA was separated at a constant 100 V for 1 h in TAE buffer (0.04 M Tris-acetate + 0.001 M EDTA, pH 8.0). DNA was visualised by transillumination with U.V. light (354 nm) after brief staining of the gel in a solution of 10 µg/ml ethidium bromide. A positive photograph of

the stained gel was taken using polaroid land film 667. Size of DNA was determined by comparing the relative mobility with those of DNA molecules of known size.

2.6.4 Southern blot analysis

The digested DNA was separated electrophoretically on a 0.8% agarose gel as in 2.6.5 (above). The separated DNA was denatured in the gel, renatured and transferred onto Hybond-N membrane (Amersham) by capillary transfer under alkaline conditions as described by Southern (1975) and updated by Sambrook *et al.* (1989). The membrane was then air dried for 30 min and the DNA cross-linked by UV exposure in a Bio-Rad® (Laboratories, Hercules, California, USA). The filter was wet in 6x SSC and incubated in hybridization solution under low stringent conditions for 6 hours at 42° C. The filter was probed with a radio active conjugated pDMu-105 DNA prepared as in 2.6.6 (below). The probe was added to the pre-hybridisation solution and incubated for a further 30 h. The filter was then washed twice in buffer (2x SSC, 0.1%) for 15 min at RT and twice in (0.1x SSC, 0.1%) for 30 min at 42° C. The filter was then exposed to X-ray film as described in 2.6.6 below.

2.6.5 Preparation of a DNA probe tagged with ^{32}P

The probe used in this experiments was a 1.56 kb (pDMu-105) fragment derived from the 2.35 kb *D. melanogaster* hemomucin DNA sequence and was provided by U. Theopold (see Theopold *et al.*, 1996). Radiolabelled probes were prepared by random primed incorporation of [α-^{32}P]dCTP using a preparative kit, Megaprime DNA labelling system (Amersham) according to the manufacturer's instructions. Briefly, 0.42 µl of the *D. melanogaster* hemomucin DNA fragment pDMu-105 (1.56 kb; 60 ng/µl) (see Figs. 62 and 63) and 44.58 µl SNDW were mixed and denatured in a boiling water bath for 5 min and chilled on ice. To the probe, 10 µl labelling buffer (2 µl DNA polymerase, 1 Klenow fragment and 5 µl [α-^{32}P]dCTP were added and incubated at 37°C for 10 min.

Un-incorparated [α-^{32}P]dCTP were removed by precipitating the labelled DNA by the following procedure. To the hybridization tube, 1 µl (10% SDS) was added and mixed by tapping the tube gently, 1 µl undenatured Herring sperm DNA, 100 µl 5 M ammonium

acetate and 100 µl isopropanol were later added, mixed and the tube allowed to stand at RT for 5 min. The tube was centrifuged for 10 min at 18,849 g and the supernatant transferred to a fresh Eppendorf tube. The pellet was dissolved in 100 µl TE and the amount of the incorporated (a-^{32}P)dCTP determined by comparison to the supernatant in a scintillation counter (Beckman LS 5000 TD). The solution was then used for hybridisation to Southern blots (see 2.6.5 above).

2.6.6 Autoradiography

Autoradiography was performed by exposure of the filter to X-ray film (Kodak Diagnostic Film, X-Omat) between 2 intensifying screens. X-ray cassettes were stored at 80°C overnight or longer where required.

2.7 Designing of oligonucleotide primers

The oligonucleotides used in this study were 20 mer synthesised from the *D. melanogaster* hemomucin protein sequence (Theopold *et al.*, 1996) by the Nucleic Acid and Protein Chemistry Unit, (Waite Campus, The University of Adelaide), using an applied Biosystems DNA/RNA synthesiser. The oligonucleotides designed were as follows:

Forward primers

Vc1 = 5'-ATC ATC GCC GAC GCC TAC TA-3'

Vc2 = 5'-GGC GAC CTC TAC TGG ACC GA-3'

Mu2 = 5'-GCG CCT GTT CCT GGC CCG-3'

Reverse primers

Vc3 = 5'-TTG TAT TTG AAG AGA CGG CC-3'

Vc4 = 5'-GTT TCG GCT ACC ACG ATG AA-3'

Vc5 = 5'-GCT CCC TTC AGG TGA TAC TT-3'

Mu4 = 5'-GAG CCC ACA ATG TTG CCG-3'

Mu7 = 5'-GGG AGC GGG TTT CTC AGA C-3'

The primers were used for the PCR reactions under the conditions described in 2.1.8. The PCR products were then analysed on a 2% agarose gel and transferred onto a membrane as described in sections 2.6.3 and 2.6.4, respectively. The membrane was then probed with ^{32}P tagged pDMu-105 DNA (see section 2.6.5) and autoradiographed as in 2.6.5 above.

Polymerase chain reactions (PCR) were carried out by following the reactions shown in section 2.1.8.

An attempt was made to clone and characterise the PCR product obtained using *V. canescens* cDNA. Unfortunately it turned out to be very similar to the *D. melanogaster* hemomucin which was probably due to a contamination of the genomic with the hemomucin DNA.

Chapter Three

Passive Immune Protection in an Endoparasitoid-Host Systems

3.1 Introduction

Endoparasitic wasps are uniquely adapted to live part of their life within other insects. They have acquired numerous adaptations through co-evolution with the host system (Stoltz and Whitfield, 1992). Hymenopteran wasps deposit their eggs into the host together with ovarian gland secretions (Stoltz and Vinson, 1979; Stoltz, 1986), which contain polydnaviruses (Krell, 1991; Fleming, 1992; Stoltz, 1993) or virus-like particles (Salt, 1965; Rotheram, 1973a; Feddersen *et al.*, 1986). In compatible parasitoid-host systems, the secretions protect the developing wasp against the host's defence reactions by manipulating the host's physiology and immune system. These ovarian secretions alter the physiological conditions inside the host to allow survival of the endoparasitoid inside the hemocoel (Beckage and Thomas, 1986; Vinson, 1990b; Pech *et al.*, 1995).

In contrast to most other hymenopteran systems analysed so far, parasitation by the ichneumonid wasp, *V. canescens* appears to exert no discernible physiological changes on its habitual host *E. kühniella* (Salt, 1970). This may be due to the fact that virus-like particles (VLPs), as opposed to viruses, are the agents of immune protection in this system. Several findings support the notion that *V. canescens* eggs are passively protected against the host defence reaction by a coat of glycoproteins and VLPs (Bedwin, 1979a; Rotheram, 1973a; Feddersen *et al.*, 1986), leaving the host defence system intact during most of the larval development of the wasp (Schmidt and Feddersen, 1989). Previous work by Feddersen (1986), demonstrated that masking of the VLPs with antibodies depleted the eggs of the protective layer. Similarly, eggs denuded of VLPs by washing with a detergent are encapsulated. When eggs devoid of VLPs were incubated with purified VLPs they were, to a large extent protected, whereas encapsulation is more frequent if the VLPs are injected separately. This suggests that the VLPs provide passive protection against the host's immune recognition but does not impair the host's immune system.

The above findings prompted several questions regarding this system: How is the cuticle of the emerging wasp larva protected against a functional host immune system? Moreover, structural features of the larval gut suggest that this endoparasitic wasp feeds

on host hemolymph throughout larval development. In this context, the question is whether the gut lining of the endoparasitoid is protected in a similar fashion? Analysis of the surface properties of host and parasitoid epidermis and gut during development of the parasitoid were carried out. Molecular surface markers specific for the epidermis and for the lining of the open circulatory system were used.

The presence of a passive protective layer on the parasitoid *V. canescens* that is effective against the immune recognition of the host *E. kühniella* is a possible explanation for a number of observations made in this parasitoid-host system. Using antisera and a specific lectin as a probe, several components were found to be involved in providing a layer of protection on the egg and larval surface of the endoparasitoid.

Two of the VLP major proteins (52 kDa and 60 kDa) were immunologically characterised using specific eluted antibodies and a related component in the host was identified by cross-reaction of the antisera. The 42 kDa protein is localised in both insect species on the surface of hemocytes and at the lining of tissues facing the hemocoel. Using antiserum against a bacterial fusion protein encoded by the PHGPX-like domain of the p40 gene (Hellers *et al.*, 1996), an additional protein was identified in both insects in the hemolymph and in the silk glands.

A third component was identified using *H. p.* lectin which binds specifically to a mucin-like glycoprotein (Theopold *et al.*, 1996) found in *D. melanogaster* at the surface of granulocytes, where it has immune functions. It is also found on the surface of sclerotised cuticle and chorion structures, where it may serve as a lubricant (Theopold *et al.*, 1996).

3.2 Results and discussion

3.2.1 VLPs on the surface of V. canescens eggs

In order to analyse the components involved in immune protection, *V. canescens* eggs were dissected from the host and analysed by scanning electron microscopy to determine the presence of VLPs on their surfaces.

Figure 2 A, shows a scanning electron micrograph of a *V. canescens* egg dissected

from the wasp's oviduct. At this magnification VLPs are not visible but at the polar ends of the egg, the chorion surface is rough due to folding and densely patched chorion outgrowth.

At a higher magnification, the egg surface shows a moderately dense layer of particles (Fig. 2 B). The density of the particles varied from egg to egg suggesting that particles are not specifically attached to the egg surface and may detach during preparation for microscopy. At this magnification, a substructure of smaller units could be detected within the particles. Similar substructures were reported after mild sodium deoxycholate treatment of purified VLPs (Bedwin, 1979a).

Eggs from the oviduct and those dissected from parasitised host caterpillars show uniform staining with anti-VLP antiserum (Fig. 3) on the chorion surface. A similar staining was observed with antiserum against the bacterial fusion protein (Hellers *et al.*, 1996), encoded by the PHGPX-like domain of the p40 gene (Fig. 4). There is no evidence for a soluble 40 kDa protein in particle-free calyx fluid. This would imply that the PHGPX-like domain of the 40 kDa protein is exposed at the surface of intact VLPs. Hellers *et al.*, (1996) stipulated that a lipophilic domain at the amino-terminus of this protein is probably used as a membrane anchor.

3.2.2 VLP protein synthesis in the wasp ovary

To localise the particle protein synthesis within the wasp ovarian tissues, sections of dissected ovaries were treated with antibodies against purified VLPs (Feddersen *et al.*, 1986) and against a bacterial fusion protein encoded by the PHGPX-like domain of p40 (Hellers *et al.*, 1996). Figure 5 shows a scanning electron micrograph of a dissected *V. canescens* calyx gland. Similar organs were sectioned and incubated with various antisera. In these sections, the calyx gland tissue can be subdivided into two parts, an anterior part adjacent to the ovarioles, which contains melanised cells (Figs. 6 and 7), and a posterior part which is connected to the oviduct. The malanization of calyx tissue is an unexpected observation, but could be related to necrosis of secretory cells after releasing most of the cellular content into the calyx lumen. Calyx glands differ in the relative proportion of melanised tissues, which may be

correlated with the age of the female. Mature oocytes, which are conventionally called eggs after having left the ovariole, enter the calyx gland passing through the calyx lumen. Frequently several eggs are seen inside the calyx (Figs. 5 and 9).

Antibodies against VLPs and the PHGPX-like domain bind predominantly to the posterior part of the calyx gland (Figs. 6, 7, 8 and 9). Overall the two antisera displayed a similar staining pattern in ovarian tissues, consisting of the posterior calyx gland and the oviduct content. However, the VLP antiserum also showed a less intensive staining of additional ovarian tissues and egg shells at a late stage in oogenesis (Fig. 9). In addition, a faint but significant staining is detectable on basement membranes surrounding the ovarioles. Some of the additional staining pattern is reminiscent to the specific lectin staining (see Chapter 5), which could suggest that the anti-VLP antiserum probably recognises an additional component comprising a mucin-like glycoprotein. This is supported by Western blots where a 100 kDa component is frequently stained by the anti-VLP antiserum (not shown). The presence of a mucin-like antigen recognised by the anti-VLP antiserum could be the result of an artefact or a co-purification of the mucin-like component during particle purification, since VLPs were obtained from ovarian tissues by CsCl gradient centrifugation (Feddersen, 1986). Alternatively, the mucin-like glycoprotein may be attached to the VLP surface at the time of assembly inside the nucleus of calyx cells or during secretion. The *H. p.* lectin staining of follicle cells and calyx gland cells is of similar intensity, suggestive that the glycoprotein which is produced in follicle cells (Theopold *et al.*, 1996) may be co-synthesised with the particles in the calyx gland and be part of the calyx fluid (see Chapter 5).

When eggs pass through the posterior calyx tissue, the secreted particles are attached to the egg surface. This can be seen in one of the calyx sections where the egg shell is located at the transition between the anterior and posterior part (Fig. 9). The egg shell is covered intensely with labelled calyx fluid on the chorion which is inside the posterior part of the calyx gland (Fig. 9), whereas the anterior chorion resembles the oocyte surface inside the ovarioles.

The ovariole tissues are not stained with anti-PHGPX-like protein antiserum. No staining is visible on the chorion of oocytes in post-vitellogenic ovarioles (Figs. 6 and

7). This pattern of ovary staining is different from *H. p.* lectin (see Chapter 5, Figs. 39 and 40), where all of the calyx gland cells and the egg chorion of late oocytes are stained (Fig. 40).

In addition to the posterior part of the calyx gland tissue, the egg surface and the surrounding calyx fluid in the oviduct are strongly labelled with antibodies against PHGPX-like domain of p40 (Fig. 10). The opaque calyx fluid between eggs inside the oviduct, seen in phase contrast as a slightly shaded region (arrow) is clearly stained, whereas oocytes in ovarioles that are located in this section next to the oviduct eggs (arrowhead) are not stained. The colour of oocyte chorions inside ovarioles (arrowhead) is due to auto-fluorescence seen under the indirect UV-light (Fig. 10). Auto-fluorescence of egg shell structures is also visible in untreated sections. Figure 11 show no staining on an ovary section treated with preserum.

This suggests that VLPs are produced in a subsection of the calyx gland and secreted into the gland lumen and transferred into the oviduct together with the eggs. In the oviduct the calyx fluid surrounds the eggs, where the VLPs appear to be more or less loosely attached to the egg surface.

3.2.3 E. kühniella defence system remains active after parasitation

Wounded *V. canescens* larva inside a super-parasitised caterpillar are encapsulated (Figs. 12, 20 and 21). This is an indication that the defence system of the parasitised host, the *E. kühniella* caterpillar, is obviously not impaired after parasitation. The initial encapsulation reaction of the wasp larva begins at the site of wounding as indicated by layers of hemocytes (h) (Fig. 12), beginning to attach locally and eventually encapsulating the whole wasp (Fig. 13). In these sections of large capsules, several layers of differential labelling can be observed (Figs. 12 and 13). This could correlate with a distinct succession of different hemocyte types being added to the object, starting with granulocytes, followed by plasmatocytes and finishing with another set of granulocytes. Sections in figures 12 and 13 were treated with FITC conjugated *H. p.* lectin and show labelled hemocytes. Figure 14 shows a section of an intact larva uniformly labelled by *H. p.* lectin. This indicates the presence of mucin-like proteins on

both the hemocytes and larval surfaces.

Other experimental evidence suggesting an intact host immune defence, consists of injection of Sephadex beads into parasitised *E. kühniella* caterpillars. These beads were as readily encapsulated as in non-parasitised caterpillars (not shown). Similarly, coagulation of hemolymph (Bohn, 1986) and spreading of hemocytes on a glass surface (Davies *et al.*, 1987) is normally regarded as an indication of an intact immune system. Both appeared to be similar in hemolymph of parasitised (Fig. 15) or non-parasitised (Fig. 44)*E. kühniella* caterpillars, and attachment to the glass surface and aggregation among granulocytes did not differ (see also Figs. 44 and 46).

3.2.4 How is the parasitoid larva protected?

The fact that the host defence system remains intact during early larval development of the parasitoid raises the question of how the cuticle of the emerging wasp larva is protected against the immune recognition by the host caterpillar. The larval cuticle is a secretion of the embryonic epidermis involving sclerotisation of proteins which are probably comparable to protein deposition of the egg shell and therefore likely to be recognised as foreign by the caterpillar.

The question is whether a passive protection mechanism of the larval cuticle exists similar to that observed on the egg chorion. If passive protection is the mode of immune protection for both the egg and the larva, where are these protective components synthesised? Are the larval protective components maternal products, as is the case on the egg, or from the embryo as cuticular secretions? Or could such molecules originate from the host caterpillar hemolymph which in *V. canescens* is continuously taken up during embryogenesis through the micropyle?

Scanning electron micrographs (SEM) of a *V. canescens* larva (Fig. 16 A) dissected from the host hemocoel, are mostly free of any VLPs but occasionally particles can be seen in cuticle folds (Fig. 16 B). This could suggest that these VLPs may have attached while the larva was wriggling out of the chorion in the process of hatching. The observed number of VLPs could not adequately explain the uniform anti-VLP antiserum staining on the larva. To ascertain whether the staining on the newly hatched larva was

present on the epidermis during embryogenesis or was acquired during cuticle formation in late embryogenesis, staining experiments were performed at different times during development. In these experiments, the layer was shown to be acquired between 42 to 48 h after oviposition. Figure 17 (a) shows a *V. canescens* egg during early embryogenesis enclosed in the egg chorion with anti-VLP staining visible on the chorion surface (see also Fig. 3). The same embryo was then teased out of the chorion and incubated with the same antiserum. The embryo did not show any labelling (Fig. 17 (c)). Figure 17 (e) shows an embryo before 48 h with the remnants of the egg shell still attached and stained with the anti-VLP antiserum, where only the chorion was labelled. However, after 48 h post-oviposition, the embryo that was separated from the egg shell was stained strongly with the anti-VLP antiserum (Fig. 18 a). Similar results were obtained using antiserum against the bacterial fusion protein encoded by the PHGPX-like domain of p40 (Fig. 19 A).

At later stages when the larva has hatched and grown considerably, the FITC-labelling on the cuticle is still visible (Fig. 20) and has probably intensified. The staining in partially encapsulated larvae is the same as in healthy larvae. The yellow auto-fluorescence can be distinguished under the microscope from the bright green staining of the antibodies. The green colour is absent in preserum controls (Fig. 21).

Since the anti-VLP and anti-PHGPX-like protein antiserum reacted to larval surface components that are probably not particles, the question is whether cross-reacting antigens exist in the wasp other than in the wasp ovary or in VLPs.

Sections of wasp larvae were treated with both antisera and the location of cross-reacting components in the hemocoel were analysed. Using the anti-PHGPX-like protein antiserum the location of the cross-reactive antigens was mainly in the silk gland (Fig. 22 A). The cross-reactive components of the anti-VLP antiserum in figure (23 A) included components on the surface of the muscle (m), fat body (f), and epidermal basement membranes facing the hemolymph. Similar results were obtained in the host caterpillar (see Chapter 4). These results suggest that hemolymph components of the wasp and caterpillar cross-react with antiserum against VLP-proteins. These cross-reacting antigens are found on the surface of hemocytes and on basement membranes at the

lining of hemolymph in the open circulatory system. The antiserum against PHGPX-like protein cross-reacted strongly with the silk gland cells and components found in the gland lumen (Fig. 22). This is reminiscent to the previous observation that anti-VLP antibodies cross-react with components of the silk gland (data not shown). In this case the cross-reacting component was the 42 kDa protein (U. Theopold, Personal communication). To test whether proteins from the hemolymph are found in the silk gland, tissue sections containing silk glands were treated with anti-hemolymph antibodies (Fig. 24 A). Under these conditions the silk gland lining was heavily labelled (arrow).

In contrast to the lepidopteran caterpillar, where only the epidermal basement membrane facing the hemolymph was labelled (see Chapter 4), the cuticle of the wasp larva is clearly labelled on the outside (Fig. 25). The outer surface of the wasp larval cuticle is covered with an amorphous layer of a conspicuous deposition which is labelled on the surface by anti-VLP antiserum.

The origin of this deposition could be from the wasp or from the hemolymph of the host caterpillar. Although the outer cuticle of the early wasp larvae contained occasional VLPs (Fig. 16 B) and cross-react with anti-VLP antibodies, it is unlikely that the antigens in later larval stages are VLPs. As the hemolymph of the host caterpillar contains components that cross-react with anti-VLP antibodies (see Chapter 4), it is more likely that the outer deposition of the wasp cuticle originated from the host hemolymph. To further explore this possibility, eggs dissected from parasitised caterpillars were labelled with antiserum against *E. kühniella* hemolymph. In eggs at a late stage of embryogenesis, both the chorion surface (Fig. 25 A, arrowhead) and the surface of the larval cuticle (Fig. 25 A, arrow) were labelled. This suggests that the antiserum is able to penetrate the egg shell. This is further experimental evidence that host hemolymph components enter the *V. canescens* egg shell, probably through the micropyle, and that the embryo feeds on the host hemolymph.

These results confirm the observations described earlier in this chapter, where components identified by anti-VLP antiserum were shown to be present on the wasp larval cuticle, while the larva is still inside the egg shell. However, it does not distinguish between wasp and host origin for these components. At later larval instars,

the cuticular surface is still labelled by the anti-VLP antiserum, although there are clearly no VLPs present any more. In figure 26, the layer (arrow) that carries components which cross-react with the anti-VLP antiserum begins to peel off and is not visible in larvae that emerge from the dying caterpillar (data not shown). This layer is obviously not required once the endoparasitoid leaves the host. The possible identity of the antigens on the larval cuticle is not clear. It could be *E. kühniella* hemolymph components (Fig. 29) or *V. canescens* larval secretions that cross-react with anti-hemolymph antibodies (Figs. 30). This anti-VLP cross-reacting components on wasp larval surface is also found on the egg and the basal membrane of host caterpillar (Figs. 27 and 28) exposed to the hemolymph.

These observations suggest that the early larva acquires a surface coating inside the egg shell consisting of components that are similar to VLPs but are probably not particles. These experiments do not distinguish between the possible origins for larval epidermis secretion or by attachment from host hemolymph that enters the egg during embryogenesis. However, at instars when the larva has grown, a proteaceous coating of the cuticle still cross-reacts with anti-VLP antibodies in the absence of VLPs, suggesting that the layer of cross-reactive components is continuously replenished on the cuticle surface.

Chapter Four

The Basement Membranes at the Hemolymph Linings

4.1 Introduction

The mechanism of immune protection is an essential requirement for animals and its inception probably precedes multicellular organisation. Even if there is a common origin, it has developed into diverse and highly specific adaptations. The most elaborate immune system exists in the vertebrates where the recognition process involves the production of numerous and diverse antibodies by blood cells (Roitt *et al.*, 1985; Male *et al.*, 1987; Roitt, 1991). Antibodies are produced in specific blood cells, the B-lymphocytes which are clonally selected to recognise structural features of pathogens and parasites, thus enabling the organism to memorise the parasite's antigens for future encounters. Invertebrates on the-other-hand, have less complex mechanisms of immune recognition that are nevertheless effective and meet the needs of these creatures. Arthropods in general, and insects in particular have a small number of hemocytes precluding a clonally selected recognition system (Klein, 1989). However, insects, like other arthropods, are capable of recognising foreign objects and micro-organisms that invade their open circulatory system (Ratcliffe, 1989). They have evolved a varied array of defence strategies that can overcome most of the potential pathogens enabling them to exploit every niche in the ecosystem but the ocean (Williams, 1960; Wigglesworth, 1964).

They utilise defence mechanisms such as phagocytosis, nodule formation and secretion of molecules like cecropin, attacins, hemolin, lectins and the phenoloxidase activating system (Dunn, 1986; Gupta, 1991a; Beckage *et al.*, 1993). Recognition molecules interact with surface determinants initiating hemolymph reaction cascades and hemocyte alterations to coagulate and aggregate around surface structures, encapsulating large objects and parasitoids, and phagocytosing small objects, like bacteria.

Lectins are a class of potential recognition molecules that have specific affinity for certain carbohydrates, thus providing a potential basis for distinction of "self" from "non-self", if a given organism produces lectins that bind to sugar components not found in its own tissues. In addition, insects may utilise adhesion-type recognition molecules with multiple or broad ligand-binding capacity, that identify a pattern of surface properties (Janeway, 1989). Given a limited number of these recognition molecules in

insects, a relatively broad pattern of recognisable structures may interact with these molecules, raising the question of specificity.

In the previous, Chapter it was demonstrated that the immune defence system of *E. kühniella* remains intact after parasitation by *V. canescens*. The wasp eggs appear to be passively protected from the defence reaction by the host caterpillar. This is in contrast to a closely related species, *Campoletis sonorensis*, where the wasp egg is protected by suppression of the host defence system (Edson *et al.*, 1981; Theilmann and Summers, 1986). The question in this context is, how *V. canescens*' progeny evade defence reaction of its host, *E. kühniella*?

If *V. canescens* protects its eggs by passive evasion, two basic processes can be postulated (Salt, 1955; Salt, 1964).

1) The host is not able to recognise the egg surface as foreign.

2) The egg surface is recognised as foreign, but the host defence system is prevented from reacting towards it.

Although nothing is known about the molecular components that are involved in the immune recognition of insects, it is possible to make certain predictions about the two alternative mechanisms. One can assume that the most likely mechanism of a passive protection inside the host, is an egg surface layer consisting of wasp components that have structural similarity to host components. The process of *V. canescens* egg protection inside the host caterpillar would thus be based on molecular mimicry.

To test this assumption, serological methods were used as a first approach. The following reasoning was applied: Antibodies, that were made against the components on the wasp egg surface as well as against the host hemolymph, were tested in reciprocal fashion on egg surface components and on host cells and tissues. If they recognise similar components, it can be concluded that the egg surface shares structural similarities with host components. This approach required antibodies against a broad spectrum of host components. It is plausible that the structural similarities related to molecular mimicry are primarily restricted to components found in the open circulatory system of the host insect. Therefore, antisera were prepared against the hemolymph of the host, *E. kühniella* caterpillar.

Antisera against purified VLPs from *V. canescens* were used as a diagnostic tool for egg surface components. In addition, antiserum against the bacterial fusion protein encoded by the PHGPX-like domain of the p40 gene (Hellers *et al.*, 1996) was used to identify potential cross-reacting proteins in the host. To address the question of specific recognition in insects, hemolymph proteins were searched for that are capable of binding foreign surface structures. Location of these hemolymph proteins on tissues within the open circulatory system were also determined.

4.2 Results and Discussion

4.2.1 Structural similarities between host and wasp

To test whether structural similarities exist between VLP proteins from the wasp and host components, tissue sections from both organisms and protein extracts on Western blots were probed with anti-VLP (Feddersen *et al.*, 1986) and anti-PHGPX-like protein (Hellers *et al.*, 1996) antibodies. Protein extracts from the fat body were electrophoretically separated and the corresponding Western blots probed with the anti-hemolymph antibodies. As shown previously by Feddersen *et al.* (1986), a 42 kDa component from *E. kühniella* caterpillar was labelled with anti-VLP antisera, similarly, anti-hemolymph antisera recognised a 42 kDa protein in a bacterial assay (see section 4.2.5), (Fig 30).

Interestingly, the anti-PHGPX-like protein antibodies also cross-reacted with a caterpillar component in the silk gland on Western blots. The size of the cross-reacting component is 49 kDa (M. Beck, personal communication).

4.2.2 Cell and tissue localisation of host-like antigens

To identify the cross-reacting components on *E. kühniella* hemocytes, cells were allowed to attach to a glass surface and incubated with specific antibodies and antiserum. A sub-population of granulocytes that might correspond to type 2 granulocytes (Ratcliffe, 1993) was labelled with the anti-VLP antibodies, whereas less label was detected on plasmatocytes and other blood cell types (Fig. 32). In comparison, no such labelling was

detected with anti PGHPX-like antisera on hemocytes (Fig. 31).

To establish the precise location of the 42 kDa protein in the open circulatory system, tissue sections of fourth instar caterpillars were incubated with anti-VLP antiserum and specific antibodies against the 42 kDa protein. The antigen was detected on basement membranes of virtually all internal tissues (Fig. 27). Closer inspection revealed, that the antigen is localised exclusively on basement membranes that are exposed to the hemolymph at the lining, but is virtually absent in connective basement membranes between tissues like muscle and epidermis (Fig. 33 arrowhead), or at basement membranes that are not exposed to hemolymph, such as inside the lumen of the gut (Fig. 34). The localisation of the antigen at the lining suggests that the 42 kDa protein or a related protein is added onto the basement membrane from the hemolymph and is probably part of the immunologically inert surface structure within the open circulatory system. Tissue sections treated with anti-hemolymph antiserum show similar patterns of labelling (Fig. 28). This is circumstantial evidence that the cross-reacting components in the open circulatory system are probably derived from the hemolymph.

4.2.3 In vivo labelling of host-like proteins

To investigate whether the anti-VLP antibodies are bound to the antigen at the hemolymph lining under *in vivo* conditions, antiserum, pre-serum, and BSA solutions, respectively were injected into caterpillars. After a short period of time, the caterpillars were frozen in liquid nitrogen and tissue sections prepared. These sections were treated with FITC-conjugated anti-rabbit antibodies made in goat, only. Under these conditions the lining of tissues in the hemocoel was labelled only in antiserum-injected caterpillar sections (Fig. 35 B), whereas pre-serum (Fig. 35 C) and BSA injections did not reveal any labelling. From this observation we conclude that the localisation of the cross-reacting components, including the 42 kDa protein at the lining of the hemocoel was a native feature and that anti-VLP antibodies bind to the protein *in vivo*.

In antiserum-injected caterpillars, the coagulation of hemolymph within cavities of the hemocoel and hemocytes attachment to the lining of tissues (Fig. 35A and 35B) was

frequently observed. This was not detected in pre-serum, BSA-injected, or in any parasitised caterpillar (data no shown). This could indicate that interaction of antibodies with hemocytes and components at the lining of the hemocoel alter hemocyte and hemolymph properties in the open circulatory system, possibly leading to "auto-immune" reactions.

4.2.4 Specific antibodies against a VLP protein

Since caterpillar proteins are structurally related to VLP-proteins, the question is whether anti-VLP antibodies and anti-hemolymph antibodies show similar tissue specific binding in the open circulatory system of the caterpillar. To test this possibility, tissue sections and hemocyte preparations were incubated with the anti-VLP antibodies. Both antiserum and specific antibodies against the 52 and 60 kDa VLP proteins (Feddersen *et al.*, 1986) produced the same staining pattern in hemocytes (Fig. 32) and on tissue sections (Fig. 33) as specific antibodies against the 42 kDa protein.

When anti-PHGPX-like protein antiserum was applied to caterpillar tissue sections, a somewhat different staining pattern was detected. Strong staining of the silk duct and its content (Fig. 22 A (a)) was observed in addition to basement membranes of epidermal tissues exposed to the hemolymph in the open circulatory system.

This suggests that several host hemolymph components that cross-react with antibodies against VLP components co-localise in the open circulatory system. The host proteins and the corresponding VLP-proteins share extensive structural similarities.

4.2.5 Bacterial recognition assay

Preliminary experiments suggest that the 42 kDa protein may be part of a soluble complex in the hemolymph and is involved in the attachment of foreign objects. To identify any hemolymph proteins that are able to attach to foreign objects, hemocyte-free hemolymph was mixed with a bacterial suspension and the bacteria recovered by centrifugation. After repeated water washes, the attached proteins were removed by salt extraction and analysed on SDS-PAGE (Sun *et al.*, 1990). Under these conditions a hemolymph protein of 42 kDa were recovered from the bacterial surface. In addition,

several high molecular weight proteins were recovered as minor bands (data not shown). Since these proteins were not visible in corresponding hemolymph aliquot, it is thought that they are either hemolymph components that are highly enriched on the bacterial surface or represent new protein complexes formed on the bacterial surface.

To confirm that the 42 kDa protein is a hemolymph protein, antibodies were raised against unfractionated hemolymph from *E. kühniella*. Total hemolymph was used for immunisation to avoid possible loss of immune reactive components in the course of a fractionation process. On Western blots most of the major hemolymph proteins are recognised by the antiserum (Fig. 29 lane c). In a similar experiment, proteins extracted from bacteria which had been incubated in *E. kühniella* cell-free hemolymph were tested on Western blots using anti-hemolymph antiserum. Among the hemolymph proteins that are visible in Coomassie blue-stained gels (Fig. 30 lane c), the 42 kDa protein cross-reacted with anti-hemolymph antisera (Fig. 30 lane a). In addition some minor protein bands are labelled that are not visible in Coomassie blue-stained gels of cell-free hemolymph (Fig. 29 lane c). Using specific antibodies eluted from Western blots from figure 29 lane (c), the major protein that bound to the bacterial surface was identified according to size as the 42 kDa protein (Fig.30 lane b).

4.2.6 The 42 kDa host protein

Previously, a 42 kDa *E. kühniella* protein was described to cross-react with antibodies against virus-like particle-proteins from the endoparasitic wasp *V. canescens* (Feddersen *et al.*, 1986). To test whether anti-VLP antibodies recognise the 42 kDa protein recovered from bacteria, aliquot of the extracts were tested on a Western blot with anti-VLP-antiserum. In this experiment, only the 42 kDa protein was labelled (Fig. 30, lane b). From these experiments, it is concluded that the 42 kDa protein binds to bacteria, either directly to a bacterial component or indirectly as part of a hemolymph protein complex. In addition, the 42 kDa protein is recognised by anti-hemolymph (Fig. 30, lane a), indicating similarities between the host hemolymph protein and wasp VLP proteins as suggested earlier (Feddersen *et al.*, 1986).

4.2.7 Conclusion

These results suggest that the VLP proteins on the egg surface share structural similarities to host components at the hemolymph lining and on the granulocyte surface. Figure 36 is a schematic representation of possible localisation of hemolymph components. The egg surface of parasitoids may not be recognised as foreign in habitual host because the egg surface is similar to components in the host's own body structures. Alternatively, with a limited number of recognition molecules at its disposal, the host insect might not have at its disposition the specific recognition molecules against the surface structures that cover the wasp egg. Since host-parasitoid interactions developed over a long period of time, it is assumed that genetic adaptations allowed the host-parasitoid interactions to evolve host recognition molecules that identify the endoparasitoid. The question can be asked then, which of the two assumptions is more stable in evolutionary terms? Since it is quite conceivable that a host could develop specific recognition molecules against a parasitoid, the lack of specific recognition molecules as a reason for the absence of a host reaction is probably not a likely explanation. Alternatively, if the host and the endoparasitoid share essential surface structures the host is probably prevented from developing a recognition capacity against egg surface components that are similar to its own body structures, since it would probably endanger its own body integrity. Thus adaptation processes by the host to distinguish between subtle differences of host and egg surface structures are probably precluded, if essential and highly conserved host-like structures are involved. Even if the host tries to develop specific criteria for the distinction of foreignness between egg and its own structures, the wasp can possibly adapt its egg surface to become even more similar to the host.

Chapter Five

The Mucin-like Glycoprotein

5.1. Introduction

Endoparasitic insect species that deposit their eggs into the hemocoel of another insect are faced with the host's immune response towards foreign objects. The egg chorion surface, like the cuticle, is an amorphous layer of cross-linked proteins that are probably recognised immediately as foreign by the host defence system and encapsulated, as discussed in chapter 1. Endoparasitic Hymenoptera have developed two ways of overcoming the defence barrier of the habitual host insect: a) an active suppression of the host defence (Stoltz and Vinson, 1979), and b) a passive evasion of immune surveillance by the host.

In the passive protection scenario, the egg surface is masked by components from the wasp that are not recognised as foreign by the host (Salt, 1955; Rotheram, 1967). Since the egg shell is initially designed to protect the embryo against physical stress in an abiotic environment, it is difficult to envision how an egg layer can possibly protect against immune recognition by another insect. However, recent findings on a mucopolysaccharide, called hemomucin, coating a non-parasitic *D. melanogaster* egg (Theopold *et al.*, 1996), suggest that this surface layer is required for the physical separation of the egg chorion from follicle cells during oogenesis and egg laying. Since *D. melanogaster* hemomucin is also found on the hemocyte surface where it is involved in immune reactions, the question is whether the same glycoprotein on the egg surface might contribute towards a protective function in endoparasitic insects. Since this assumption can be examined experimentally, it was decided to search for evidence of a glycosylated protein layer on *V. canescens* egg and larval surfaces.

The *D. melanogaster* hemomucin is a glycoprotein found in intracellular vesicles, and on the surface of hemocytes (Theopold *et al.*, 1996). In addition, the glycoprotein is found on the surface of sclerotised structures, like peritrophic membranes, vitello-membranes and egg shells. How common or different the functional features of mucin-like glycoproteins in invertebrates and vertebrates is not clear at this stage, but some indications suggest that they are involved in the modulation of hemocyte surface properties and specific recognition processes (Shimizu and Shaw, 1993). Three examples, from mammalian and *D. melanogaster* mucin-like glycoproteins might support

this notion:

1. A glycocalyx around lymphocytes created by CD43 glycoproteins prevent specific cell-adhesion with other cells and tissues (Manjunath *et al.*, 1995), thereby avoiding cell aggregation and allowing the cell to be mobile within tissues.

2. In *G. mellonella* the hemomucin is found on granulocytes and spherulocytes which are the first blood cells to encounter foreign objects (U. Theopold, personal communication). It is possible to speculate that these cells contain adhesive surface components which are covered by a glycoprotein umbrella and activated in case of an encounter with a foreign object.

3. Hemomucin in *D. melanogaster* is involved in activation of the immune defence reactions causing the production of defence-related proteins after exposure to micro-organisms and LPS. Hemomucin is clustered on the hemocyte surface after stimulation creating a 'speckled' phenotype (U. Theopold, personal communication).

In *D. melanogaster*, the location of mucin-like molecules on the surface of eggs and cuticular membranes, implies a functional role in separating and lubricating cells and tissues from polymerised non-cellular structures (Theopold *et al.* 1996). This is particularly suggestive in "cardia cells" which are involved in producing the peritrophic membranes in the gut. This membrane is secreted as a polymerised, cuticle-like, thin layer at or towards the inner lining of the gut lumen. Both the gut cells exposed to the inner lining and the peritrophic membrane are covered by the mucin. The peritrophic membrane is steadily moves from the site of synthesis along the gut epithelial and eventually separates from the gut cells.

D. melanogaster hemomucin is also detected on the surface of vitello-membranes which are produced by the follicle cells, at the end of vitellogenesis and, at a later stage, on the surface of the egg shell (Theopold *et al.* 1996). The vitello-membrane separates the oolemma from the egg shell and during cellularisation and gastrulation allows cell movements of the embryo along the egg shell. The mucin found on the egg chorion surface could facilitate egg movements within the ovary as the egg shell has to separate from the follicle sheath to move through the oviduct.

The two functional aspects of mucin-like glycoproteins consisting of physical separation or lubrication and modulation of immune reactions are not mutually exclusive. It is therefore reasonable to assume that the mucin itself, or in conjunction with other components, might be a lubricant on polymerised insect surface structures and at the same time have an immune function on the surface of hemocytes.

In this context, the putative function of a mucin-like glycoprotein, on the egg surface of a non-parasitic insect, like *D. melanogaster,* is probably restricted to lubrication of the egg shell during egg maturation and deposition. However, if the glycoprotein contribute to modulation of immune reactions, such a coating in the context of an endoparasitoid would facilitate egg survival after oviposition.

In *D. melanogaster*, *H. p.* lectin proved to be a specific indicator for the mucin-like glycoprotein (hemomucin) (Theopold *et al.*, 1996). This lectin was therefore use for identification of similar glycoproteins in the parasitoid *V. canescens.*

The presence of glycoproteins on the egg surface of *V. canescens* as part of VLPs was suggested previously (Rotheram, 1973a; Bedwin, 1979a). The first positive evidence of the presence of carbohydrates in VLPs was carried out by Bedwin (1979a). He later determined the ratio of proteins to carbohydrates of VLPs found on the egg surface to be 100 µg protein to 17 µg carbohydrates. He also showed that the two components are covalently linked with the protein part consisting of a single polypeptide chain. However, Bedwin (1979a) did not show whether additional glycoproteins other than the VLPs are found on the egg surface or their possible functions.

To investigate the assumption that glycoproteins in *V. canescens* are involved in immune protection of the egg and larva in the host, the location of mucin-like components were analysed in *E. kühniella* and *V. canescens* using *H. p.* lectin as a diagnostic indicator of mucin-like glycoprotein. These components were compared in both organisms. An attempt was also made to characterise and clone the mucin in *V. canescens.*

5.2 Results and discussion

5.2.1 Identification of mucin-like protein in V. canescens ovarioles

To identify the location of mucin-like components on the egg surface, wasp ovaries were dissected and sectioned. The staining of tissue sections treated with FITC-conjugated *H. p.* lectin were compared with those treated with anti-VLP and anti-PHGPX-like proteins antisera. In contrast to the VLP proteins, which are only synthesised in posterior calyx gland cells, the mucin-like glycoprotein was localised on a number of ovarian tissues and sclerotised structures. Early ovarioles of the wasp are surrounded by a thin, translucent sclerotised sheath which was labelled by the *H. p.* lectin (Fig. 37, arrow). The labelling around ovarioles disappeared in later vitellogenic stages of ovarioles. *H. p.* lectin staining is also visible over follicle cells (Fig. 37, arrow head). At the final stages of vitellogenesis, a faint labelling is visible between the follicle cells and oocyte (Fig. 38 A, arrow). Since this staining pattern coincides with the deposition of the vitello membrane (Fig. 38, arrow) and chorion deposition, it is likely that the follicle cells produce the glycoprotein as part of the egg shell.

At later stages of oogenesis, the now visible egg shell is clearly labelled by the *H. p.* lectin (Fig. 39, arrow). Although most of the ovarioles are labelled with *H. p.* lectin, some follicle cells (in egg shell producing ovarioles) are more intensely stained than others. Compared to follicles cells that are in the process of chorion synthesis, the calyx gland cells are stained with the same intensity (Fig. 40, arrow). This could suggest that the calyx gland cells, like follicle cells, express and secret mucin-like proteins for deposition onto the egg surface. In contrast to the anti-VLP antiserum, which only labelled the posterior part of the calyx gland (See Chapter 3), the *H. p.* lectin labelling is equally distributed over the calyx gland.

5.2.2 Identification of mucin-like protein in V. canescens eggs and larvae

Oviduct tissue sections of *V. canescens* incubated with FITC-conjugated *H. p.* lectin show that the chorion structure of the egg on its passage through the calyx gland (Fig. 41,

arrow) and inside the oviduct is clearly labelled with the *H. p.* lectin. The observation that follicle cells which are known to produce hemomucin in *D. melanogaster* (Theopold *et al.*, 1996) and calyx gland cells are labelled equally strongly would suggest a site of synthesis in calyx cells in *V. canescens*. This is supported by the observation that calyx fluid inside the oviduct is strongly labelled by *H. p.* lectin (Fig. 42, arrow). This indicates that a mucin-like glycoprotein may be secreted by calyx cells as an independent fluid component or as a part of the VLPs. This agrees with the observation that a mucin-like glycoprotein can be found in cell-free hemolymph of *E. kühniella* (see Fig. 52).

V. canescens eggs from wasp oviduct (Figs. 41 and 42) and eggs dissected from parasitised host caterpillar, also show similar labelling (Fig. 43). This indicates that, the mucin-like glycoproteins acquired during oogenesis and on its passage through the calyx, remain on the egg surface after deposition inside the host.

5.2.3 Identification of mucin-like protein in V. canescens and E. kühniella hemocytes

Hemocytes of *V. canescens* and *E. kühniella* attached to the surface of glass slides were treated with FITC-conjugated *H. p.* lectin to determine the location of the mucin-like glycoprotein on hemocyte types. A sub-population of the *E. kühniella* hemocytes are heavily labelled by the *H. p.* lectin as indicated by the green colour (Fig. 44 arrow) whereas other hemocytes are not. In some areas of the blood smear a diffuse labelling of the glass surface was visible (Figs. 44 arrow head, 45). It is interesting to note that the differential labelling of hemocyte types are similar to that observed for anti-VLPs antiserum (Chapter 4).

When hemocytes from parasitised caterpillar were stained with *H. p.* lectin the pattern of labelling was similar to that in non-parasitised caterpillars. However, the labelling showed a more speckled pattern and the granulocytes had a higher tendency to aggregate (Figs. 46 and 47). At this juncture, it is not clear whether this represents variations between individual caterpillars or is a characteristic feature of parasitised caterpillars.

A different pattern of staining was observed in hemocytes from *V. canescens*

adults. In the hemocytes from *V. canescens* larvae, the *H. p.* lectin was evenly localised among hemocytes. In adult wasps, the hemocytes were not divided into readily noticeable morphotypes (Fig. 48) as seen in lepidopteran hemocytes (Fig. 44). All hemocytes appeared to spread on the glass surface in a fashion intermediate to lepidopteran granulocytes and plasmatocytes (Figs. 48 and 49). The star-like appearance of spreading was usually observed accompanied by shedding of vesicles, which were labelled by *H. p.* lectin (Fig. 49, arrow head).

From these observations, it can be concluded that hemocytes in the lepidopteran species *E. kühniella* and the hymenopteran species *V. canescens* are labelled by the *H. p.* lectin. In contrast to *D. melanogaster* and *V. canescens* hemocytes, which are rather homogenously labelled by *H. p.* lectin, the hemocytes from lepidopteran caterpillars show distinct labelling of granulocytes whereas plasmatocytes show much less staining.

5.2.4 Identification of mucin-like protein in V. canescens embryo

V. canescens embryo that were separated from the egg shell and stained with *H. p.* lectin showed segment-specific staining initially in certain regions of the segment anlagen. This is an indication that the mucin-like components originated from within the larval epidermal cells (Figs. 50 and 51). At the stage when the larva is free living within the coelomic cavity of the host, the mucin-like components are uniformly distributed on the cuticle surface (Fig. 14). One of the other major sites of *H. p.* lectin-binding is found in the silk duct.

5.2.5 Identification of mucin-like proteins on Western blots

In order to characterise the lectin-binding components in tissues of the two species, Western blots were analysed using peroxidase-conjugated *H. p.* lectin as a probe. *H. p.* lectin-binding proteins from *E. kühniella* hemocytes, cell-free hemolymph and silk gland as well as *V. canescens* larva, ovaries and calyx glands were identified (Fig. 52). Proteins were extracted from these tissues dissected from the two organisms and analysed side by side, to determine whether the corresponding glycoproteins are similar in size.

Both insect species showed a major component that is strongly labelled by *H. p.*

lectin in various tissues. However, the size of the respective glycoproteins differed as identified by the respective electrophoresis migration properties. The major glycoprotein labelled by *H. p.* lectin from total *V. canescens* larva appears to be around 100 kDa. There is a slightly less prominent band at 200 kDa (Fig. 52, lane e), which corresponds to the hemomucin protein sizes in *D. melanogaster* (Fig. 52, lane g) (Theopold *et al.*, 1996). However, additional minor bands are also labelled in *V. canescens* larva (Fig. 52, lane d), whereas in *D. melanogaster*, only the two bands are detected (Theopold *et al.*, 1996). It is not clear whether the additional bands in *V. canescens* are due to degradation of the glycoproteins or represent different sized glycoproteins that are coded by different genes. Nevertheless, the major band is a 100 kDa component and the overall *H. p.* lectin labelling pattern appears to be similar between *V. canescens* and *D. melanogaster* suggesting the presence of a glycoprotein of similar size.

In contrast, the major *E. kühniella* glycoproteins labelled with *H. p.* lectin are different in size (Fig. 52, lanes a-c) compared to both *V. canescens* and *D. melanogaster.* Instead of the major glycoproteins around a 100 kDa and 200 kDa, a strong labelling is detected at 80 kDa in hemocytes and silk gland (Fig. 52, Lanes a, c), whereas, a soluble hemolymph component at approximately 76 kDa in size is detected in cell-free hemolymph (Fig. 52, lane b).

This suggests that with the *D. melanogaster* hemomucin-specific lectin, a similar component is recognised in *V. canescens*, in terms of its tissue-specific localisation, size and glycosylation. In *E. kühniella*, a glycoprotein is present in similar tissues but differs in size from that of the other two species. This difference in protein size may reflect a variation in glycosylation or in the gene product. This can be investigated further by molecular cloning of the corresponding gene(s).

5.2.6. The V. canescens gut lining

Since previous observations suggest that the defence system of *E. kühniella* remains intact by the time *V. canescens* larvae emerge, the question also arises as to how the gut lining is protected against internalised *E. kühniella* hemolymph. Although the mouthparts of *V. canescens* larva consist of a sclerotised mandibles (Figs. 16 A and 20),

it is likely that the wasp feeds on the hemolymph and the gut would thus require some type of immune protection when host hemolymph enters the gut lumen. Therefore, surface properties of the host and parasitoid endodermis and the morphology of the gut during development of the parasitoid were analysed and compared using molecular surface markers specific for the internal gut lining (*H. p.* lectin) and for the lining of the open circulatory system (anti-VLP antiserum).

Tissue sections of a *V. canescens* larva that has emerged from the egg chorion were incubated with anti-VLP antiserum and visualised under indirect UV light. The anti-VLP antibodies cross-react with basement membrane components located at the lining of the hemocoel (Fig. 53). This cross-reactive property of the anti-VLP antibodies is obviously due to conserved components since it is detected in tissue sections of lepidopteran (see Chapter 4) and hymenopteran species.

To obtain some insight into the development of the molecular lining of the larval gut, tissue sections of early and late wasp larvae were analysed using antisera and lectin. In all early larval stages a unique morphological feature of the gut lumen was observed. Instead of an empty gut lumen lined by a peritrophic membrane, a spongy tissue was observed within the gut lumen in early larval stages (Figs. 53 and 54). In later larval stages and by the time the larva emerges from the caterpillar, the spongy tissue is altered becoming auto-fluorescent under indirect UV-light, an indication that the cells become necrotic (Figs. 55 and 56).

This observation suggests that the parasitoid larva does not digest solid food but may absorb liquid nutrients from host hemolymph throughout larval life. This observation is supported by the absence of physical damage to tissues of the host caterpillar. The presence of the spongy gut tissue throughout most of the larval life is an indication that hemolymph uptake and absorption is probably the only way of food acquisition by this endoparasitoid. The presence of a parasitoid inside the caterpillar may metabolically resemble an open wound where hemolymph is continuously lost through feeding. A metabolic feedback system must exist, where the continuous absorption of nutrients by the parasitoid creates a signal to regenerate certain hemolymph components from host tissues like fat body.

If the host hemolymph is the source of nutrients for the wasp larva, the question is whether the lining of the gut lumen is similar to the lining of the hemolymph in the open circulatory system. To test whether cross-reactive components are present inside the gut lumen, sections of early wasp larvae were treated with antibodies raised against VLPs. These antibodies cross-reacted with the wasp lining of the hemocoel, the cuticle surface inside and outside (arrow), and with the lining of internal tissues (Fig. 53). The lining of the gut lumen of *V. canescens* larva is labelled with anti-VLP antibodies. This suggests that the internal lining of the gut is coated with antigens recognised by the anti-VLP antibodies. The origins of these antigens may be derived from ingested host hemolymph or secreted from the wasp gut cells. It appears as if the inside lining of the gut in the endoparasitoid is labelled in a similar fashion as the lining of tissue in the open circulatory system of the hemocoel. This may be necessary to protect the gut from immune attack since the larva feeds on the host's hemolymph which may also include hemocytes. In contrast, the mucin-like component which is normally found at the gut lining of other insects (Theopold *et al.*, 1996) is absent at the lining, and only the spongy tissue inside the gut is somewhat labelled with the *H. p.* lectin (Fig. 54).

To test whether the internal gut lining changes at a later stage of the endoparasitoid development, the anti-VLP antiserum was applied to sections of the wasp larvae that had already left the host caterpillar. As seen from light micrographs, the inside of the wasp gut undergoes dramatic changes during this stage of development. Figure 55 shows a section of a *V. canescens* shortly after leaving the host, stained with anti-VLP antibodies and visualised with FITC-conjugated secondary antibodies. The section shows the basement membrane of tissues labelled as seen in a muscle (Fig. 55, (m)) and the gut surface that is exposed to the hemolymph (Fig. 55, arrow). The spongy cells inside the gut are in the process of malanization as can be seen from the yellow colour due to auto-fluorescence, but importantly the internal lining of the gut at this stage of development is not stained.

To test whether host hemolymph is still present inside the gut, sections of the late larval stage of *V. canescens* were also incubated with anti-hemolymph antibodies from *E. kühniella* visualised with FITC-conjugated secondary antibodies (Fig. 56 A). The internal

spongy tissue of the gut was strongly melanised and no labelling of the inside was observed. This suggests that at this stage the interior structure of the gut has probably changed in preparation for adult life when the food would be different and therefore require changes in the protection of the gut epithelium.

Interestingly, the antiserum against host hemolymph cross-reacted with antigens in tissue sections from the wasp larvae. In fact the localisation of cross-reacting antigens in *E. kühniella* and *V. canescens* tissue sections are similar and correspond to the localisation of antigens detected with the anti-VLP antibodies (Fig. 55), including the basement membranes of tissues that are exposed to the hemolymph. This confirms the notion that some of the structures to which the wasp anti-VLP antibodies react in the caterpillar hemolymph are conserved among lepidopteran and hymenopteran species, and therefore are visible in wasp sections using antibodies against lepidopteran hemolymph.

5.2.7 The tissue lining during metamorphosis

An unexpected observations was made when the tissue lining of the open circulatory system was analysed in the pupal stages of *E. kühniella*. At early pupal stages, the basement membranes of some of the fat body tissues disappeared and the fat body cells changed in morphology and cell surface staining. Instead of its normal translucent appearance during the larval instars, these fat body cells become crystal-shaped with a bright appearance under phase-contrast. At later pupal stages, the fat body cells (or its remnants) undergo a change in tissue distribution in relation to other tissues. In contrast to fat body tissue in earlier larval stages, these fat body cells are found along the lining of tissues, like epidermis and unsclerotised cuticle structures (Fig. 57).

When advanced pupal stages (brown puparium) were sectioned and incubated with FITC-conjugated *H. p.* lectin, the bright fat body cells were intensively stained. Instead of being evenly spread between tissues, these fat body cells dissociate from their former location and attach to the surface of epidermal tissues (arrowhead) or muscle (arrow) (Fig. 57). These cells were also found on the surface of newly formed epidermal structures (Fig. 58, arrow). The cells are clearly seen in light clumps in figures 57 (B) and 59 (B).

Similar labelling patterns were obtained using anti-VLP and anti-PHGPX-like protein antiserum. In early pupal sections (Fig. 59) intact fat body tissue, that are still surrounded by a basement membrane, are seen side by side with parts of fat body tissue where individual fat body cells are intensely stained (arrow head). This suggests that fat body cells undergo morphological changes during metamorphosis, which includes the expression of mucin-like glycoproteins on the cell surface, dissolution of fat body tissue, and reassembly of fat body cells along the lining of other tissues (Fig. 60). At this stage these fat body cells also contain antigens on the surface which cross-react with anti-VLP and anti-PHGPX-like protein antisera. Figure 61 show a schematic representation of *V. canescens-E. kühniella* interaction and the location of wasp and host components involved in immune protection.

5.3.1 Southern hybridization analysis

To identify the hemomucin-homologous DNA in *V. canescens* genome, Southern blot hybridization analysis was carried out. A probe derived from the cDNA pDMu-105 coding for *D. melanogaster* hemomucin gene was used to hybridize a Southern blot containing *D. melanogaster* and *V. canescens* genomic DNA, double digested with restriction enzymes *Apa*I + *Sac*I, *Bam*HI + *Hin*dIII, and *Eco*RI + *Pst*I. Although a signal in *V. canescens* lane was difficult to see, some weak hybridization signals were detected in films exposed for a longer period of time. This was however difficult to document in a picture due to increased background staining. Figure 62 show a radiograph of restriction enzymes digest of *D. melanogaster* (lane, a, b, and c) while (d), (e) and (f) are similar digests of *V canescens* genomic DNA. The Southern blot was exposed for 30 min. Longer exposure showed a light band on lane (f) of *V. canescens* genomic DNA digested with *Eco*RI + *Pst*I (data not shown)

5.3.2 PCR analysis of *D. melanogaster* and *V canescens* genomic DNA

Since the genomic digest Southern blot data were somewhat ambiguous, genomic and cDNA (Hellers *et al.*, 1996) from *V. canescens* as well as genomic DNA from *D. melanogaster* was used for amplification using primers designed from the hemomucin

(Figs. 63 and 64). Figure 63 shows a schematic representation of *D. melanogaster* hemomucin and the position of the probe, pDMu-105 used for hybridization as well as the primers used in the PCR reactions. Figure 64 is a similar representation in relation to the hemomucin protein sequence. The PCR products using genomic DNA from both insects showed similar results when hybridized to pDMu-105 probe (Fig. 65). The primers used in this reaction were, forward primer Mu2 and reverse primers Mu4a and Mu7. The film was exposed for 30 min in case of *D. melanogaster* and 24 hours for *V. canescens*.

When *V. canescens* cDNA was used with different sets of primers, products very similar in size to *D. melanogaster* genomic were obtained (Figs. 66 and 67). When the PCR products were cloned, the DNA sequence was almost identical to the hemomucin sequence. This led to the belief that a contamination with *D. melanogaster* genomic DNA had occurred. A repeat of these experiments was not possible within the scope of this study. Further investigation would be a basis for understanding the immune interaction between *V. canescens and E. kühniella*.

Chapter Six

General Discussion

Discussion

The mechanism of immune evasion in endoparasitoid-host interactions has fascinated researchers for many years. Investigations into the molecular aspects of these relationships are only at the beginning, but reveal a diversity of strategies utilised among hymenopteran parasitoids to overcome the defence reactions of habitual host organisms (Whitfield, 1993).

The immune protective mechanism of *V. canescens* eggs inside the host caterpillar, *E. kühniella*, was long ago recognised to constitute a special mode of protection (Salt, 1955; 1970). It is still not possible to experimentally determine whether the egg is not recognised by the host as foreign or whether the egg is recognised but prevented from being attacked. Whatever the case, the immune defence of the host caterpillar remains intact long after parasitation and emergence of the wasp larva. Therefore the egg and the larva must be protected by a unique mechanism without the need of actively suppressing the host defence system.

In most of the hymenopteran endoparasitoids analysed, the wasp symbiotic viruses infect the hemocytes and, together with the ovarian proteins, provide immune protection to the developing larva by inactivating the host defence system (Vinson, 1990a; Stoltz, 1993). However, in the *E. kühniella*-*V. canescens* system, components from the calyx gland provide passive protection and the host immune system, including the metabolic and hormonal status, remains virtually unchanged (Bedwin, 1979b; Salt, 1980; Feddersen *et al.*, 1986). Earlier studies have shown that the wasp, *V. canescens,* produces VLPs in the calyx gland (Salt, 1968; Rotheram, 1973a), which are able to protect the egg against encapsulation (Rotheram, 1973a; Bedwin, 1979b; Feddersen *et al.*, 1986). In this system the question is therefore, how does the wasp larva avoid encapsulation after it has emerged from the protecting egg shell? It was suggested earlier that, just like the egg surface, VLPs are present on the larval surface (Rotheram, 1973a).

In this study scanning electron micrographs revealed that the surface of the egg is covered by VLPs, whereas the larval surface is not. In a few cases where occasional VLPs are found in segmental groves it is likely that these particles were picked up by the larva

in the process of hatching and moving out of the egg shell. Despite this observation, both the entire egg and larval surfaces show similar staining with anti-VLP antiserum. This suggests that the two sclerotised surfaces are covered with components that are antigenically similar and therefore may be protected by a similar mechanism.

The importance of mucin-like glycoproteins in cell-cell interaction is well documented in the literature as exemplified in the case of the egg and sperm attachment during fertilisation (Gahmberg *et al.*, 1992; Springer, 1994; Lasky, 1995). Moreover, some evidence suggests that vertebrate blood cells are modulated in their adhesive behaviour by mucin-like glycoproteins (Shimizu and Shaw, 1993; Springer, 1994). In the case of *D. melanogaster*, a mucin-like glycoprotein was discovered which is specifically recognised by *H. p.* lectin (Theopold *et al.*, 1996). Like LPS, the lectin induces cecropin, a known anti-bacterial protein in mbn-2 *D. melanogaster* cells (Samakovlis *et al.*, 1992), after binding to the mucin on the membrane of these cells (Theopold *et al.*, 1996). Interestingly, hemomucin was also found on *D. melanogaster* chorion surface, being synthesised by follicle cells at a late stage in oogenesis (Theopold *et al.*, 1996).

Biochemical studies on *V. canescens* VLPs had indicated that the particles contained a glycoprotein (Bedwin, 1979a). It was therefore interesting to investigate the possible existence of mucin-like glycoproteins on surfaces of *V. canescens* egg and larva. The results from Western blots and tissue sections probed with *H. p.* lectin show that a glycoprotein of similar size to *D. melanogaster* hemomucin is localised in oocytes and calyx cells. The intensity of the staining in wasp follicle cells, where hemomucin is produced in *D. melanogaster*, is comparable to the staining in *V. canescens* calyx glands, which suggests that the mucin-like glycoprotein is also produced in this organ. This could explain the staining with the lectin of the calyx fluid that is visible inside the oviduct. It is therefore possible, but requires further experimental proof, that hemomucin is a structural part of the VLP surface. This assumption is supported by the observation that anti-VLP antiserum has a background staining on tissue sections that is reminiscent to the *H. p.* lectin staining.

The mucin-like glycoprotein is secreted by epidermal cells of the wasp larva during a stage in embryogenesis when the segmental anlagen are formed in a characteristic stripe pattern. Shortly before the larva hatches, the larval cuticle is completely covered with the glycoprotein. The egg and larval surfaces are covered by the glycoprotein which is probably coded by a wasp gene as the host glycoprotein is of a different size. Since this is the component found to be common to both the egg and the larval surface, the question is whether the glycoprotein is the basis for the protective properties on both surfaces, and whether it is necessary and sufficient for the egg and larval protection.

Several observations suggest that the mucin-like glycoprotein is not sufficient for protection against the host defence. It was shown (Feddersen *et al.*, 1986) that oocytes and eggs that are devoid of VLPs are encapsulated when injected into the host caterpillar. Although it is not clear from these experiments whether the detergent was able to remove the glycoprotein together with VLPs, the results here suggests that the mucin-like glycoprotein is difficult to remove from sclerotised surfaces. In any case, oocytes dissected from mature ovarioles (before entering the calyx gland) contain mucin-like glycoproteins. Nevertheless, they are encapsulated when injected into caterpillars whereas, oocytes are protected when incubated with VLPs prior to injection (Salt, 1955; Feddersen, 1986). More recent observations using mucin-coated Sephadex beads suggest that, the hemomucin coating may delay the cell attachment process in some cases but is not sufficient to prevent cellular encapsulation *in vitro*. Thus the glycoprotein layer on the chorion surface may be a prerequisite for the protective coating but crucial components are required in addition and are provided to the egg surface by the calyx fluid, possibly using the mucin-layer on the egg surface as a receptor.

The hemomucin-like glycoprotein is present on both the egg and the larval surfaces. However, unlike the egg, the larval cuticle has only a small number of VLPs coating its surface, nevertheless it cross-reacts strongly with specific antibodies against VLP proteins. This could suggest that the wasp secretes non-particle proteins similar to VLP proteins during embryogenesis. Alternatively, the cross-reacting components could be derived from the hemolymph taken up by the early embryo. These host components

could bind to the hemomucin in a similar fashion as calyx gland products attached to the egg surface and thus confer immune protection. Recent experimental evidence that lipophorin, among other hemolymph components, is specifically bound to mucin-like glycoprotein has kindled scientific interest in hemolymph-mucin interactions (Theopold, personal communication).

The general concept of passive protection that is emerging from initial observations suggests that *V. canescens* egg and larva surfaces are covered with a highly conserved glycoprotein produced by the parasitoid. This protein consists of a mucin-like component but is not restricted to this type since ligands with wheat germ agglutinin binding properties are also found on *D. melanogaster* egg surfaces (U. Theopold, personal communication). From these studies, it is evident that the glycoprotein can only be immune protective in conjunction with other components that are added to it. These could be hemolymph components like the 42 kDa protein or lipophorin. The observation that the mucin-like glycoprotein is also part of the calyx fluid, suggests that VLPs are covered with the glycoprotein as well. This is an important aspect of the putative VLP properties which require further experimental investigation. If mucin-like glycoproteins are shown to be part of the VLP surface, the protective layer of the egg surface would consist of the glycoprotein in conjunction with hemolymph proteins. The protective principle would therefore be similar for both the egg chorion and for the VLP surface. In both cases, the glycoprotein would be necessary but not sufficient to confer immune protection. Complete protection might depend on binding of soluble components either from the wasp calyx gland or the host hemolymph. Lipophorin is a good candidate as indicated by preliminary results that lipophorin binds to hemomucin (U. Theopold, personal communication).

From observations that the *H. p.* lectin binds to a *V. canescens* glycoprotein that is similar in size to *D. melanogaster* hemomucin molecule, it can be implied that the immune mechanism would be similar in both organisms. That is, certain components such as lipophorin, present in the host hemolymph, would bind the hemomucin-like glycoprotein on the larva and thus confer "self-like" properties. On-the-other-hand, "non-self" objects without the glycoprotein components would be opsonised and recognised as foreign.

The ichneumonid *V. canescens* appears to exert no discernible physiological changes on its habitual host *E. kühniella* (Salt, 1970). The egg is passively protected against the immune response of its host by a coat of glycoprotein and VLPs, leaving the host defence intact (Schmidt and Feddersen, 1989). The advantage of this strategy is that, the parasitoid inside the host caterpillar is protected against microbial infections that might otherwise affect the host caterpillar and thereby endanger the parasitoid development. Since the VLPs are devoid of nucleic acids (Bedwin, 1979a; Feddersen *et al.*, 1986), they are regarded as defective PDVs which lost their DNA through the process of evolutionary adaptation (Whitfield, 1993). The observation that the p40 gene consists of highly conserved PHGPX-like domain (Hellers, *et al.*, 1996) could suggest that ancestral pro-viral DNA has been rearranged in the wasp genomic DNA to express the PHGPX domain in the calyx cells. Thus the function of the *V. canescens* pro-virus can be stipulated to modify and alter expression of wasp genes in the calyx gland.

Research in some braconid systems has shown that mixed protection strategies exist, which include passive protection of the egg (Asgari and Schmidt, 1994) and active suppression of host hemocytes (Asgari *et al.*, 1996). Polydnaviruses inactivate immune system of the host in the initial three days post-oviposition, after which the immune response recovers (Asgari *et al.*, 1996; Lavine and Beckage, 1996). It is not only essential to protect the emerging wasp larva in an initial period, but it may be of an evolutionary advantage to the parasitoid to leave the host immune system intact. This process is necessary to protect the emerging larva and maintain the integrity of the parasitoid in an initial period while it is resident in the host. It also implies that the wasp larva is protected by the time the host immune system is restored. The fact that *V. canescens* is capable of maintaining this balance from the onset of oviposition and is able to avoid immune response, would place it at an advantage and an advanced evolutionary level over other parasitoids which utilise both passive and active immune suppression.

Structural features of the larval gut suggest that this endoparasitoid feed on host hemolymph. Given a functional hemolymph defence, the question is whether the gut lining of the endoparasitoid might be protected in a similar manner? Morphological and physiological data from this study suggest that the wasp feeds on the host hemolymph

throughout larval development, exposing the internal gut lining to the host defence system. Observations in *V. canescens* show that the gut lining of the early parasitic larva differs from non-parasitic insects. It contains a molecular coating similar to the surface coating of the larva. This protective feature inside the gut lumen would allow the wasp to absorb molecular nutrients from the host hemolymph without being exposed to the defence reaction of the host.

In conclusion, the present work provides substantial information on the mechanism of immune protection of the wasp egg and larva. It has been shown that a hemomucin-like glycoprotein, produced by the wasp ovaries, form part of the chorion onto which the VLPs attach. In the case of the parasitoid larva, hemomucin-like glycoprotein is produced by the larval epidermal cells during late embryogenesis, which is then exposed to the host hemolymph components that pass through the micropyle and may bind onto the hemomucin-like glycoprotein. This complex of wasp glycoprotein and host hemolymph components may then be responsible for the passive immune evasion observed on the larval surface after hatching.

Based on these findings, future research avenues can be proposed to elucidate the *V. canescens-E. kühniella* interactions. The cloning of the *V. canescens* hemomucin-like glycoprotein could provide the basis for molecular studies of its specific role in immune protection. Determining the identity of putative host components, with which the glycoprotein forms a complex responsible for immune evasion, will facilitate further investigation of passive protection in parasitoid host interactions.

Figures

Fig. 1 Virus-like particles from dissected calyx and oviduct tissue of *V. canescens* purified by caesium chloride gradient centrifugation (1.77g/5 ml PBS). The particle proteins are usually detectable in normal light as a single opaque band.

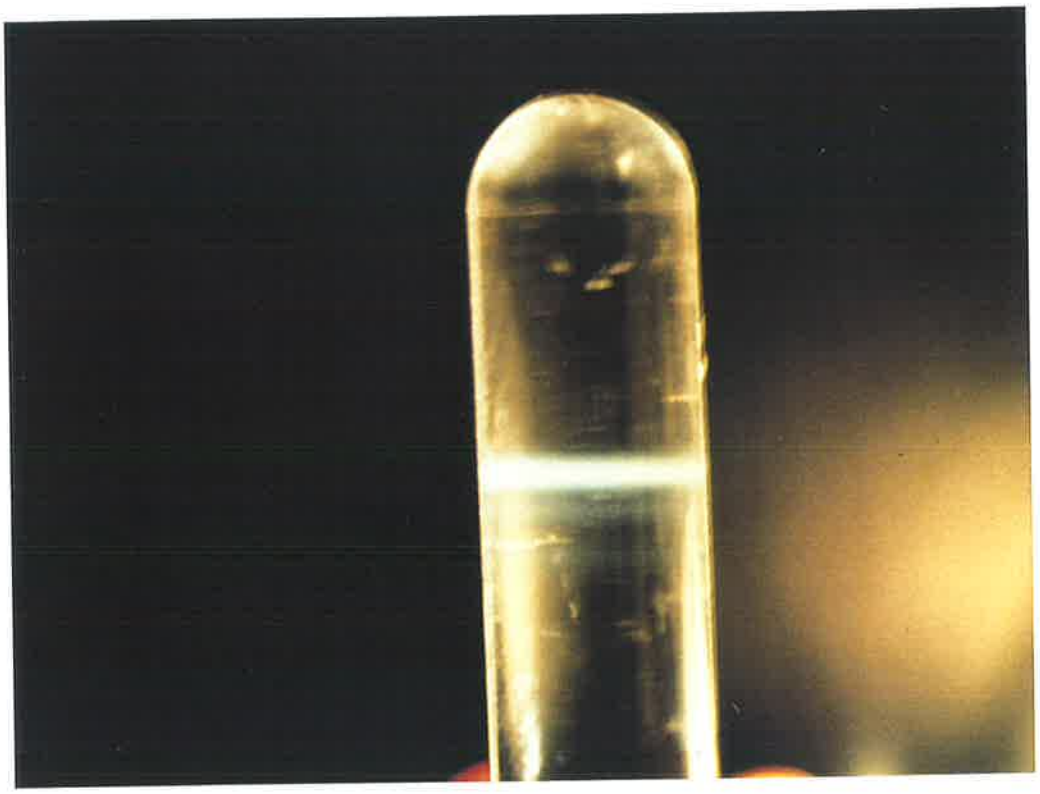
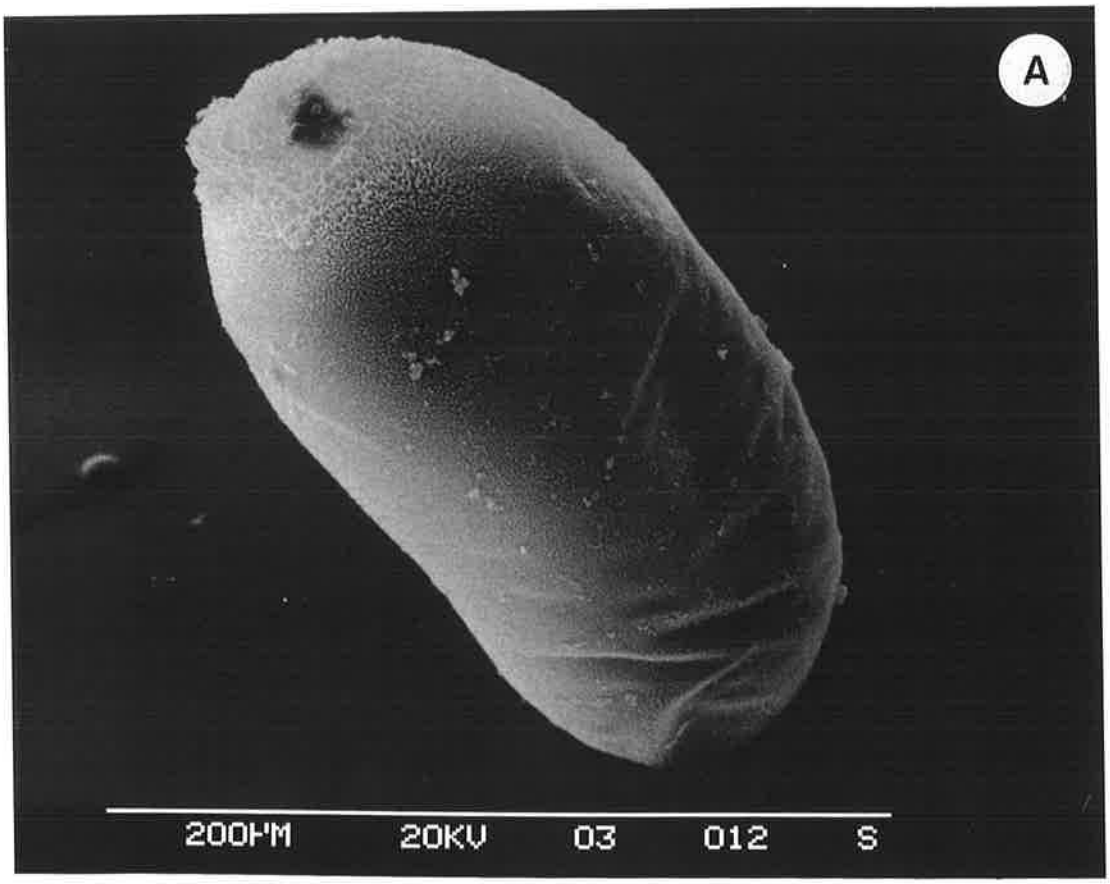


Fig. 2 Scanning electron micrograph of a *V. canescens* egg, dissected from a parasitised caterpillar. (A) Whole egg seen from the micropyle end. 1 cm = 19.4 µm. (B) Enlargement of the egg surface showing the VLPs attached to the chorion. Note the faint substructures noticeable on some of the VLPs. 1 cm = 100 nm .


A
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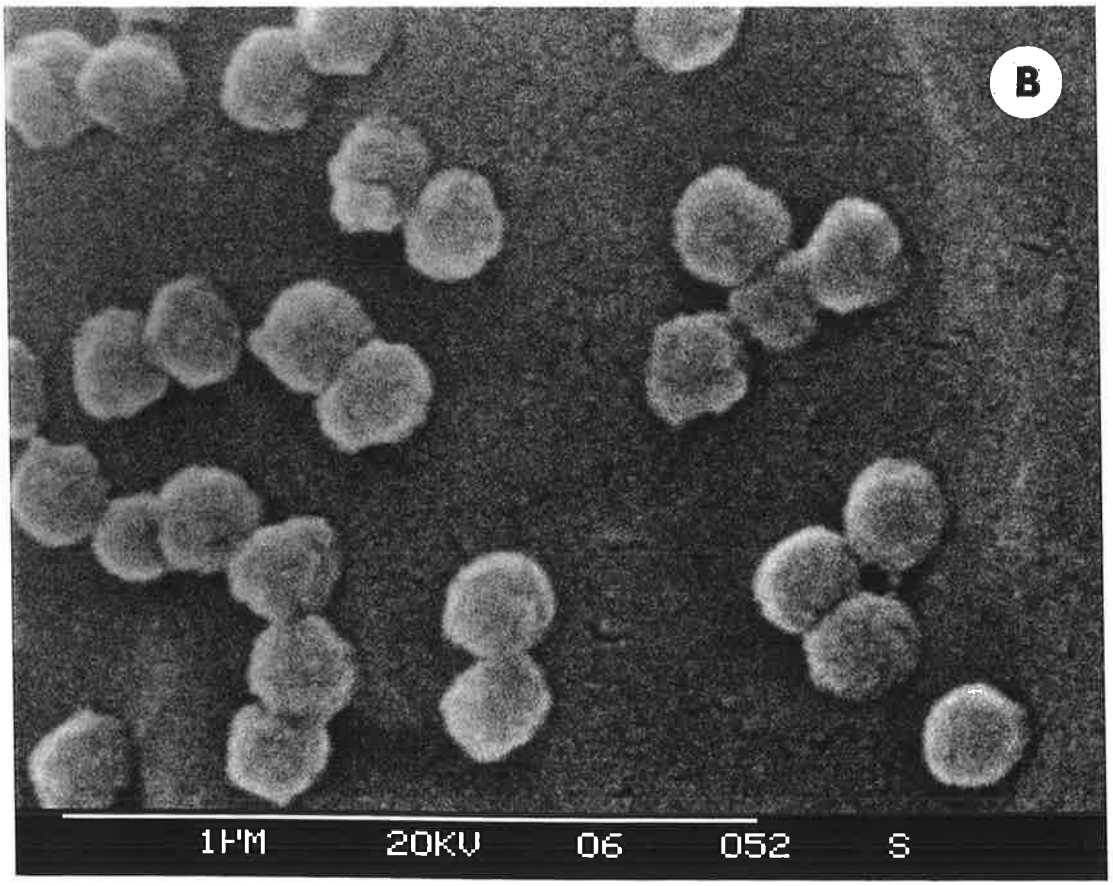
B
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Fig. 3 *V. canescens* egg dissected from a parasitised caterpillar and incubated with anti-VLP antibodies, visualised by FITC-conjugated secondary antibodies.

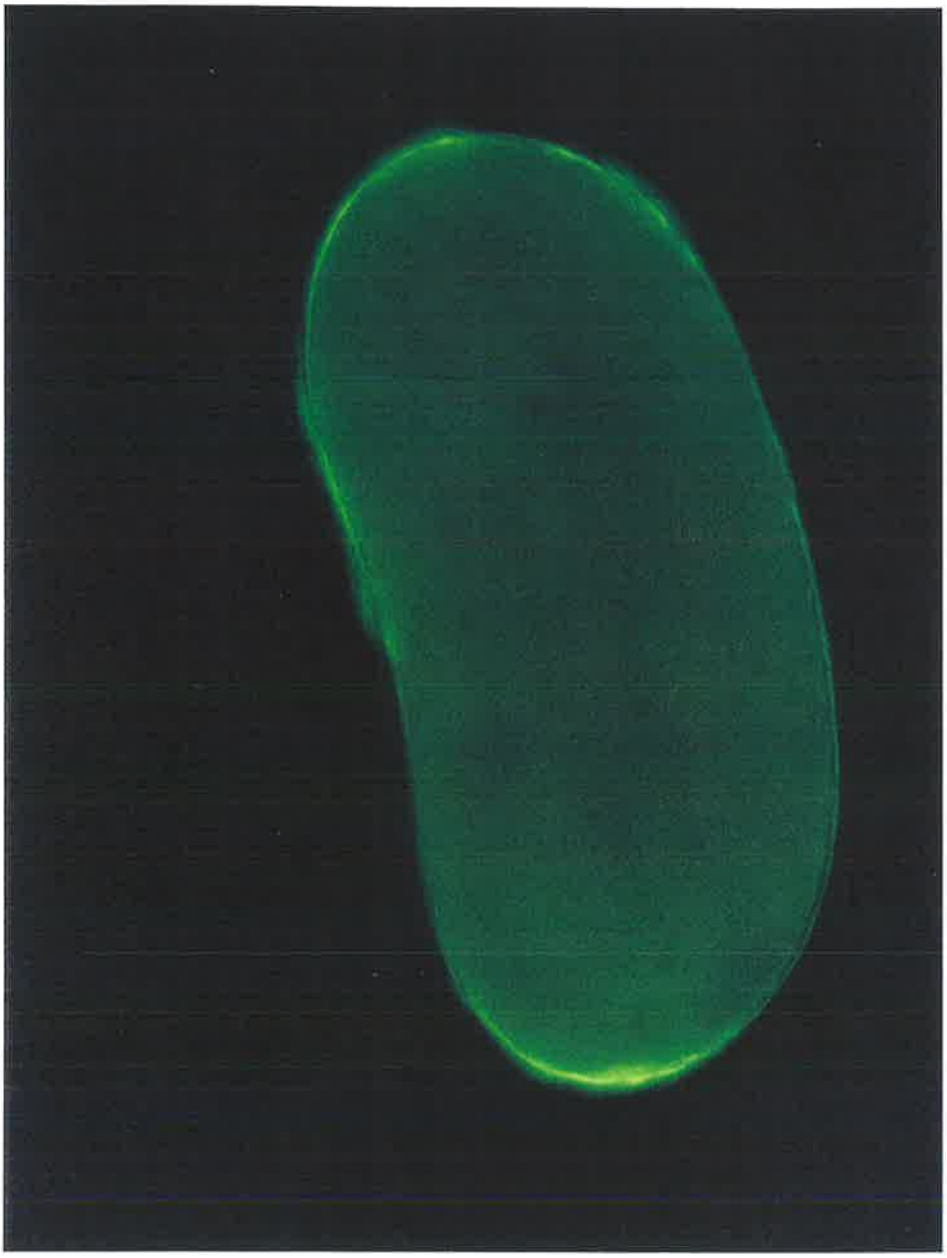


Fig. 4 (A) *V. canescens* egg dissected from a parasitised caterpillar and incubated with antibodies against the bacterial fusion protein encoded by the PHGPX-like domain of p40, visualised by FITC-conjugated secondary antibody. Similar staining was obtained on eggs that were dissected from the oviduct of wasp females. (B) The same specimen under phase contrast.

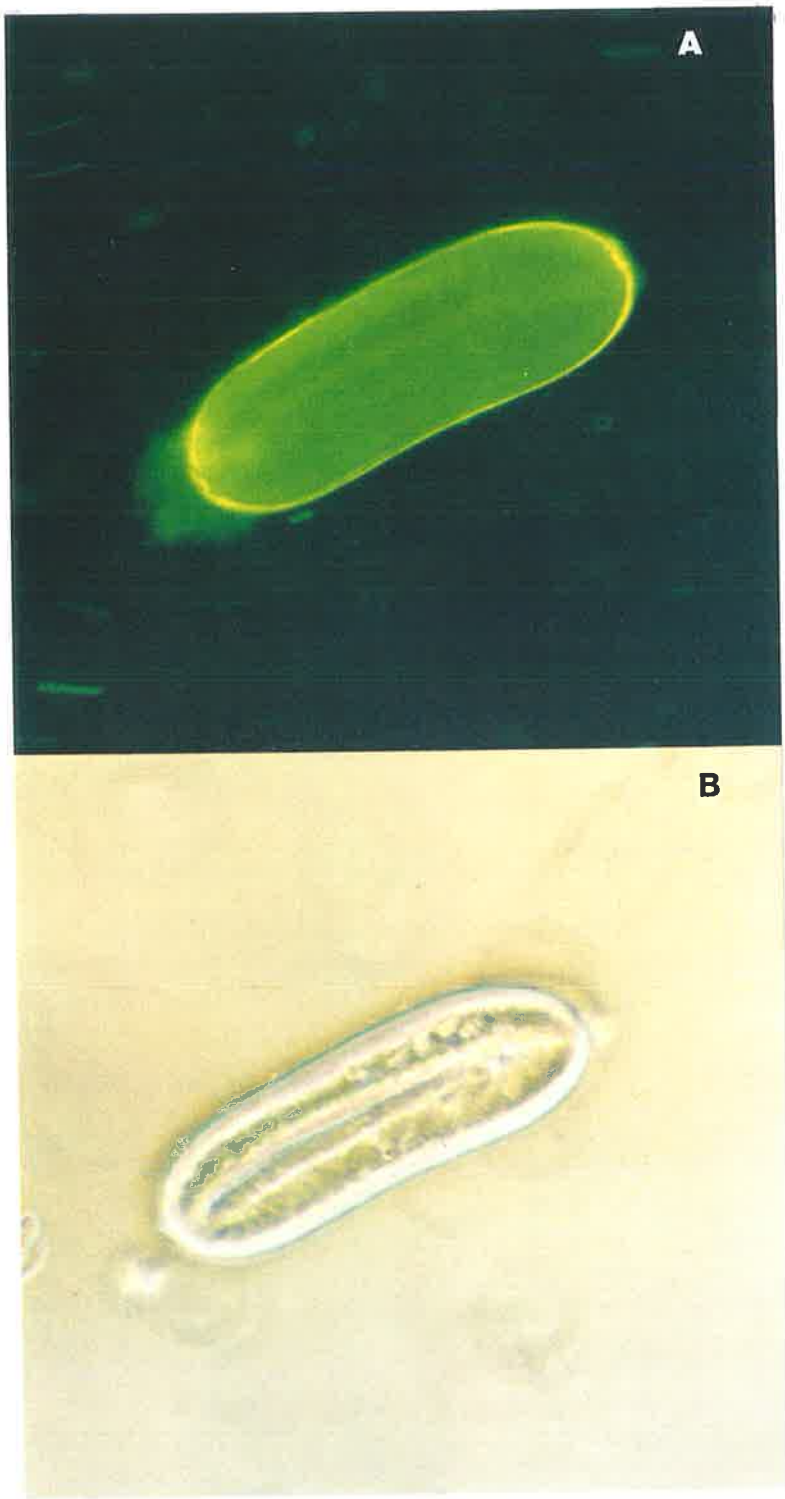
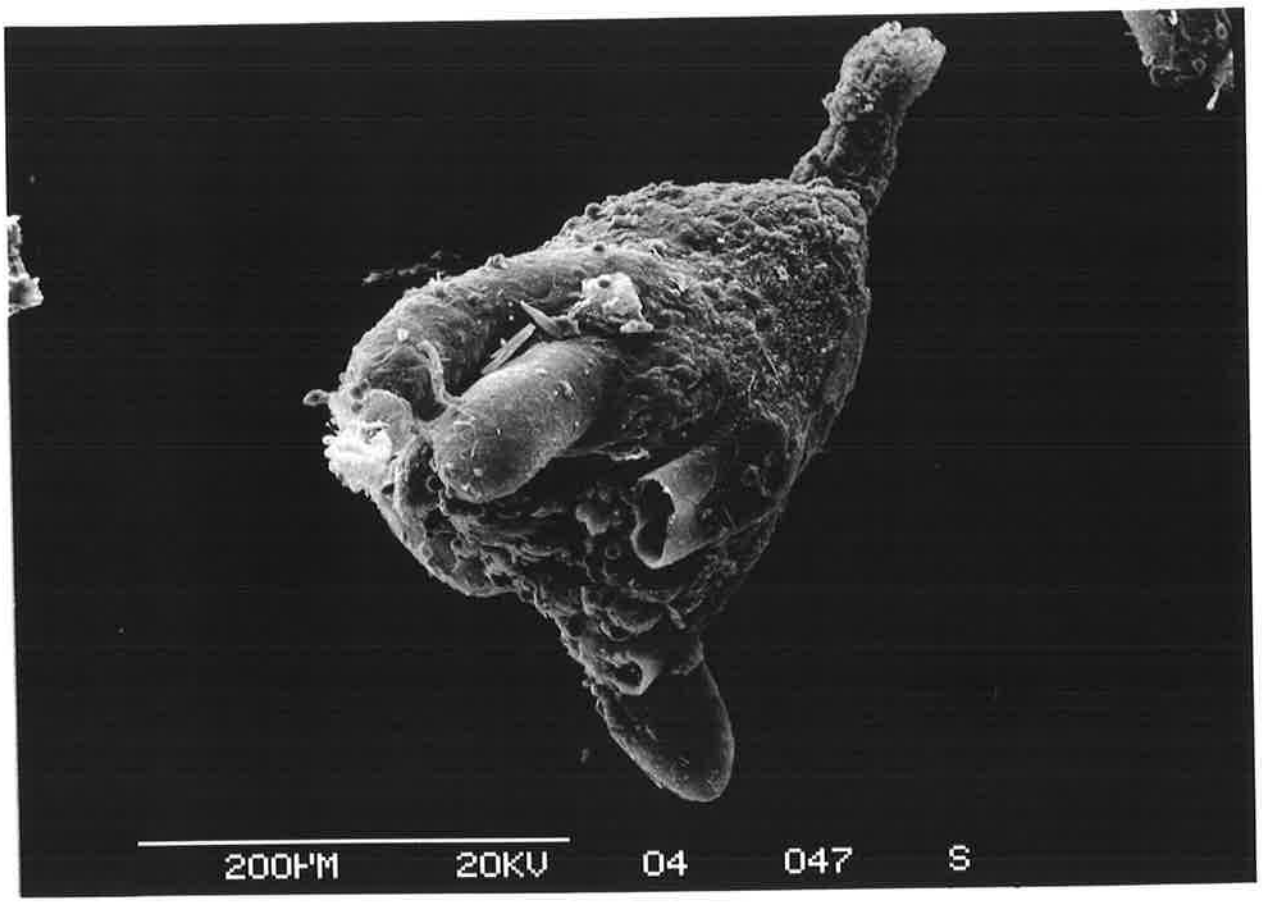

A
B

Fig. 5 Scanning electron micrograph of a *V. canescens* calyx gland showing several eggs during their passage through the calyx lumen. The tube-like structure at the upper right end of the gland is the beginning of the oviduct. 1 cm = µm


200UM
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S

Fig. 6 Tissue section of the ovary dissected from the wasp, *V. canescens*, treated with antibodies against a bacterial fusion protein encoded by the PHGPX-like domain of p40. Bound antibodies were visualised with FITC conjugated secondary antibodies. (A) The yellow-orange dots in the anterior part of the calyx gland (arrow) are probably due to melanisation of calyx gland tissue. Since these dots are seen under indirect UV-light in the absence of FITC, they are due to auto-fluorescence of unknown substances produced in this part of the calyx gland. The two parts of the calyx gland show slight morphological differences, with the anterior part showing signs of disintegration of surrounding epithelial cells (arrow), where the posterior part (arrowhead) does not. (B) The same section under phase contrast.

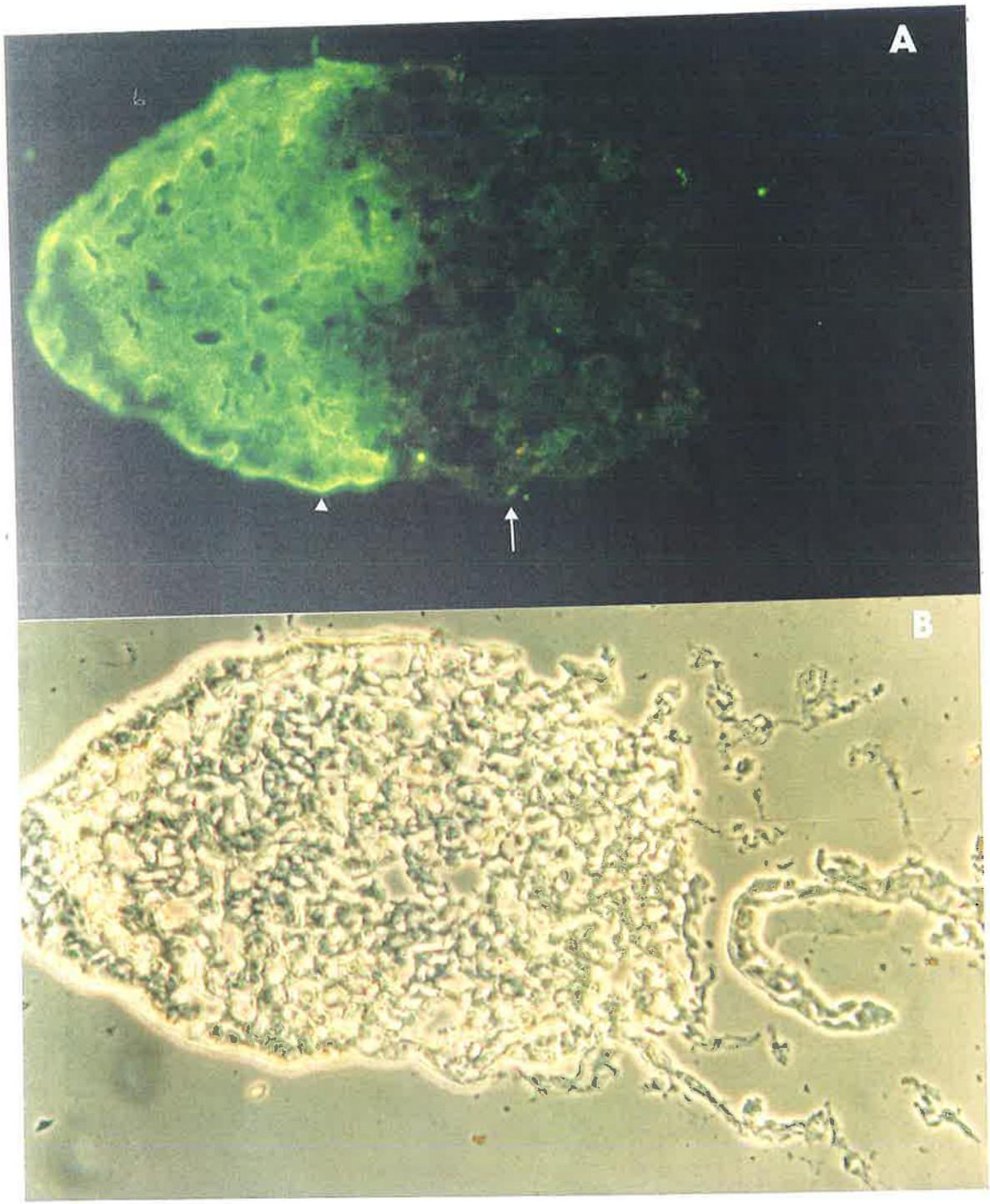

A
B

Fig. 7 Tissue section of the ovary dissected from the wasp *V. canescens*, incubated with antibodies against a bacterial fusion protein encoded by the PHGPX-like domain of p40. Bound antibodies were visualised with FITC conjugated secondary antibodies. (A) The oocytes passing through this part of the calyx gland are not stained by the FITC-conjugated antibodies. Since the yellow-grey colour is also detectable in untreated sections under phase contrast, the colour of the egg shell (arrow) is probably due to auto-fluorescence of the chorion. The yellow-grey colour in the anterior part of the calyx gland is also detectable in untreated sections and is due to auto-florescence of the chorion, (compare the bright green colour seen in FITC conjugated *H. p.* lectin sections in figures 8 and 40). (B) The same section under phase contrast.

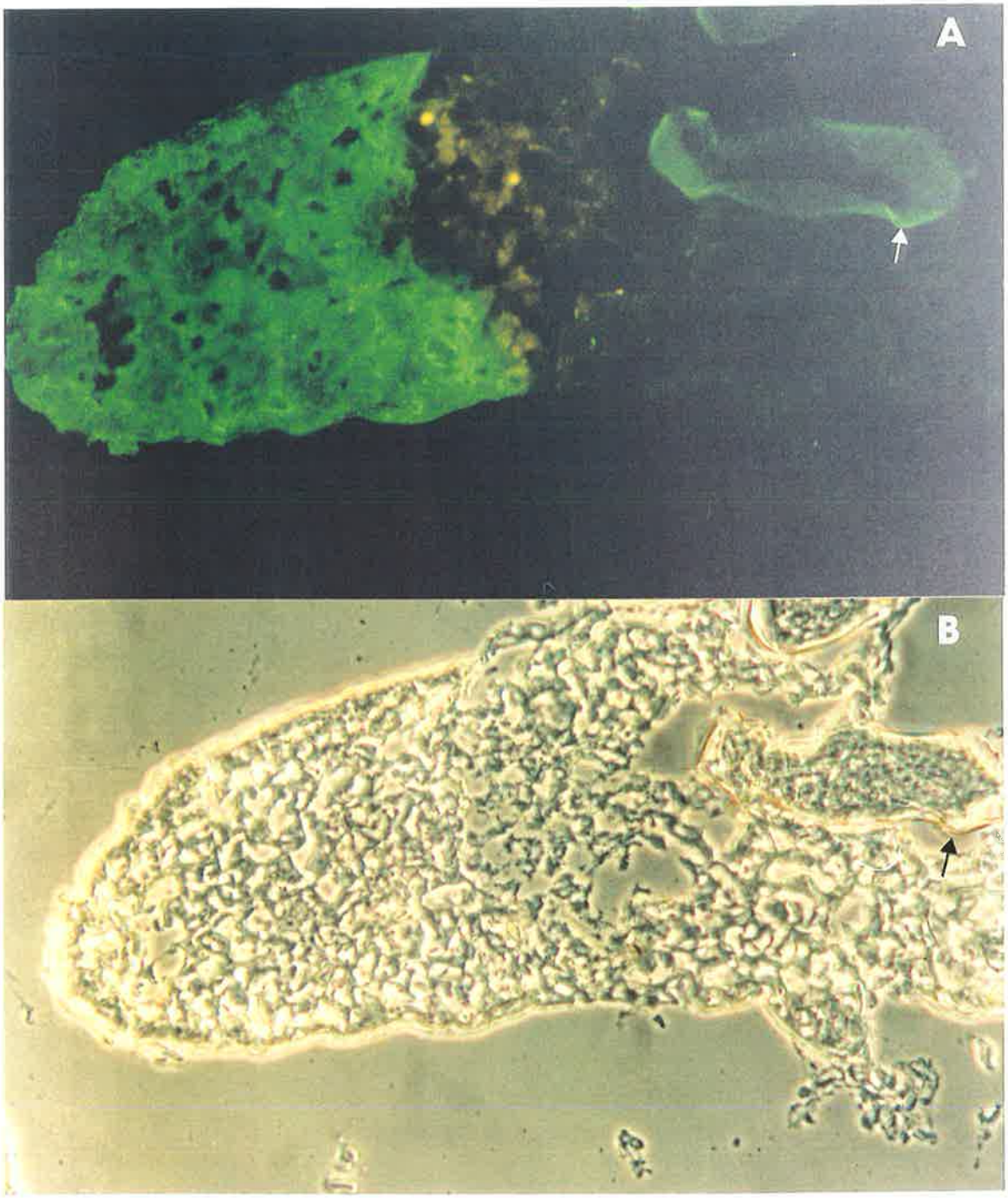

A
B

Fig. 8 Tissue section of a dissected ovary from the wasp, *V. canescens* treated with antibodies against VLPs. Bound antibodies were visualised with FITC conjugated secondary antibodies. (A) A slight background staining in the ovarioles is visible (arrow) and the oocyte chorion surfaces are slightly stained with a distinct green (top left) compared to the yellow colour caused by auto-fluorescence. (B) The same section under phase contrast.

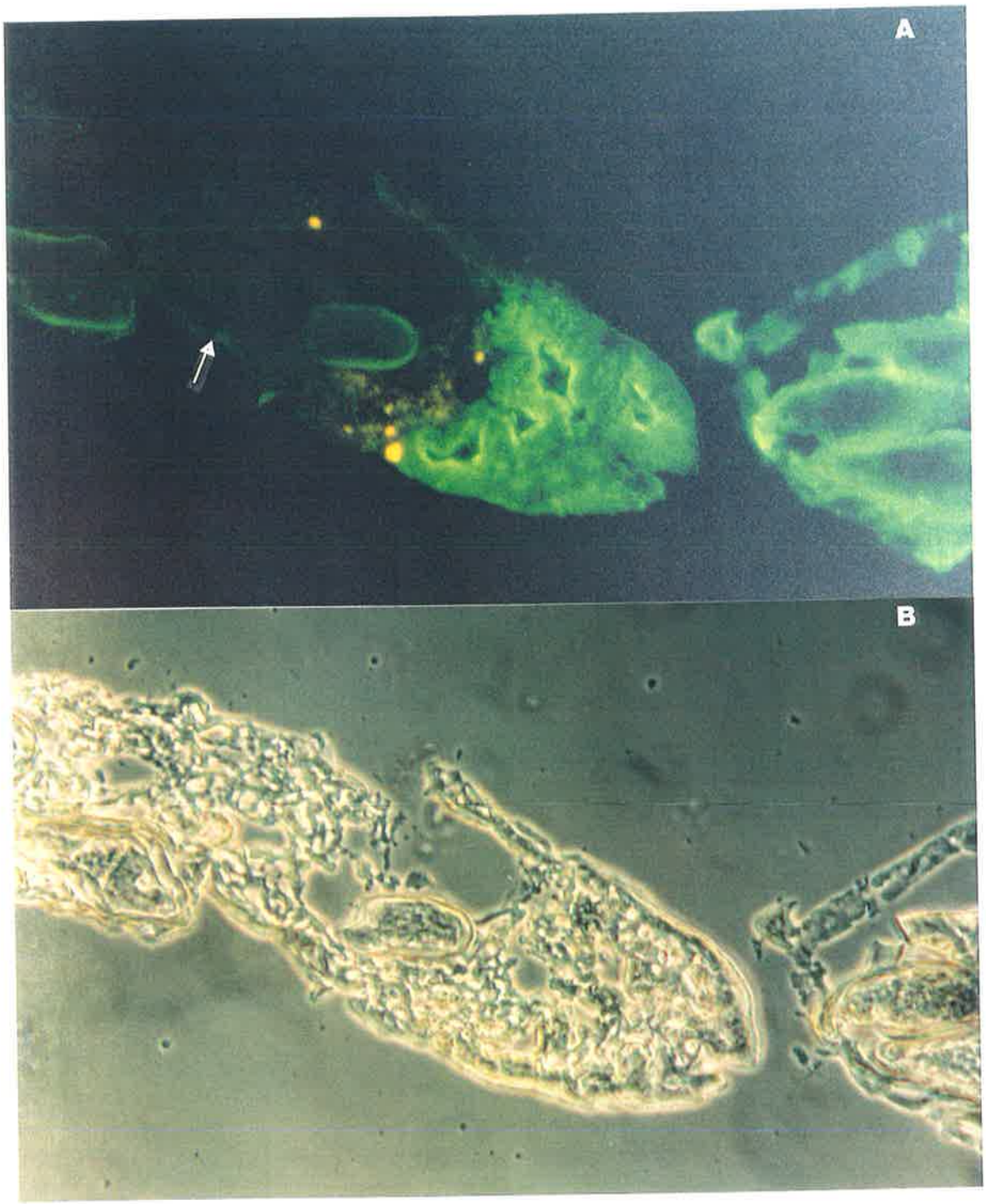

A
B

Fig. 9 Tissue section of dissected oviduct and ovarioles of the wasp, *V. canescens*, treated with antibodies against VLPs. Bound antibodies were visualised with FITC conjugated secondary antibodies. (A) The posterior part of the calyx is strongly labelled. The faint colour of the egg shell is caused by auto-fluorescence. (B) The same section under phase contrast.

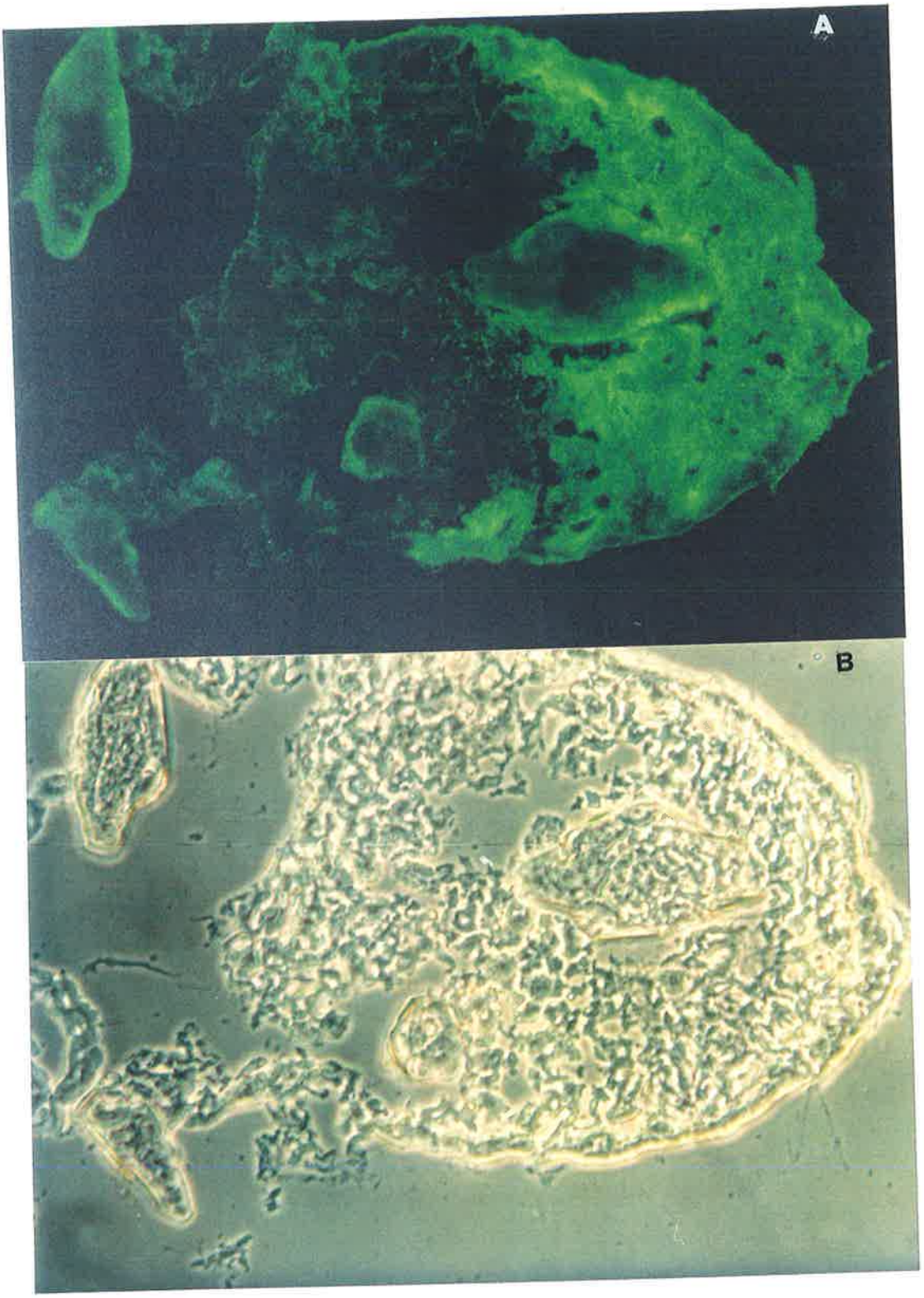

A
B

Fig. 10 Tissue sections of dissected oviduct of the wasp, *V. canescens* incubated with antibodies against a bacterial fusion protein encoded by the PHGPX-like domain of p40. Bound antibodies were visualised with FITC conjugated secondary antibodies. (A) The oviduct content is brightly stained including the egg surface and the calyx fluid between the eggs inside the oviduct (arrow). The egg content is probably not labelled, although it is difficult to rule out a possible labelling of the oolemma. The calyx fluid can be clearly seen in phase contrast (B) as a greyish opaque substance (arrow). The section was chosen because two ovarioles were located in this section (arrow head) showing the contrast of labelling between ovariole tissues and oviduct content. The faint colour of the oocyte chorion (arrowhead) is also detected in non-treated sections under UV-light and is caused by auto-fluorescence. (B) The same section under phase contrast.

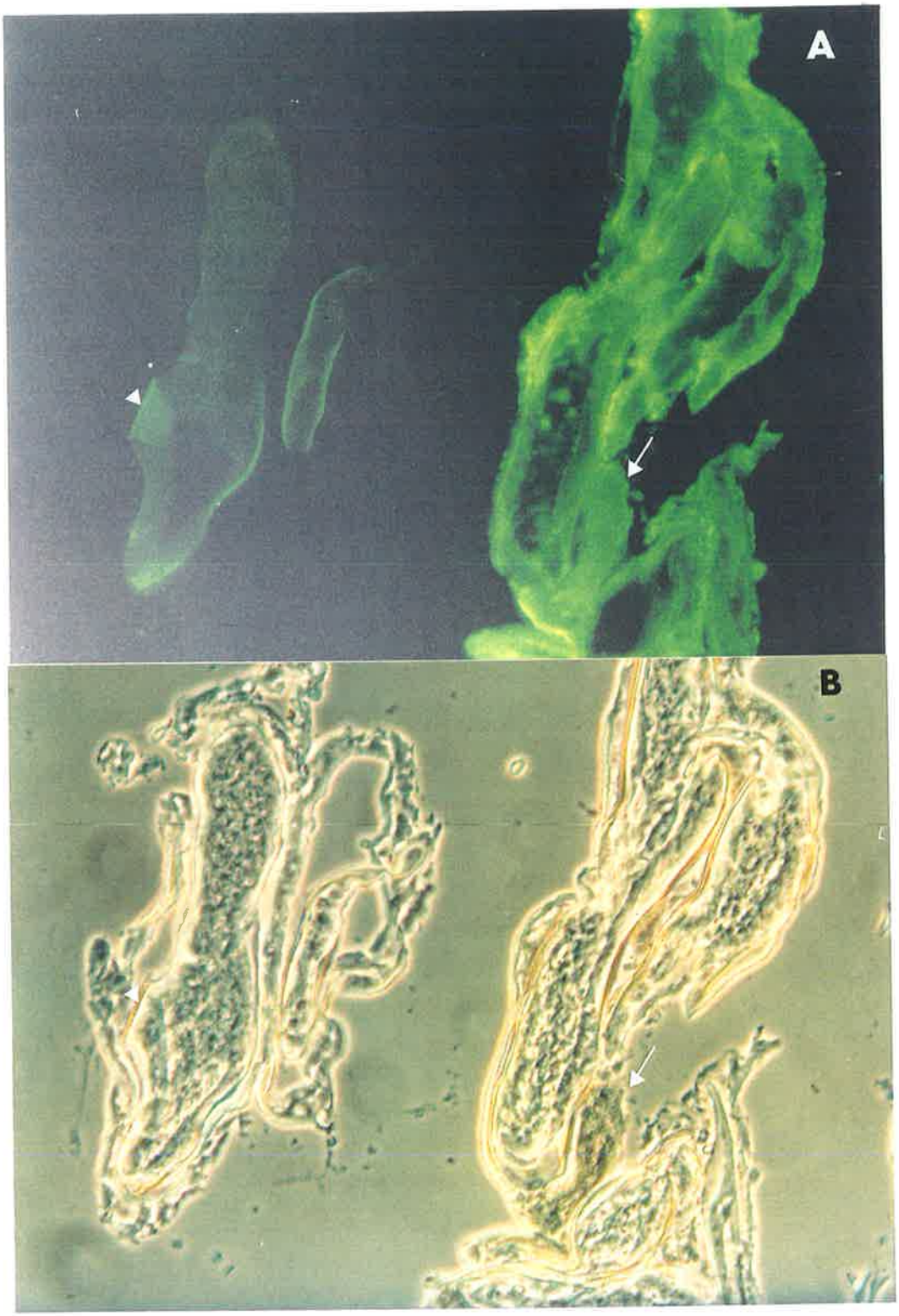
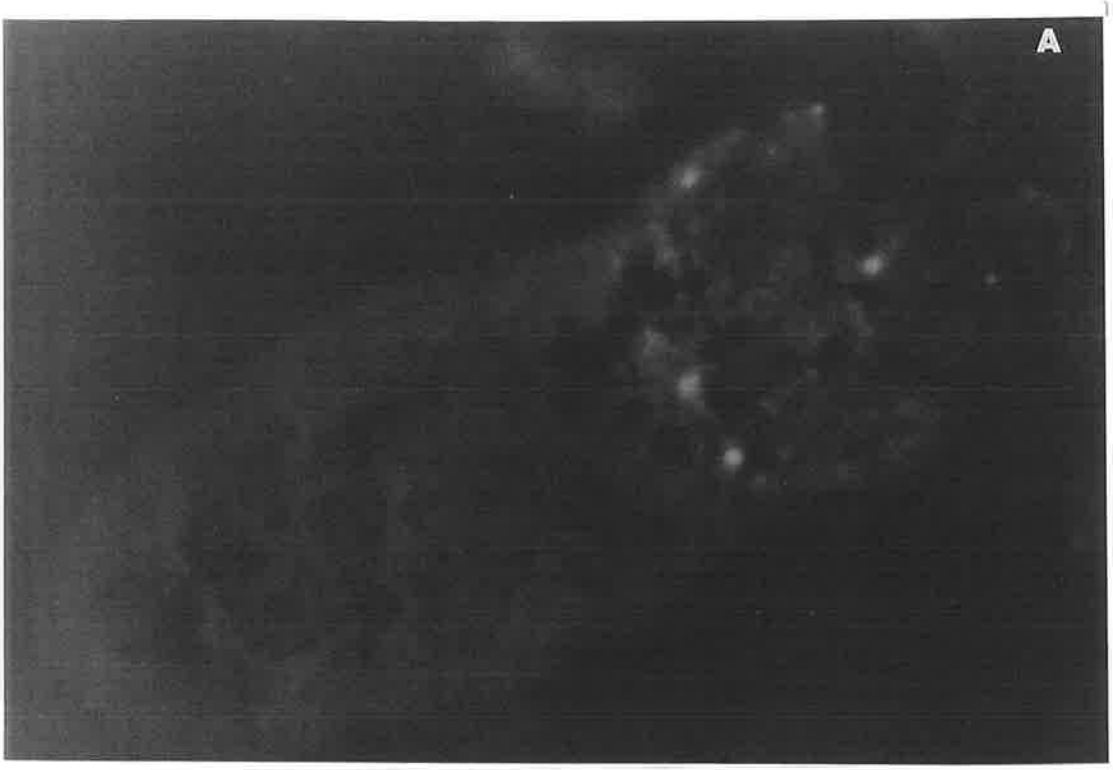

A
B

Fig. 11 Tissue section of a dissected ovary from the female wasp, *V. canescens*, treated with the preserum of a rabbit which was later immunised with a bacterial fusion protein encoded by the PHGPX-like domain of p40. The faint colour of the oocyte chorion (arrowhead) is also detected in non-treated sections under UV-light and is caused by auto-fluorescence. (B) The same section under phase contrast.


A

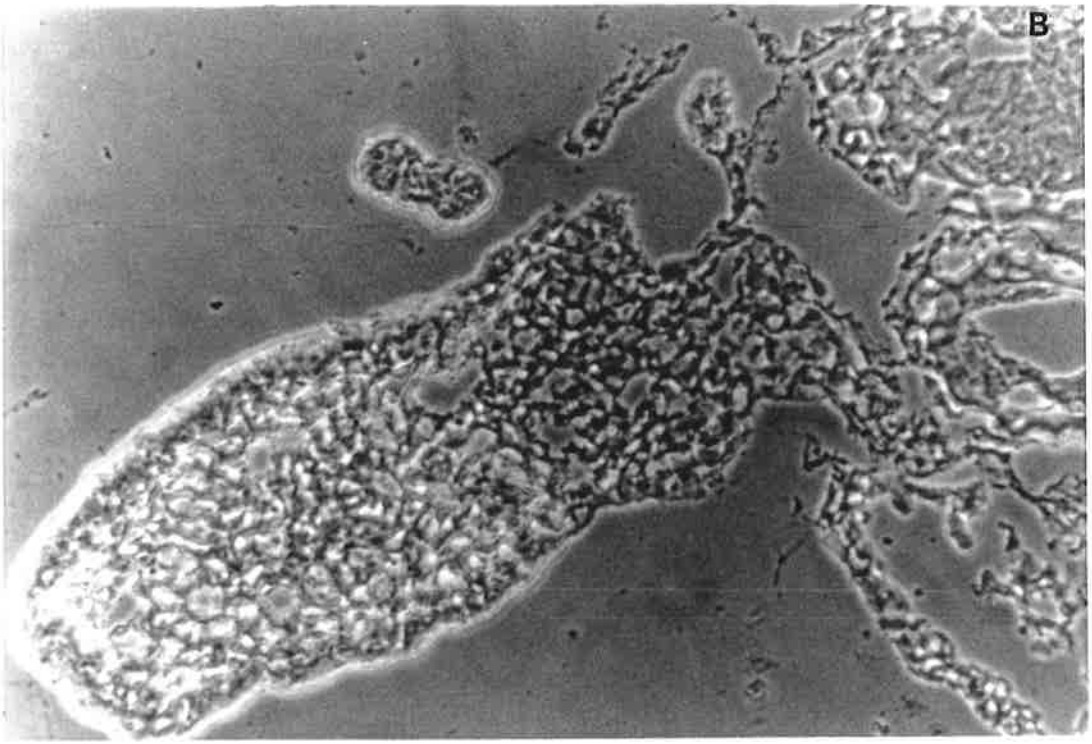
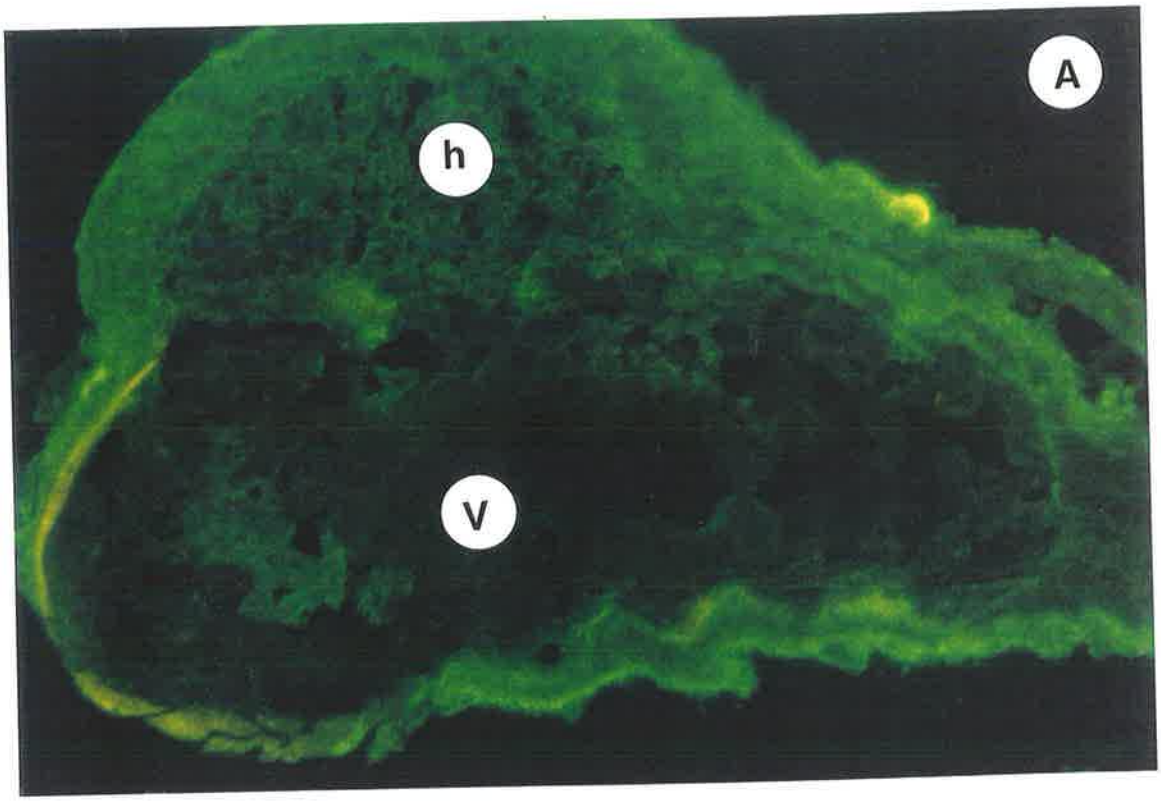

B

Fig. 12 Tissue section of an injured *V. canescens* larva (V) in the process of being encapsulated by hemocytes of the host, *E. kühniella*. The section was incubated with FITC-conjugated *H. p.* lectin and inspected under indirect UV-light. (A) Encapsulation started at the site of wounding. Note several layers of the hemocytes (h) in the capsule are clearly visible due to staining of hemocytes. (B) The same section under phase contrast.


A
h
V

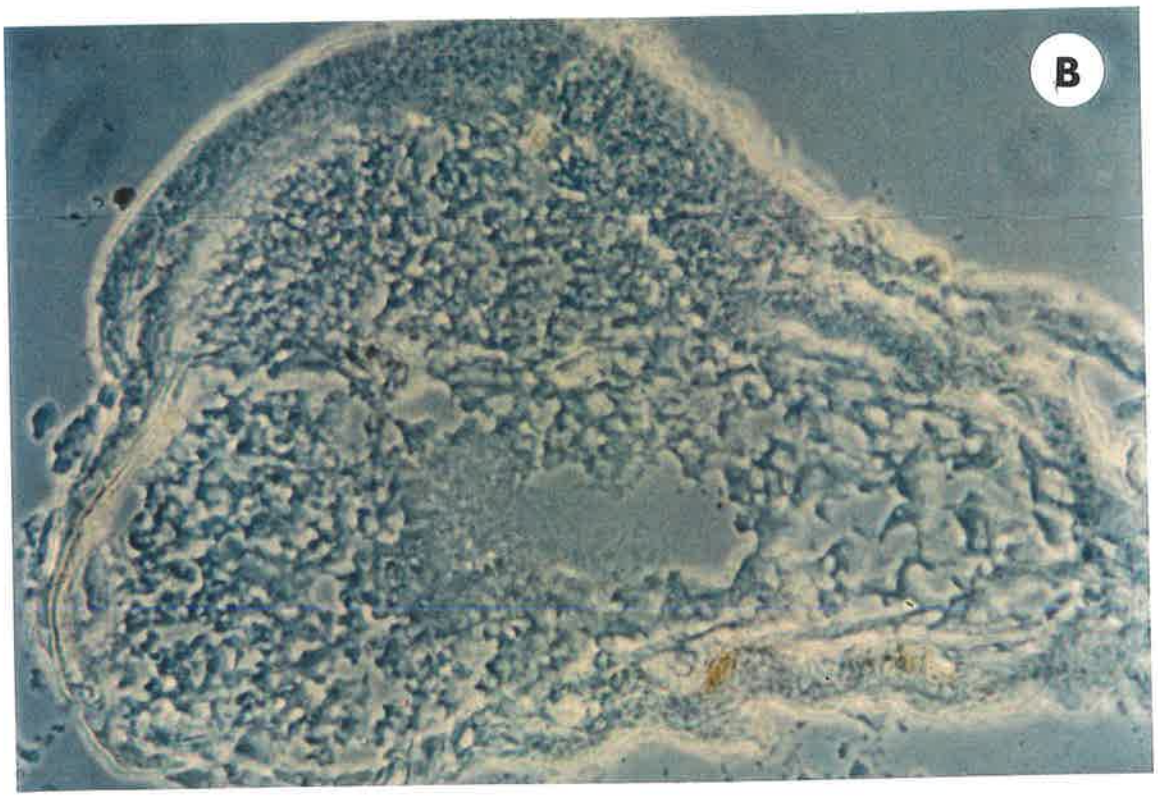
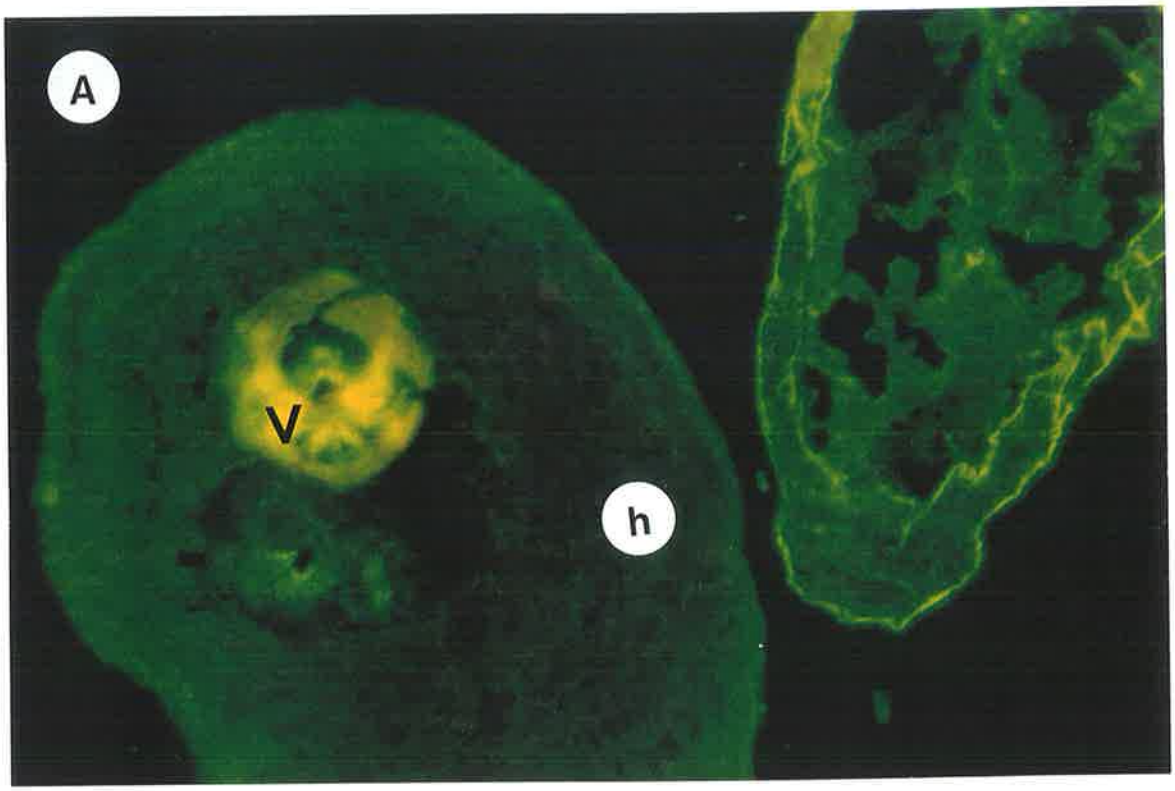

B

Fig. 13 Tissue section of a *V. canescens* larva fully encapsulated by the host, *E. kühniella,* caterpillar hemocytes. The sclerotised head capsule (V) shows the yellow colour of auto-florescence. (A) The section was incubated with FITC-conjugated *H. p.* lectin and inspected under indirect UV-light. Note the layers of the hemocytes (h) in the capsule are visible and labelled with the antisera. No intensive labelling is detected on the surface of the capsule, whereas the larval cuticle is labelled on the inside and outside (arrow). (B) The same section under phase contrast.


A
V
h

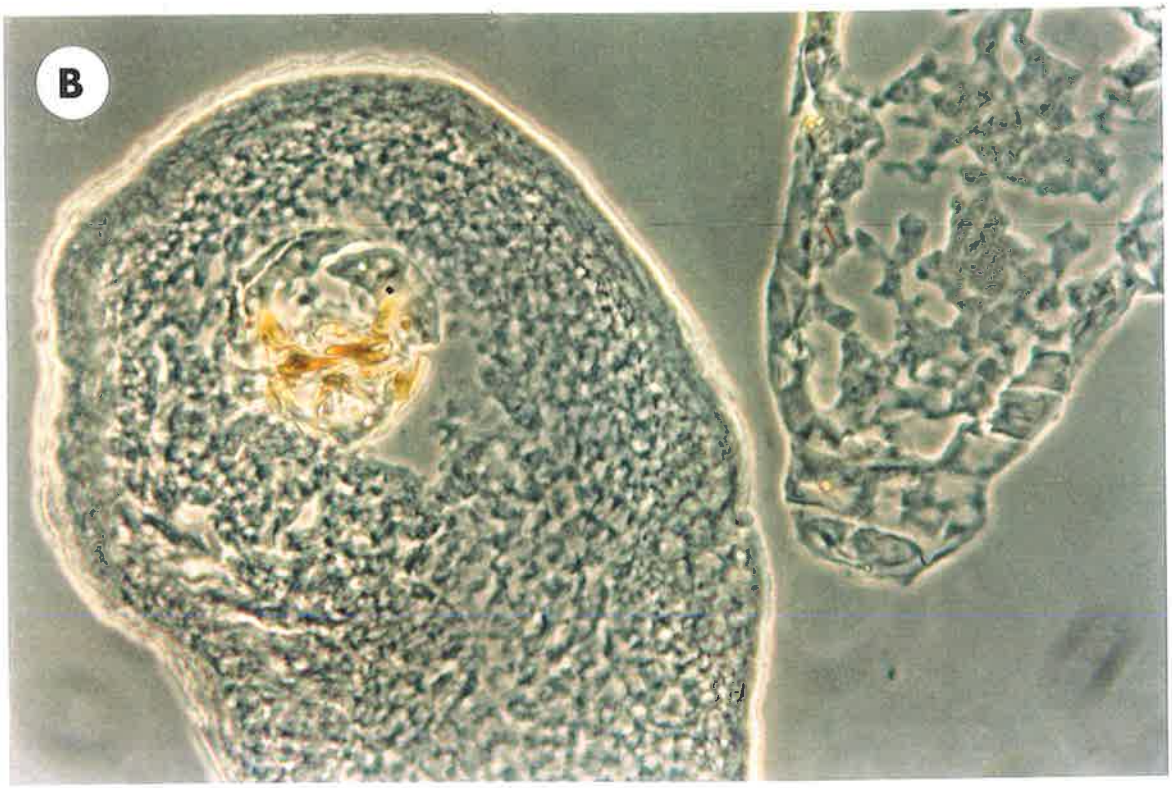

B

Fig. 14 Tissue section of a *V. canescens* larva shortly after emergence from the egg shell, incubated with FITC-conjugated *H. p* lectin. Note the labelling is visible on the yet unsclerotised surface of the larval cuticle. Damage of the epidermis (arrow) is probably due to fighting of larvae in super parasitised caterpillars.

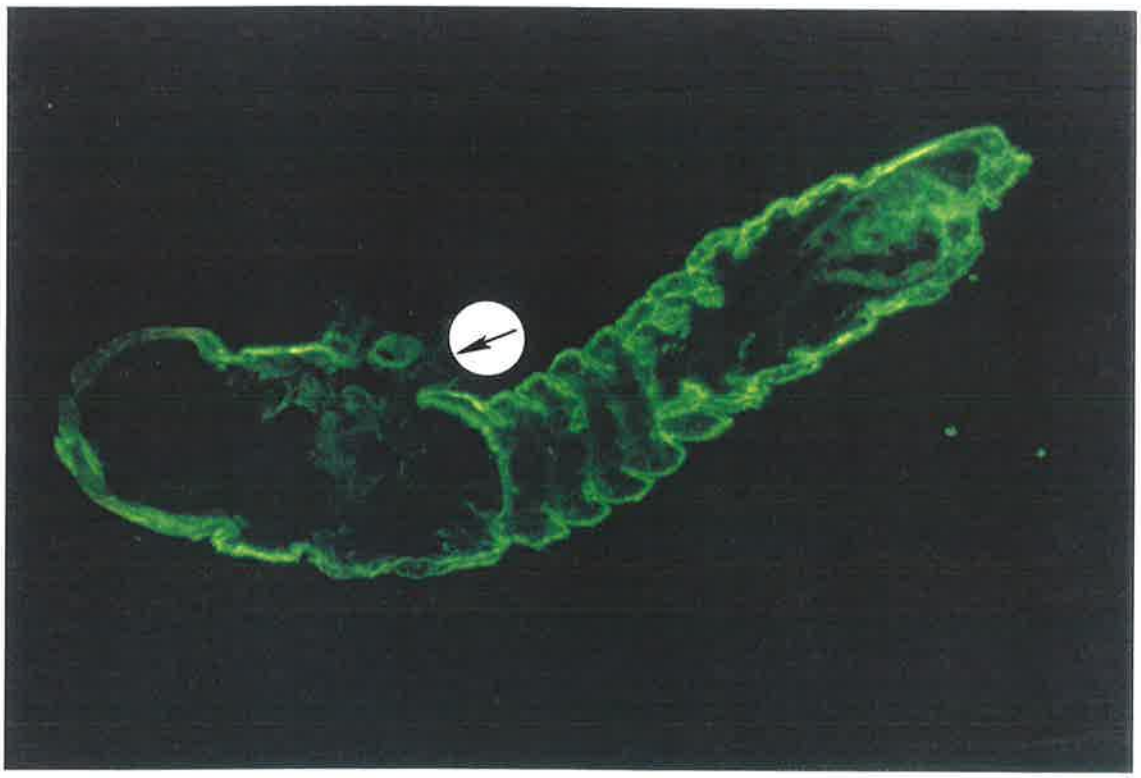


Fig. 15 Hemocytes from a parasitised *E. kühniella* caterpillar allowed to attach to the surface of a glass slide. (A) Hemocytes were fixed, incubated with anti-VLP antiserum and visualised with FITC-conjugated secondary antibodies under indirect UV-light. Hemocytes are morphologically similar to hemocytes from unparasitised caterpillars and appear to be functionally intact since they are forming occasional clumps of cells which consist mainly of granulocytes (arrow). (B) The same specimen under phase contrast.

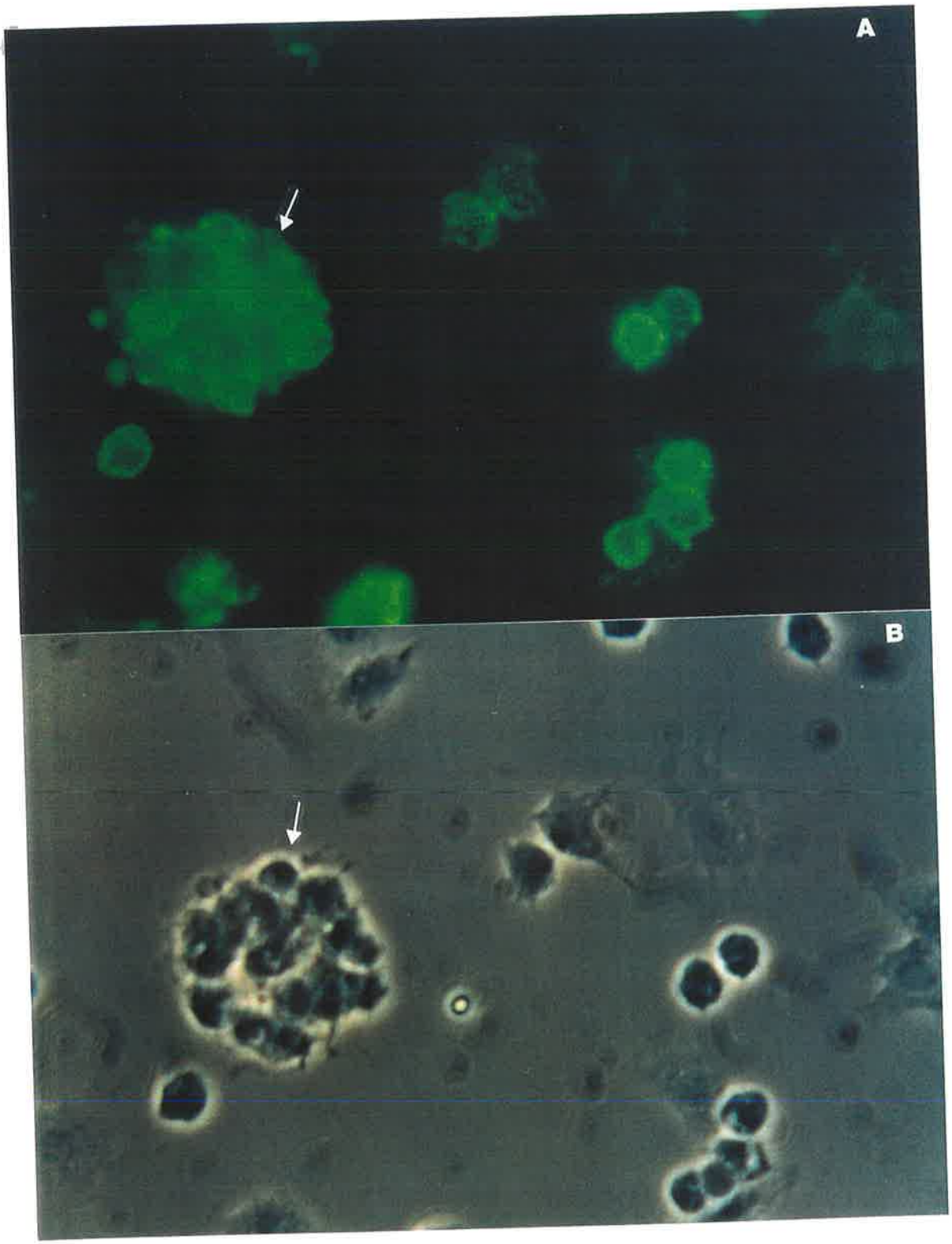
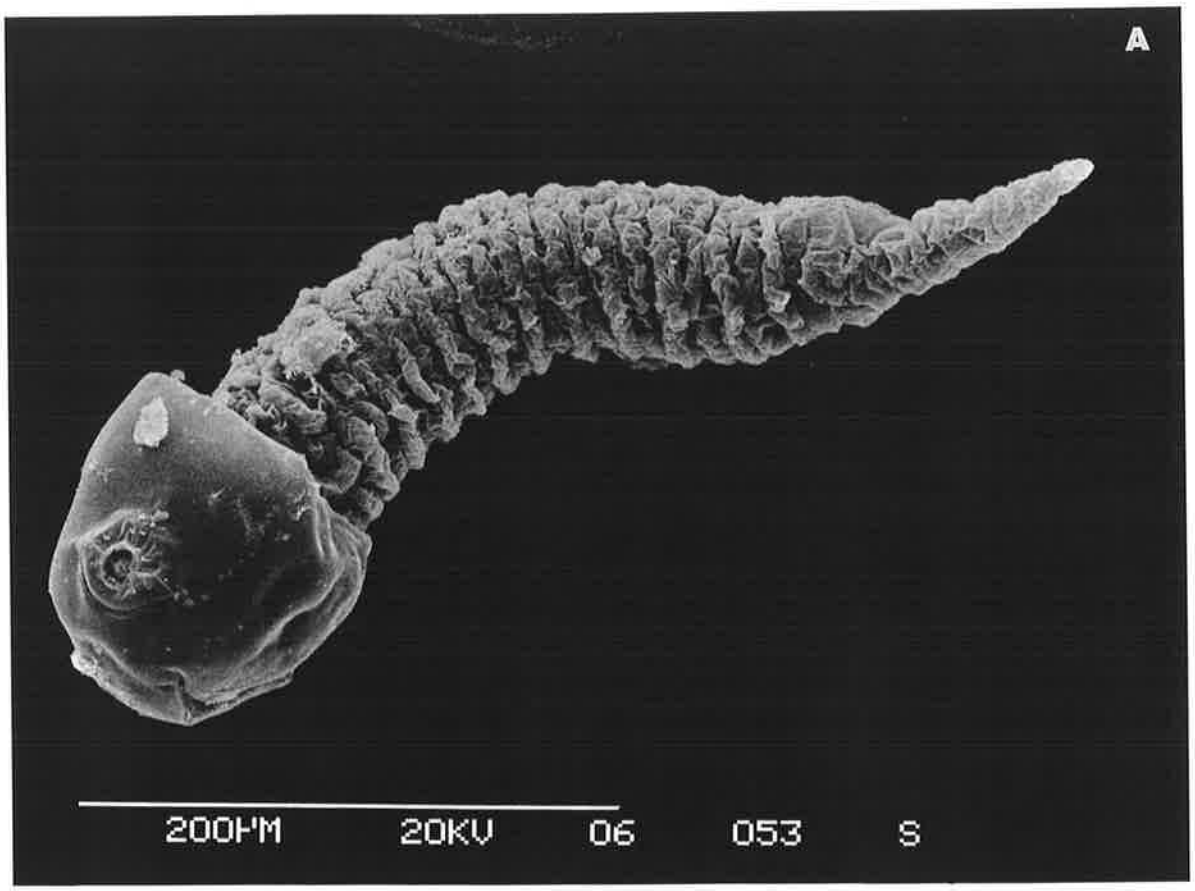

A
B

Fig. 16 (A) Scanning electron micrograph of a *V. canescens* larva. 1 cm = 28,2 µm. (B) Enlargement of the larval surface showing scanty VLPs attached to the cuticular folds. The VLPs are not uniformly located on the cuticle and their presence could possibly be a contamination by the chorion during hatching. 1cm = 56.2 nm.


A
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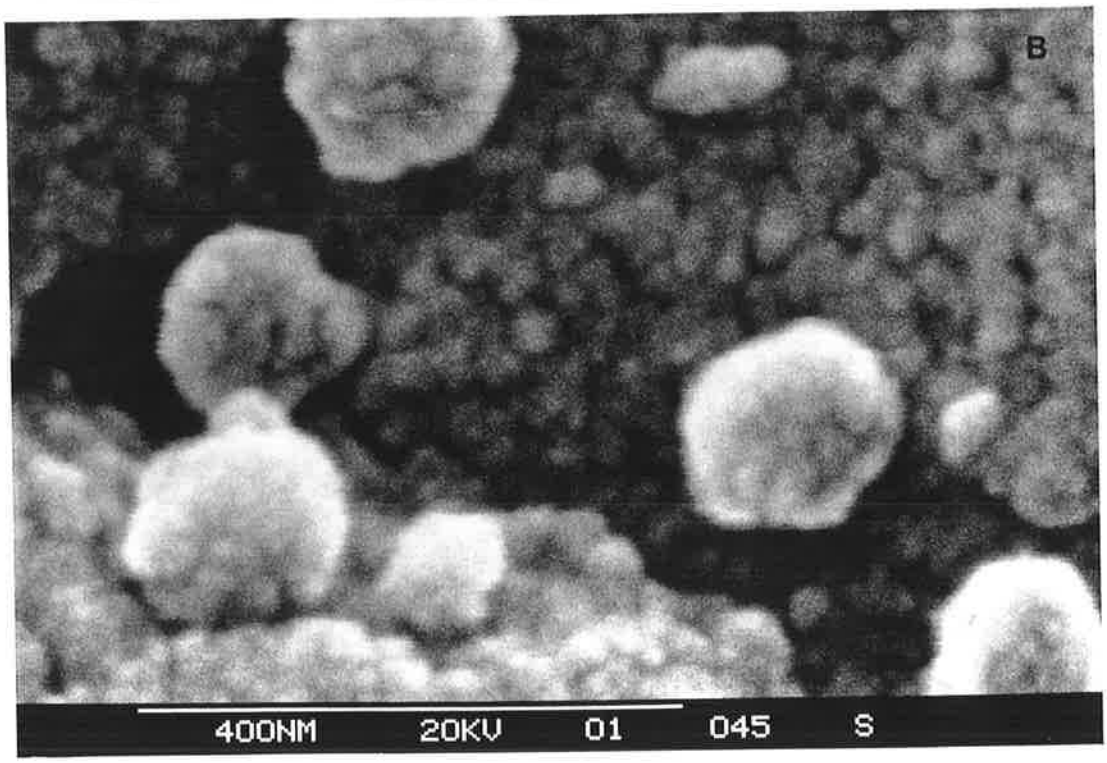

B
400NM 20KV 01 045 S

Fig. 17 Egg chorion of *V. canescens* and late embryo surface properties. The egg and larva separated from its egg shell were incubated with anti-VLP antibodies and visualised with FITC-conjugated secondary antibody under indirect UV-light. a) Intact egg surface showing cross-reaction with anti-VLPs antibodies. Early larva (c) teased out of chorion. In the case where the chorion is still attached to the embryo (e) labelling is only visible on the chorion. The same specimen (b), (d) and (f), respectively, under phase contrast.

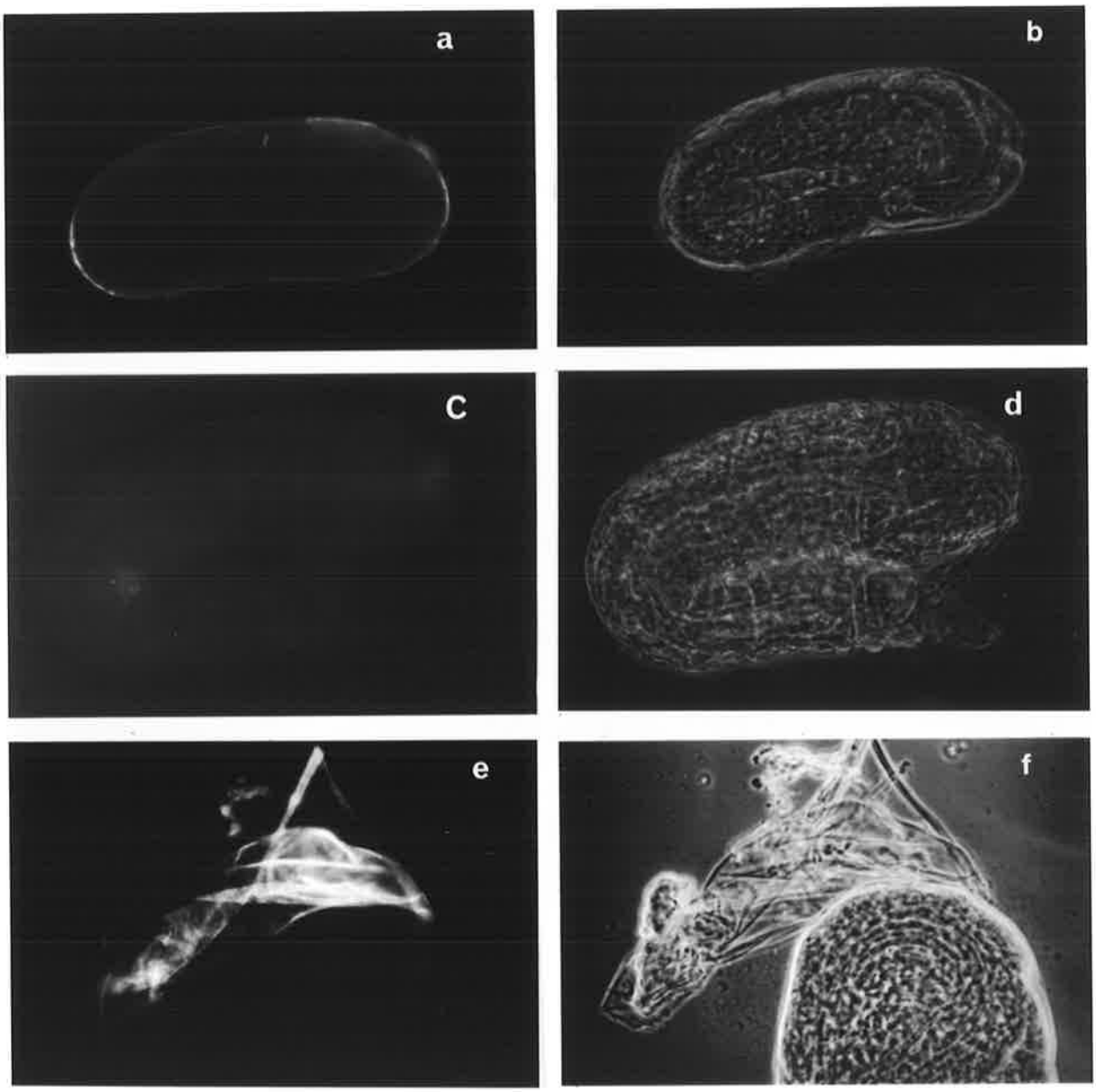

a
b
C
d
e
f

Fig. 18 *V. canescens* larva ready to hatch was separated from the chorion and incubated with anti-VLP antibodies. (a) Bound antibodies were visualised with FITC conjugated secondary antibodies. At this stage, the larva clearly shows a cross-reaction to the anti-VLP antibodies particularly at the segment folds. Note the labelled piece of chorion still attached to the larva (arrow). (b) The same specimen under phase contrast.

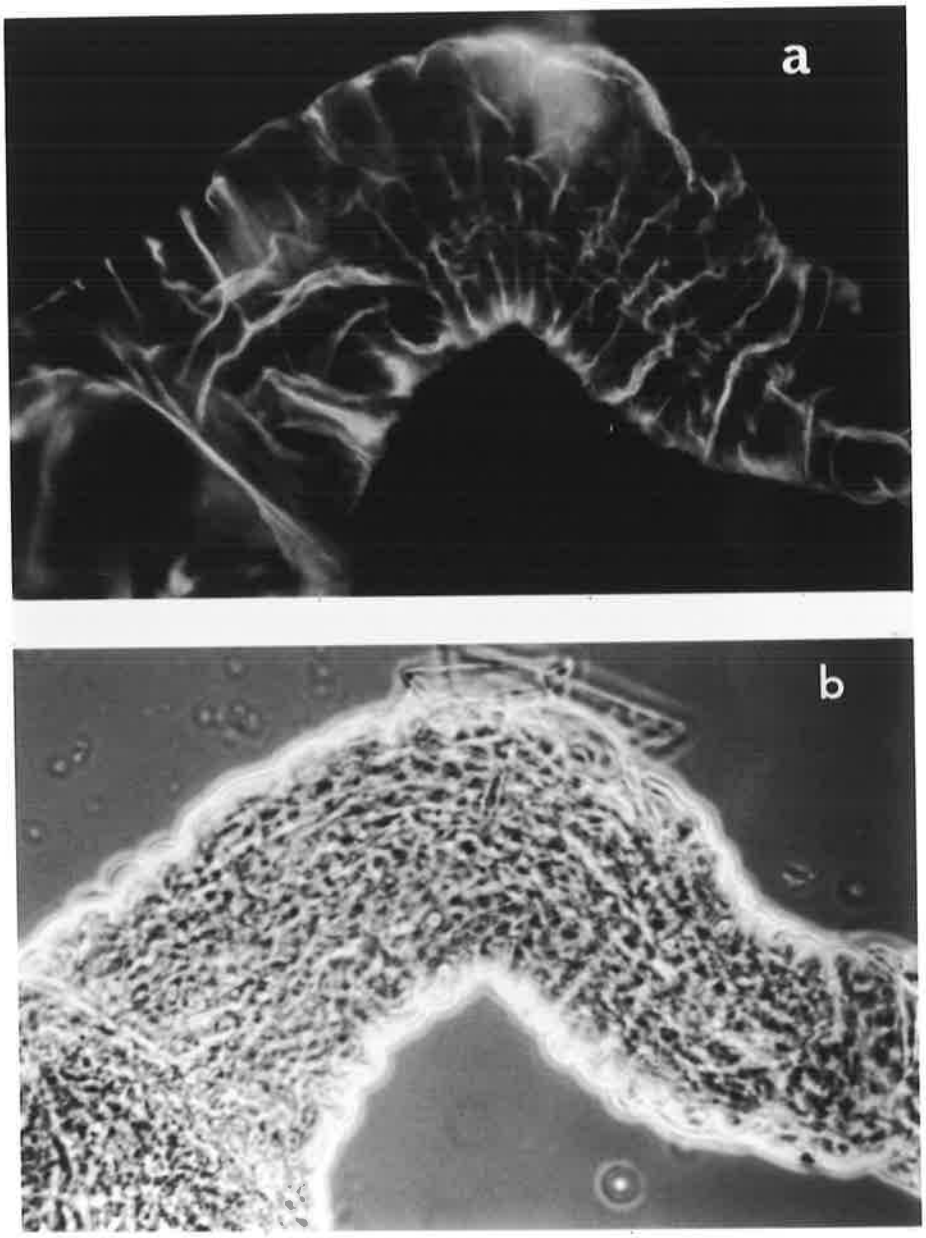

a
b

Fig. 19 *V. canescens* larva ready to hatch was teased from the chorion and treated with antibodies against a bacterial fusion protein encoded by the PHGPX-like domain of p40. (A) Bound antibodies were visualised with FITC conjugated secondary antibodies. The larva shows much less staining than the chorion. The yellow colour is probably due to auto-fluorescence as seen in the head capsule. (B) The same specimen under phase contrast.

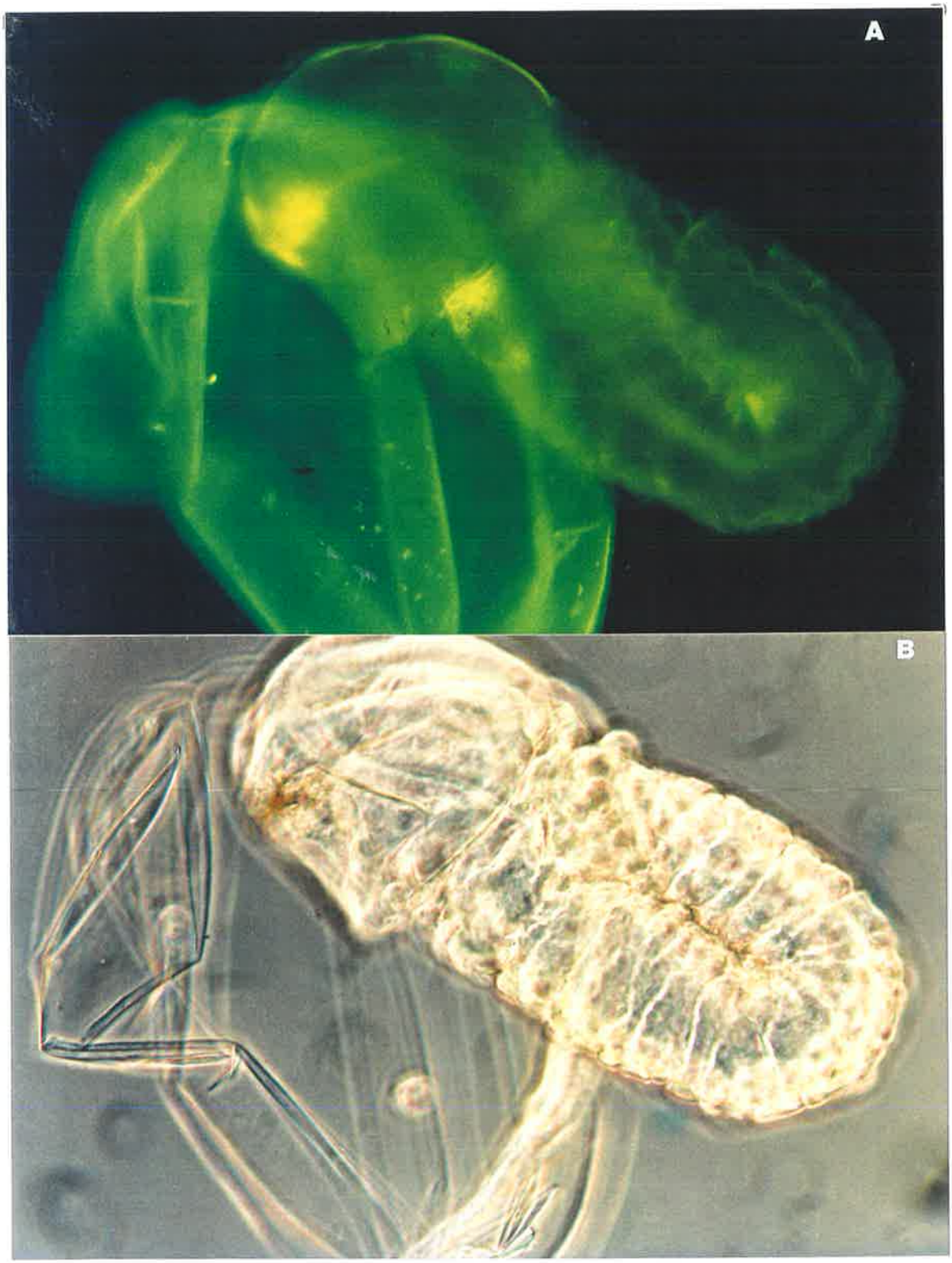

A
B

Fig. 20 *V. canescens* larva treated with antibodies against a bacterial fusion protein encoded by the PHGPX-like domain of p40. (A) Bound antibodies were visualised with FITC conjugated secondary antibodies. The larva is partially encapsulated and probably dying, nevertheless it is still stained with the antiserum. The bright yellow colour is due to auto-fluorescence of sclerotinised or melanised tissue of the head capsule and of encapsulation material. (B) The same specimen under phase contrast.

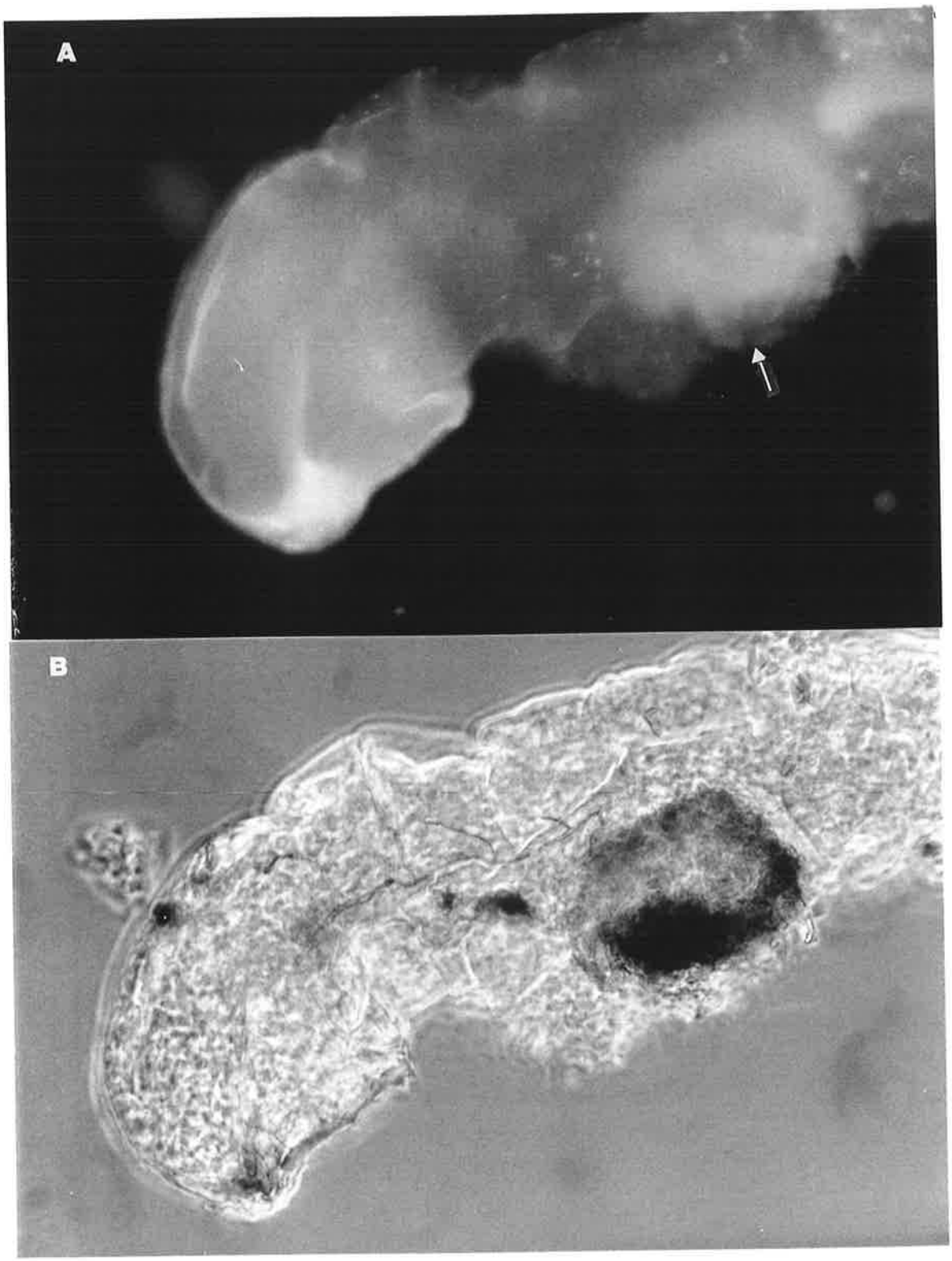
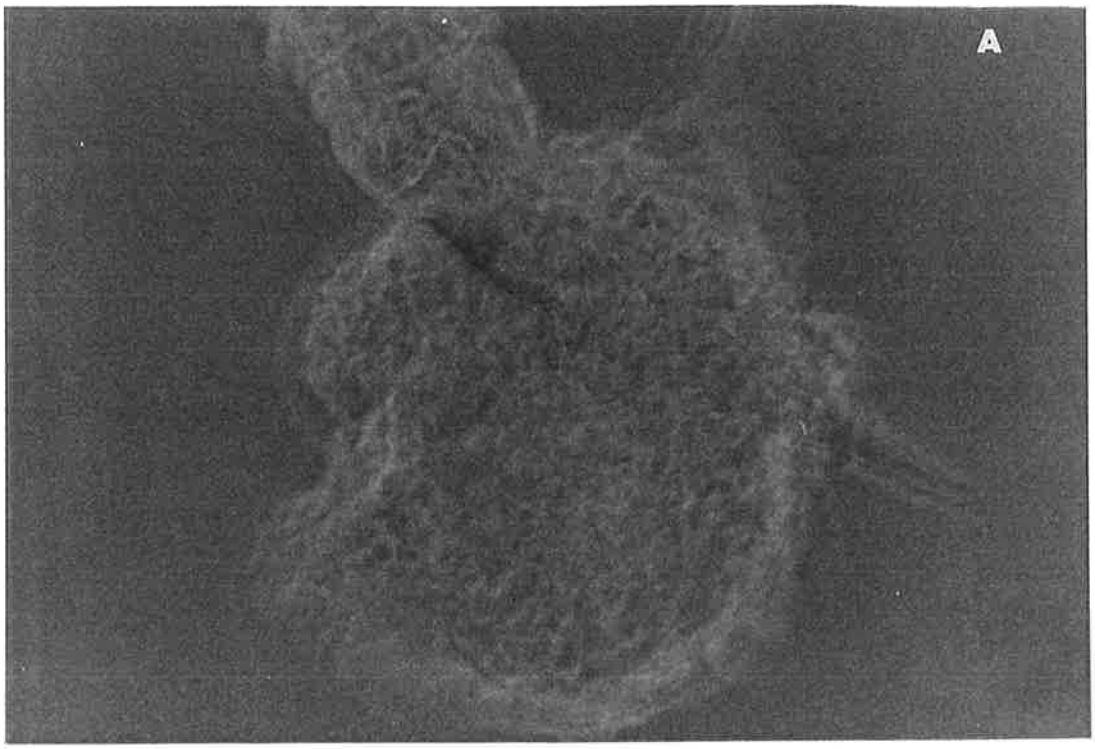

A
B

Fig. 21 *V. canescens* larva treated with preserum of a rabbit which was later immunised with a bacterial fusion protein encoded by the PHGPX-like domain of p40. (A) The larva is partially encapsulated. There is no labelling with the preserum. (B) The same specimen under phase contrast.


A

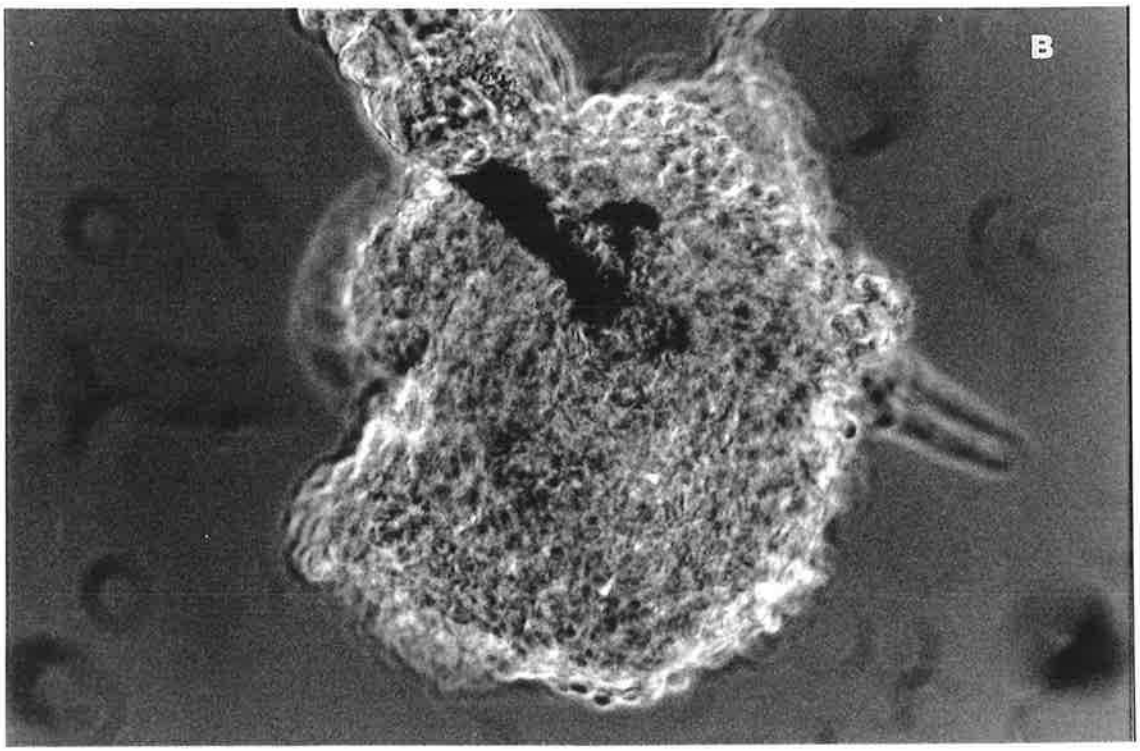

B

Fig. 22 Tissue section of a late *V. canescens* larva incubated with antibodies against a bacterial fusion protein encoded by the PHGPX-like domain of p40. (A) Bound antibodies were visualised with FITC conjugated secondary antibodies. The section show a silk gland tissue and the silk duct, (s). Both the silk gland cells and the duct contents are labelled by the antiserum. In preserum-treated sections no labelling is seen in gland tissue. (B) The same section under phase contrast show slight auto-fluorescence over the oviduct content, which is also detected in non-treated sections.

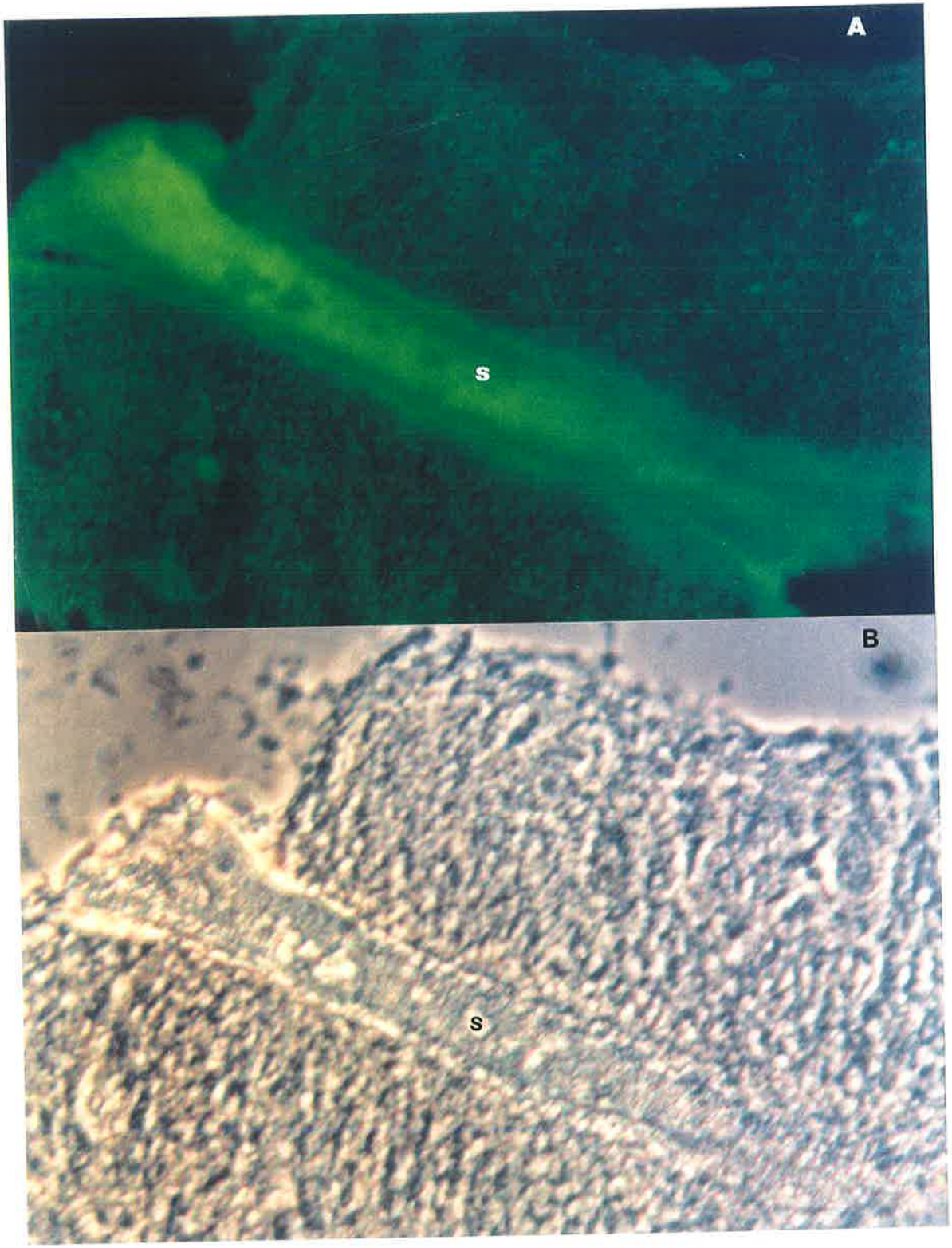
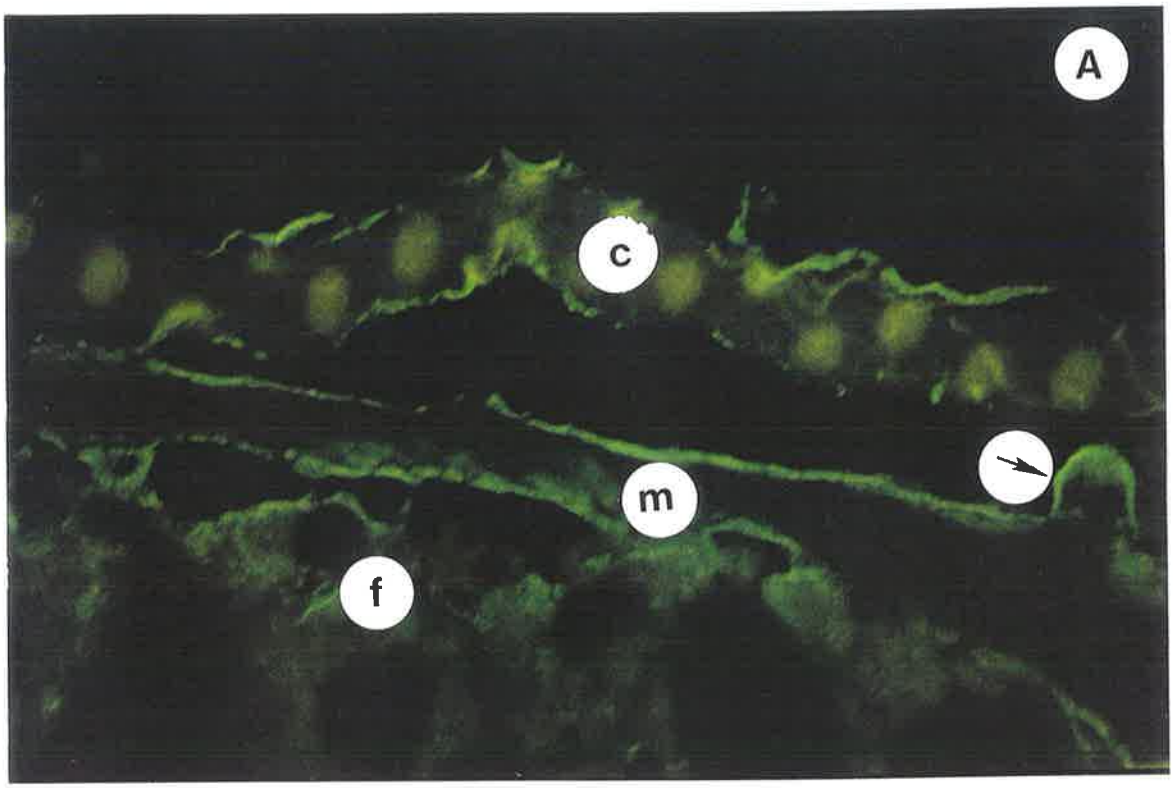

A
S
B
S

Fig. 23 Tissue section of a *V. canescens* larva shortly after leaving the host caterpillar. The section was incubated with antibodies against purified VLPs. (A) Bound antibodies were visualised with FITC-conjugated secondary antibodies. The cuticle (c) is in the process of sclerotisation (yellow dots). Note; the hemocytes (arrow) attached to the muscles (m) cross-react with the anti-VLP antisera, and the crystalline fat body cells (f) are not labelled. (B) The same section under phase contrast.


A
c
m
f

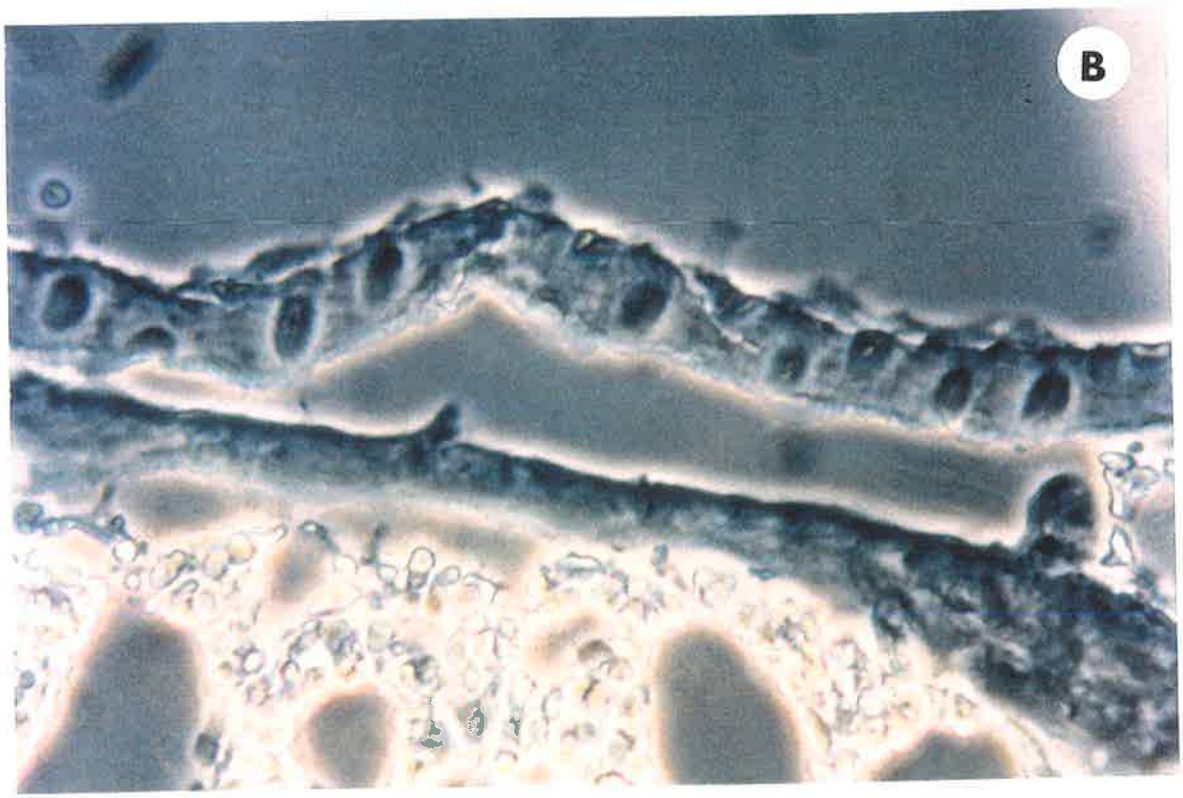
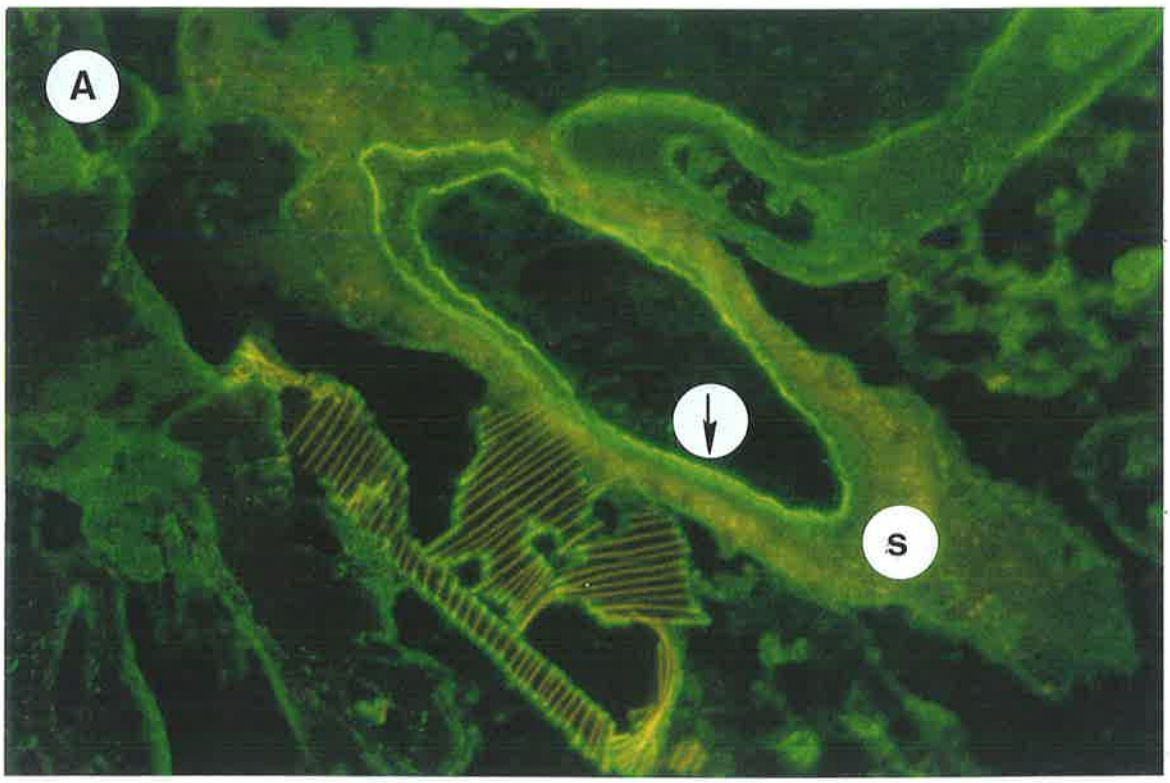

B

Fig. 24 Tissue section of *E. kühniella* caterpillar incubated with anti-hemolymph antiserum. (A) Bound antibodies were visualised with FITC-conjugated secondary antibodies. Basement membranes of tissues and of tracheal structures are labelled. Note that the lining (arrow) of the silk gland (s) lumen is strongly labelled. (B) The same section under phase contrast.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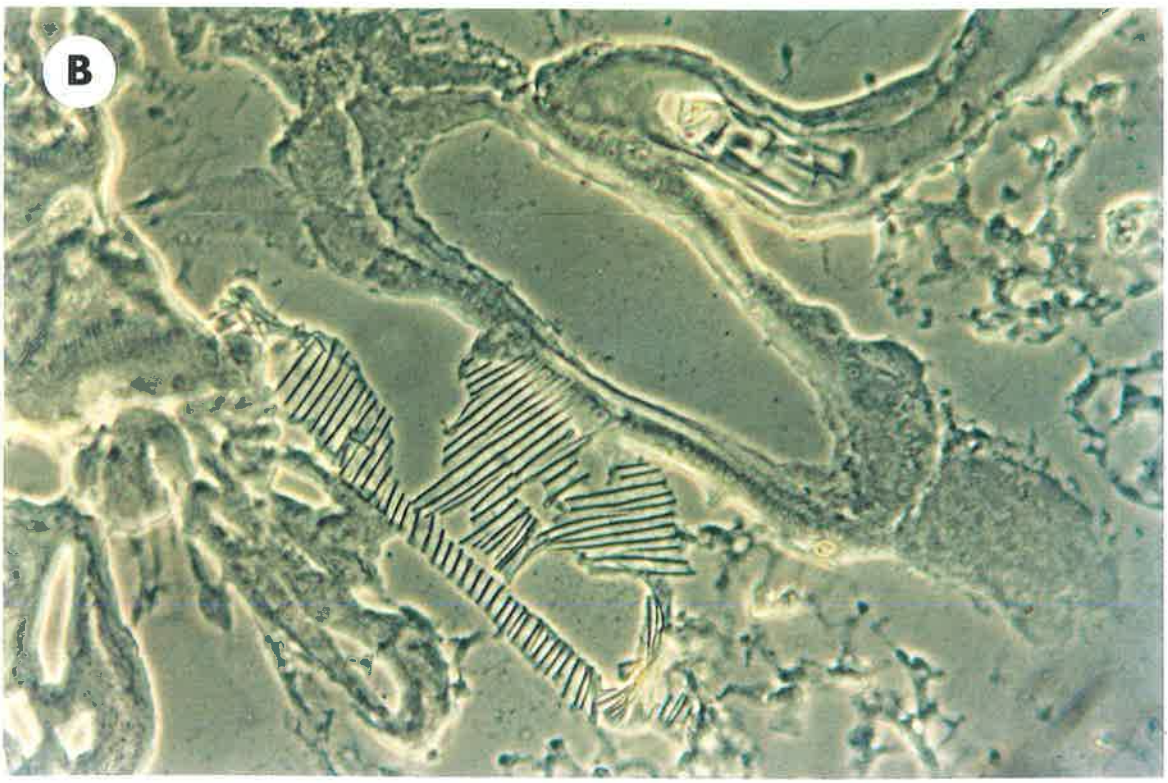

B

Fig. 25 *V. canescens* embryo inside the chorion. The embryo was incubated with anti-hemolymph antiserum. (A) Bound antibodies were visualised with FITC-conjugated secondary antibodies. Both the chorion (arrowhead) and the larval cuticle inside the chorion (arrow) are labelled, indicating that the antiserum is able to reach antigens inside the chorion possibly through the micropyle (blank arrow). (B) The same specimen under phase contrast.

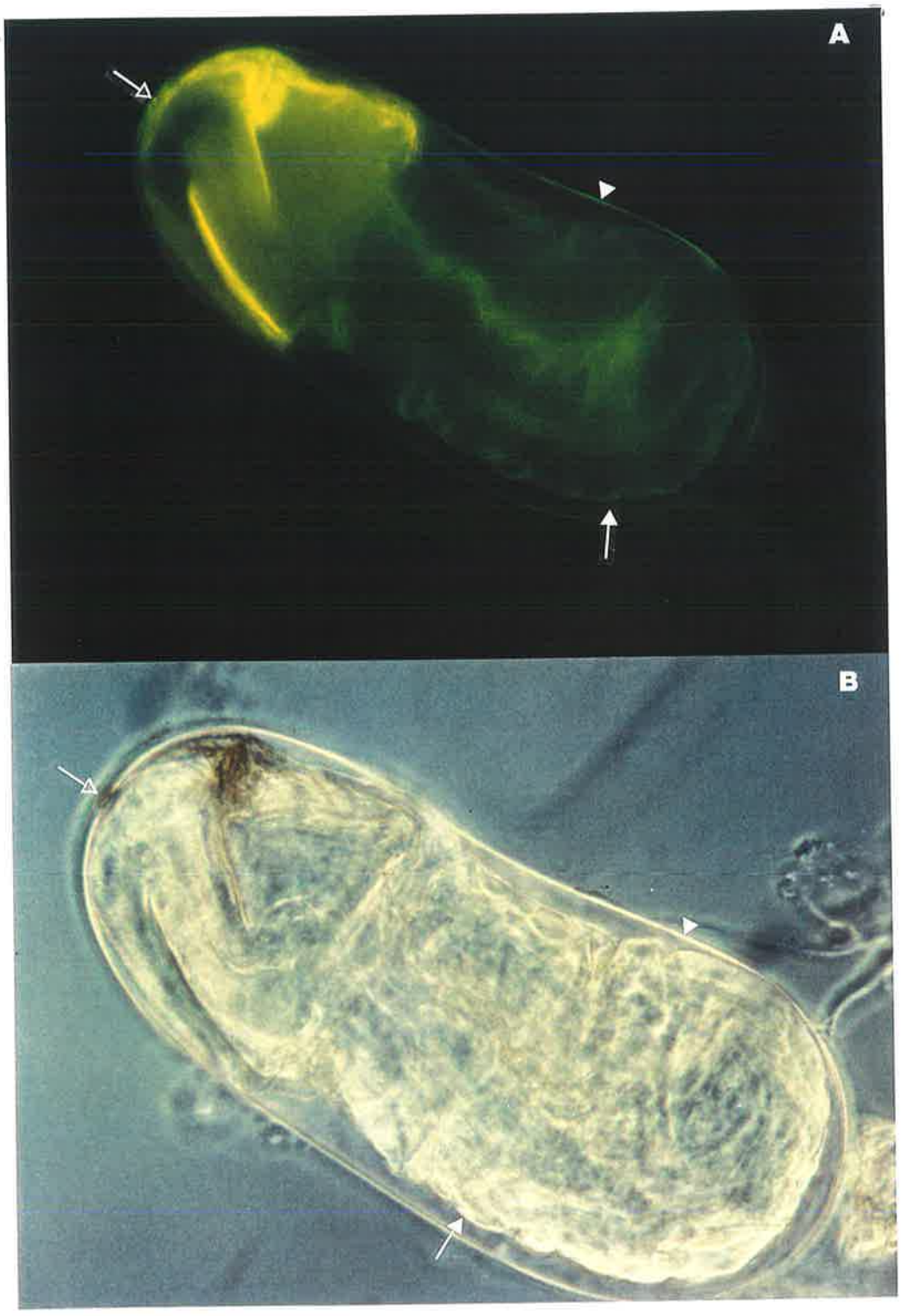
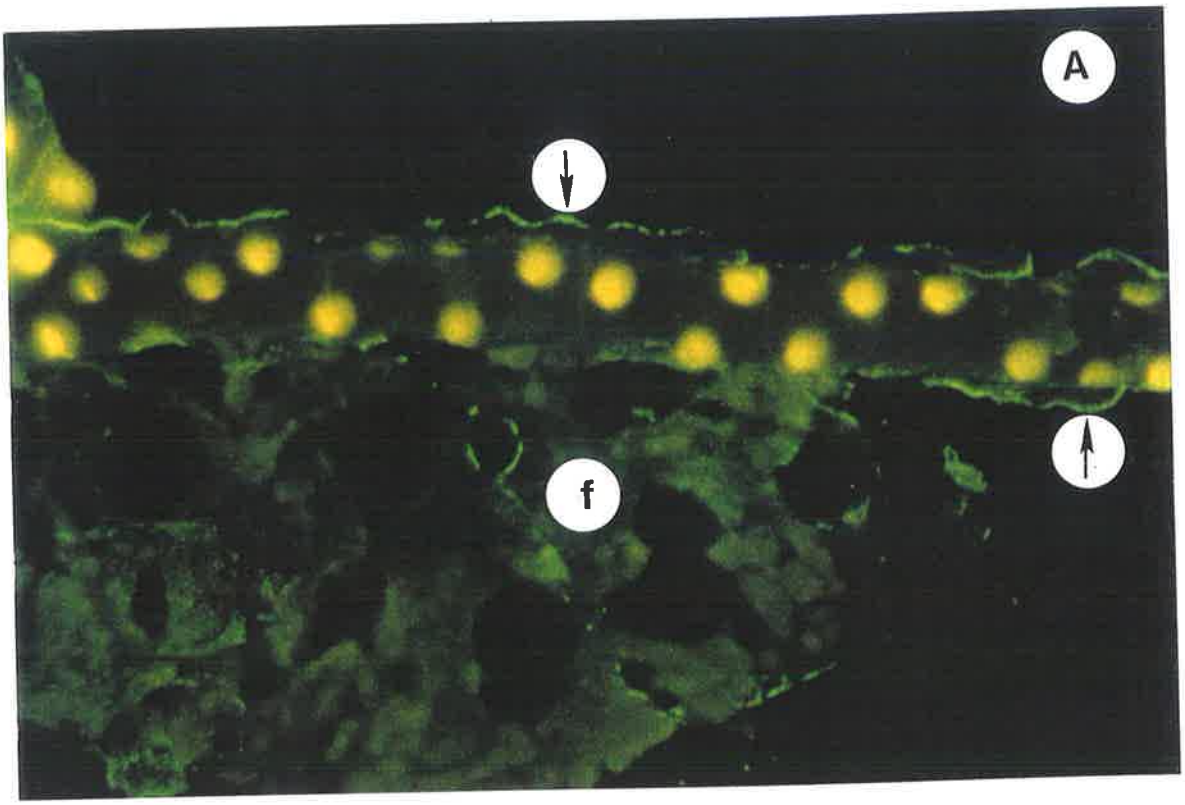

A
B

Fig. 26 Tissue section of a *V. canescens* larva shortly after leaving the host caterpillar. (A) The cuticle is in the process of sclerotisation (yellow dots). The tissue was incubated with anti-VLP antibodies and bound antibodies were visualised with FITC-conjugated secondary antibodies. Note the thin layer of labelling inside and outside of the cuticle (arrows), and fat bodies (f) showing some auto-florescence at the basement membrane. Some of the labelled basement membranes around the fat body are in the process of dissolution. The yellow dots inside the cuticle are due to auto-florescence, probably caused by sclerotisation foci. (B) The same section under phase contrast.


A
f

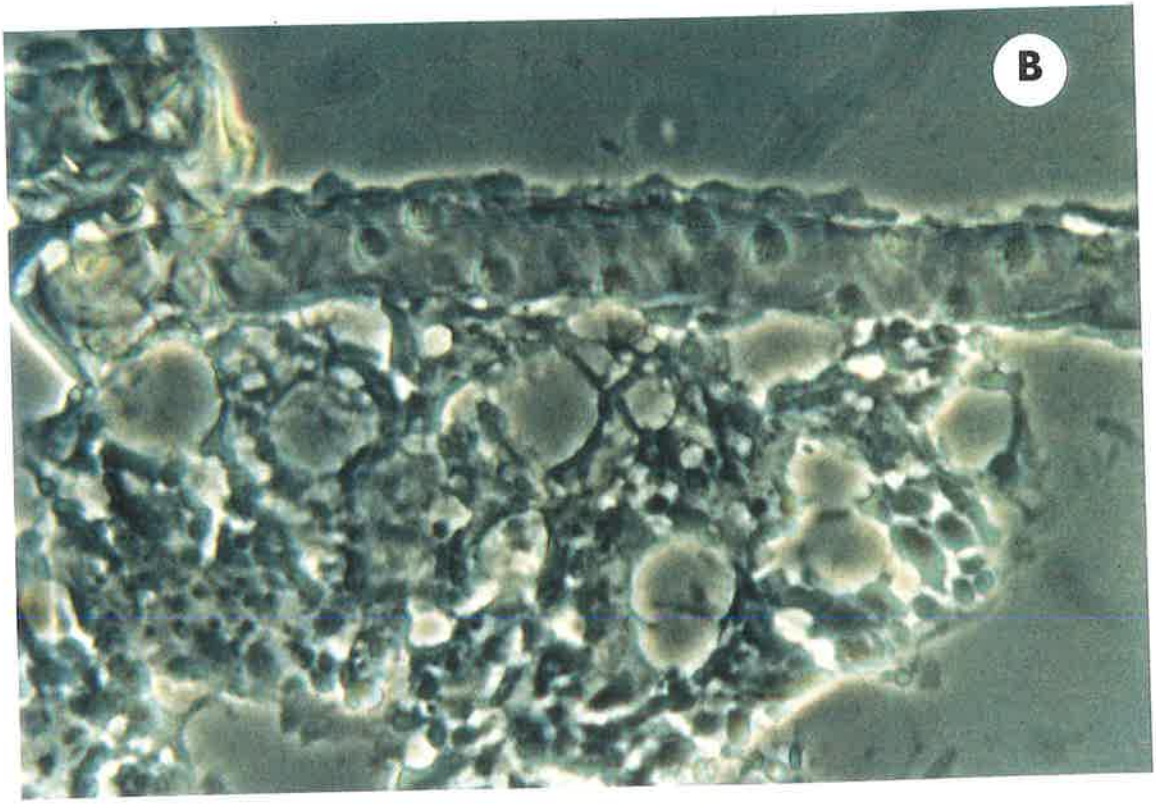
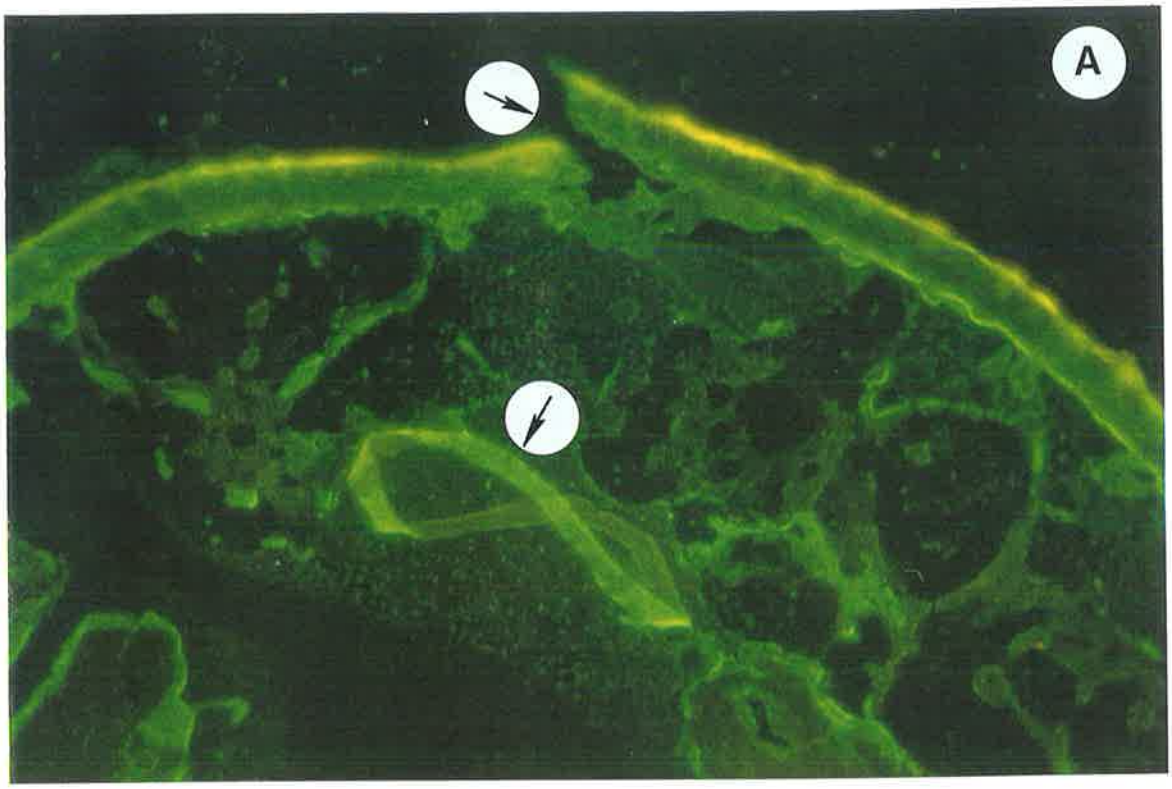

B

Fig. 27 Tissue section of *E. kühniella* caterpillar after parasitation treated with anti-VLP antibodies. (A) Bound antibodies were visualised with FITC-conjugated secondary antibodies. A possible empty chorion structure (lower arrow) can be seen inside the caterpillar hemocoel. The oviposition site (upper arrow) on the cuticle of the caterpillar is still visible. (B) The same section under phase contrast.


A

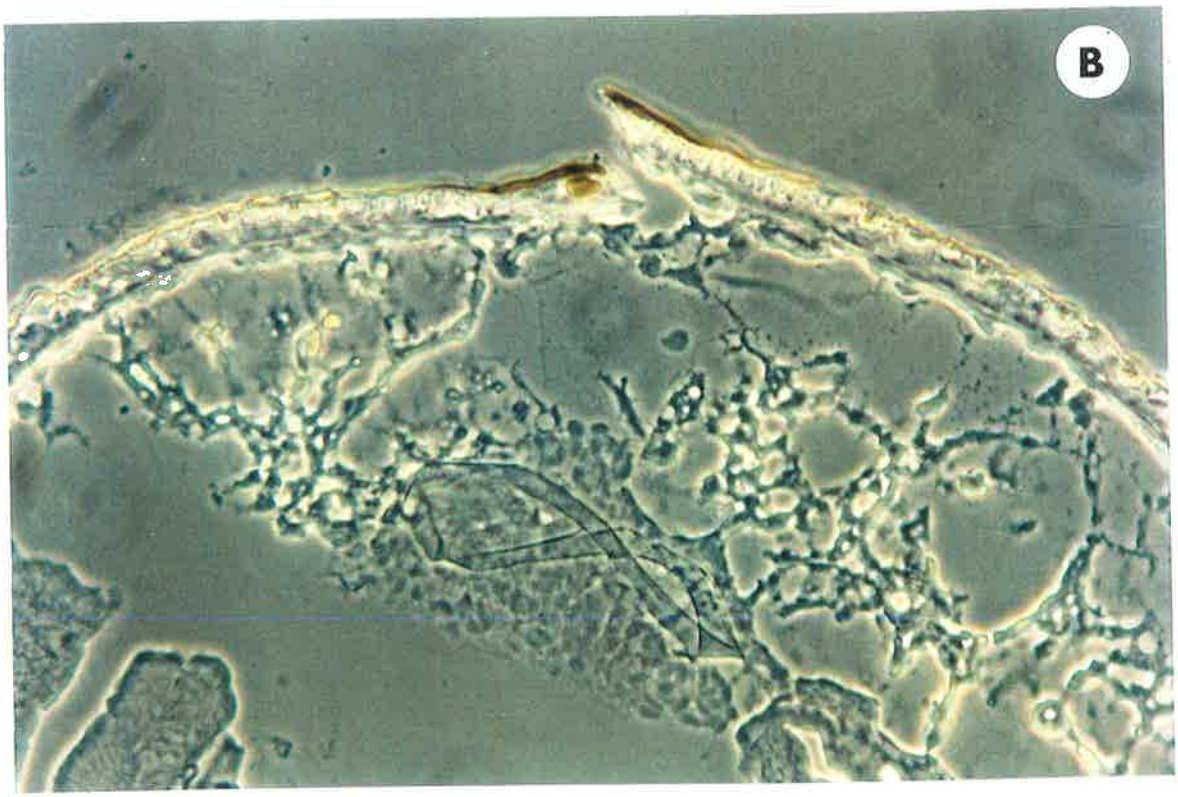
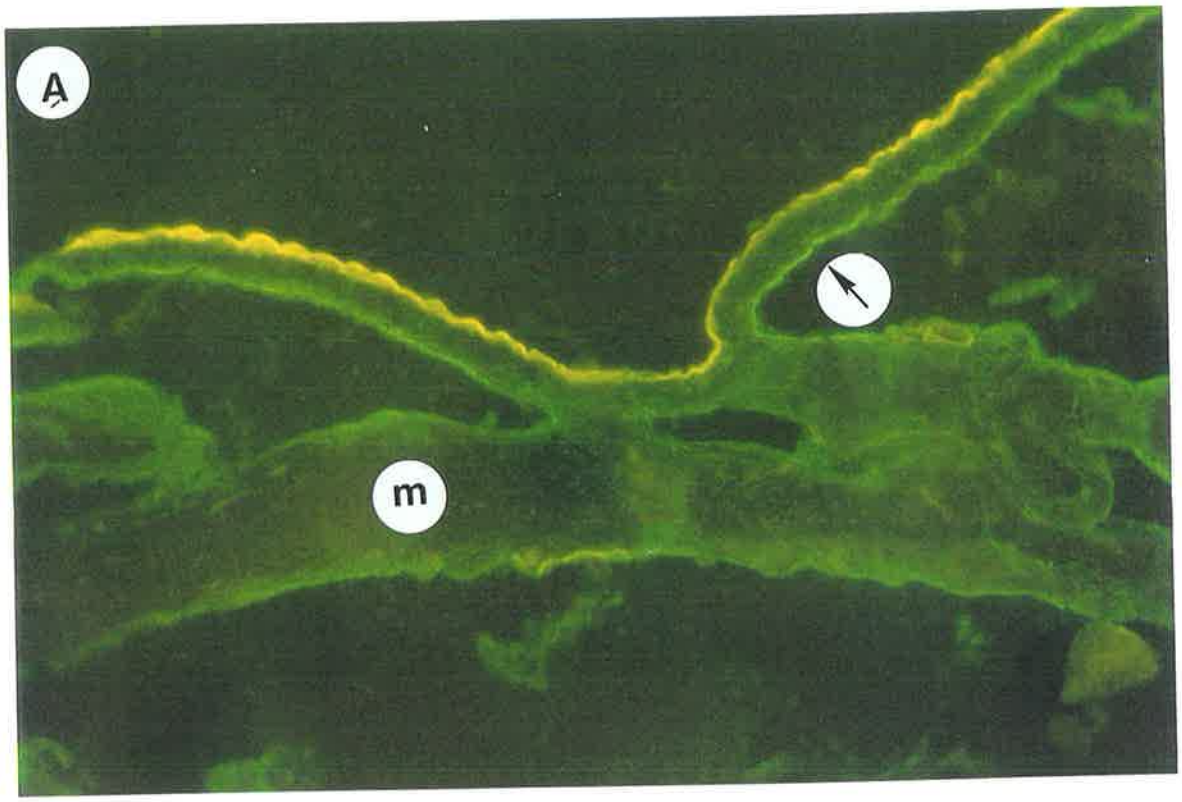

B

Fig. 28 Tissue sections of *E. kühniella* caterpillar incubated with anti-*E. kühniella* hemolymph antibodies. (A) Bound antibodies were visualised with FITC-conjugated secondary antibodies. The basement membranes of muscles (m) that are exposed to the hemolymph are labelled. The labelling on the muscle and epidermis attachment site follows the lining exposed to the hemolymph (arrow), suggesting that the labelled component is not a structural part of the basal membrane but an addition on the surface of the basal membrane at a later stage. (B) The same section under phase contrast.


A
m

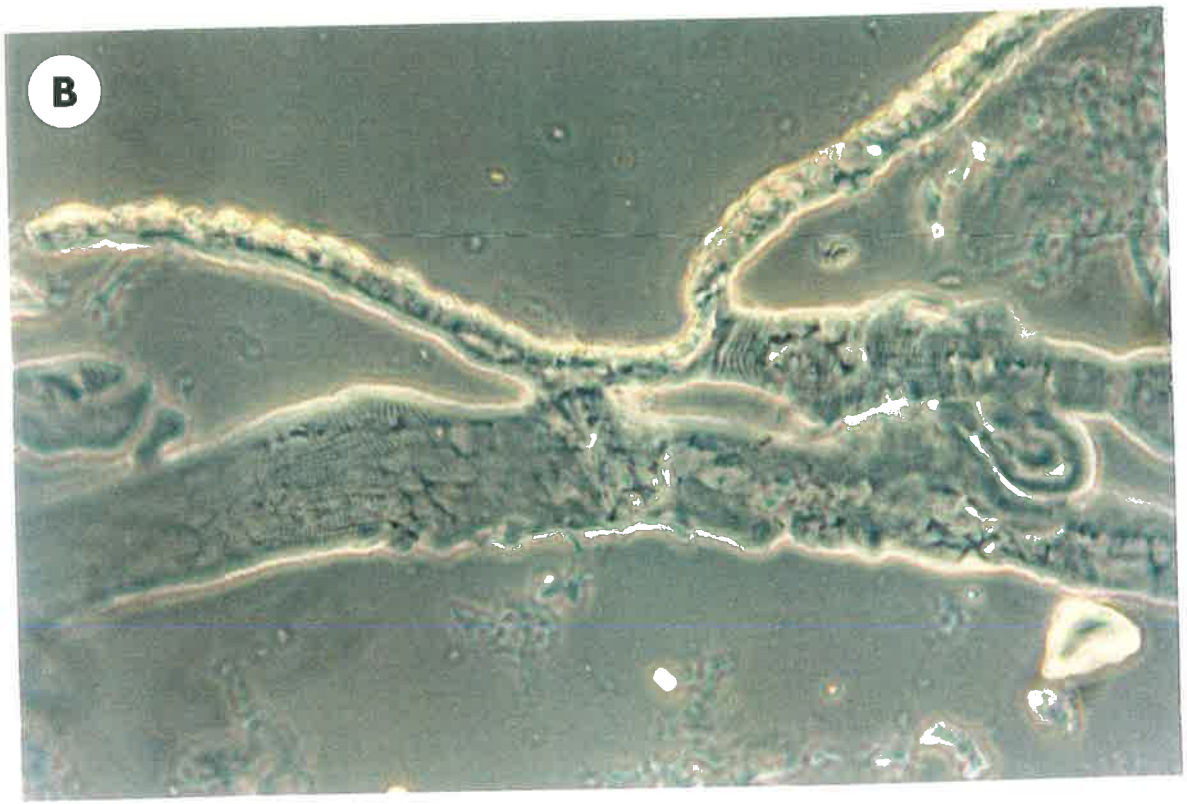

B

Fig. 29 Protein extracts from fat body of bacteria-infected (a) and uninfected (b) *E. kühniella* caterpillars separated on 10% SDS-PAGE and stained with coomassie brilliant blue. (c) Western blot of fat body protein extract of uninfected caterpillars were incubated with anti-hemolymph antisera. Dots indicate the protein bands eluted for specific antibodies. The abundant proteins at 70-80 kDa are treated as one band and probably represent the storage proteins (Wyatt and Pan, 1978; Kanost *et al.*, 1990). The 42 kDa and 47 kDa proteins are probably single proteins. The molecular weight markers used are 85.5 kDa (fructose-6-phosphate kinase), 55 kDa (glutamate dehydrogenase), 39.2 kDa (aldolase), and were from Bio-Rad®.

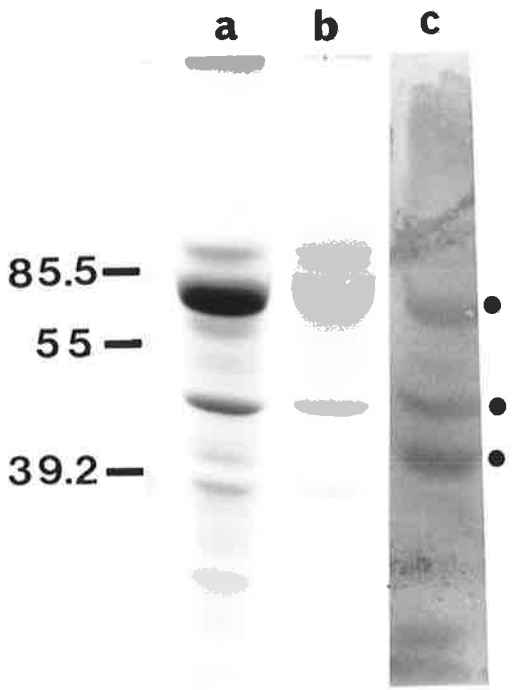

a
b
c
85.5
55
39.2

Fig. 30 *In vitro* bacterial recognition assay. Cell-free hemolymph was mixed with bacteria and the proteins attached to the bacterial surface were recovered in the presence of 0.5 M ammonium formate and separated on 10% SDS-PAGE. The Western blots were analysed using anti-hemolymph antisera (a) and anti-VLP antisera (b). Cell-free hemolymph (c) separated under similar conditions and stained with Coomassie blue. The position of pre-stained molecular weight markers (Bio-Rad®), are indicated on the right: 204 kDa (myosin), 132 kDa (ß-galactosidase), 65 kDa (bovine serum albumin), 42.6 kDa (carbonic anhydrase), 29.9 kDa (soybean trypsin inhibitor), and 17.1 kDa (lysozyme).

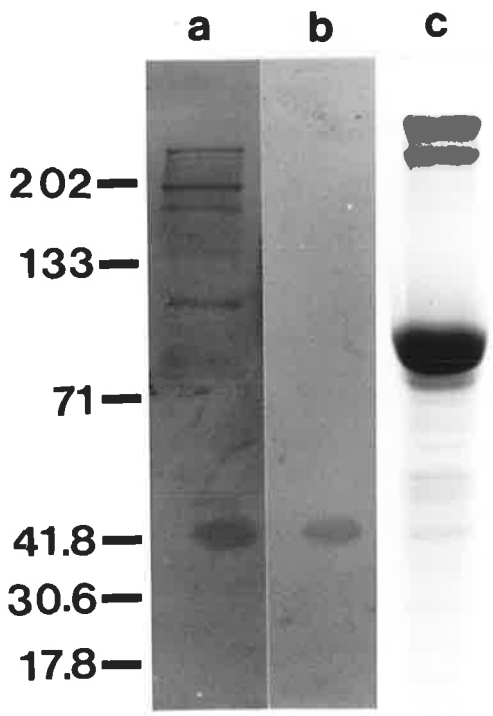
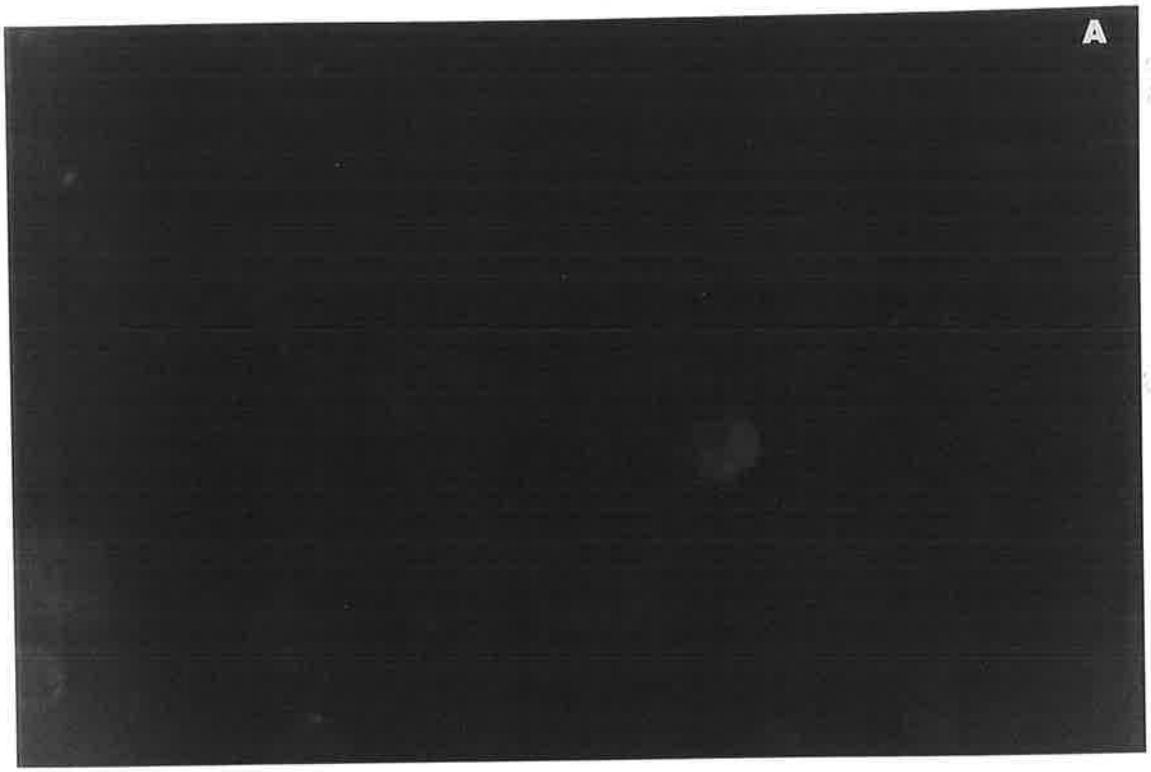

a
b
c
202
133
71
41.8
30.6
17.8

Fig. 31 *E. kühniella* hemocytes using antibodies against a bacterial fusion protein encoded by the PHGPX-like domain of p40. (A) Hemocytes are not labelled with this antiserum. (B) The same specimen under phase contrast.


A

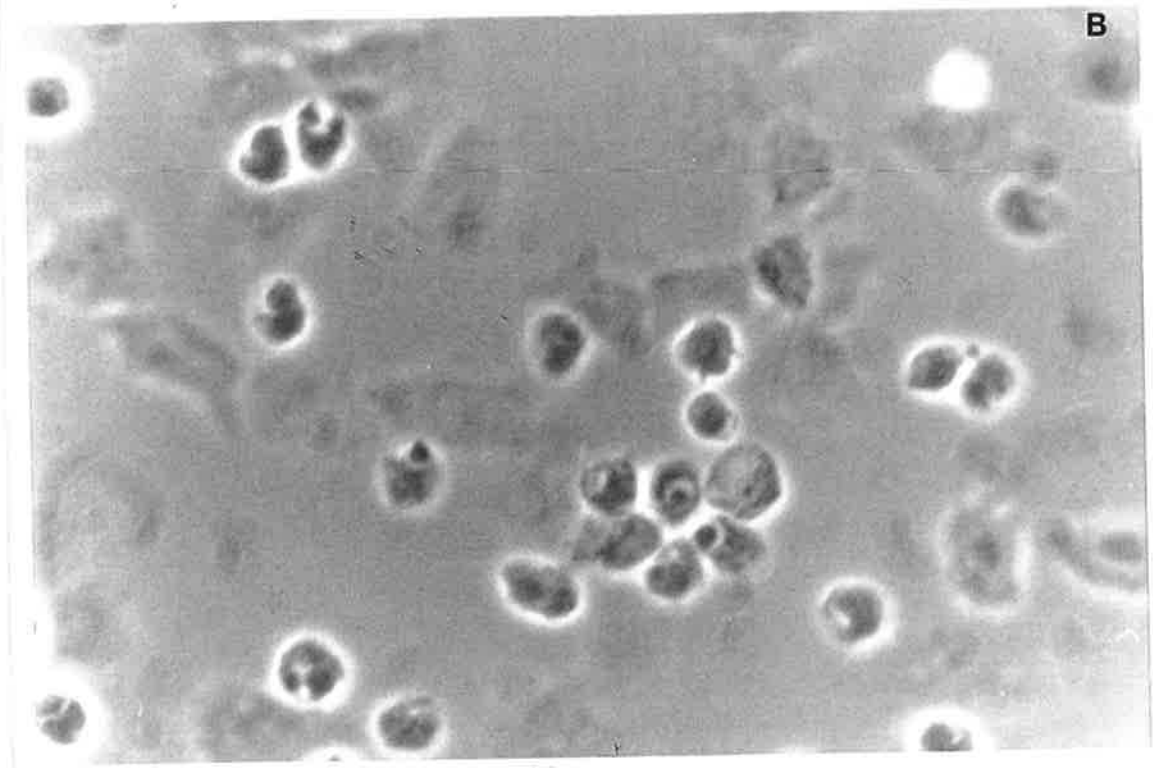

B

Fig. 32 *E. kühniella* hemocytes treated with specific antibodies against the 42 kDa *E. kühniella* hemolymph protein. (B) Indirect immuno-fluorescence staining of FITC-conjugated secondary antibodies, with granulocytes specifically labelled with antibodies against hemolymph proteins. No labelling was observed with pre-serum. Among the two morphologically distinct granulocyte types in *E. kühniella* only one is labelled (arrowheads), probably representing granulocyte type 2. Apart from slight differences in background staining, labelling of hemocytes is virtually identical to those treated with anti-VLP antibodies. (A) The same specimen under phase contrast.

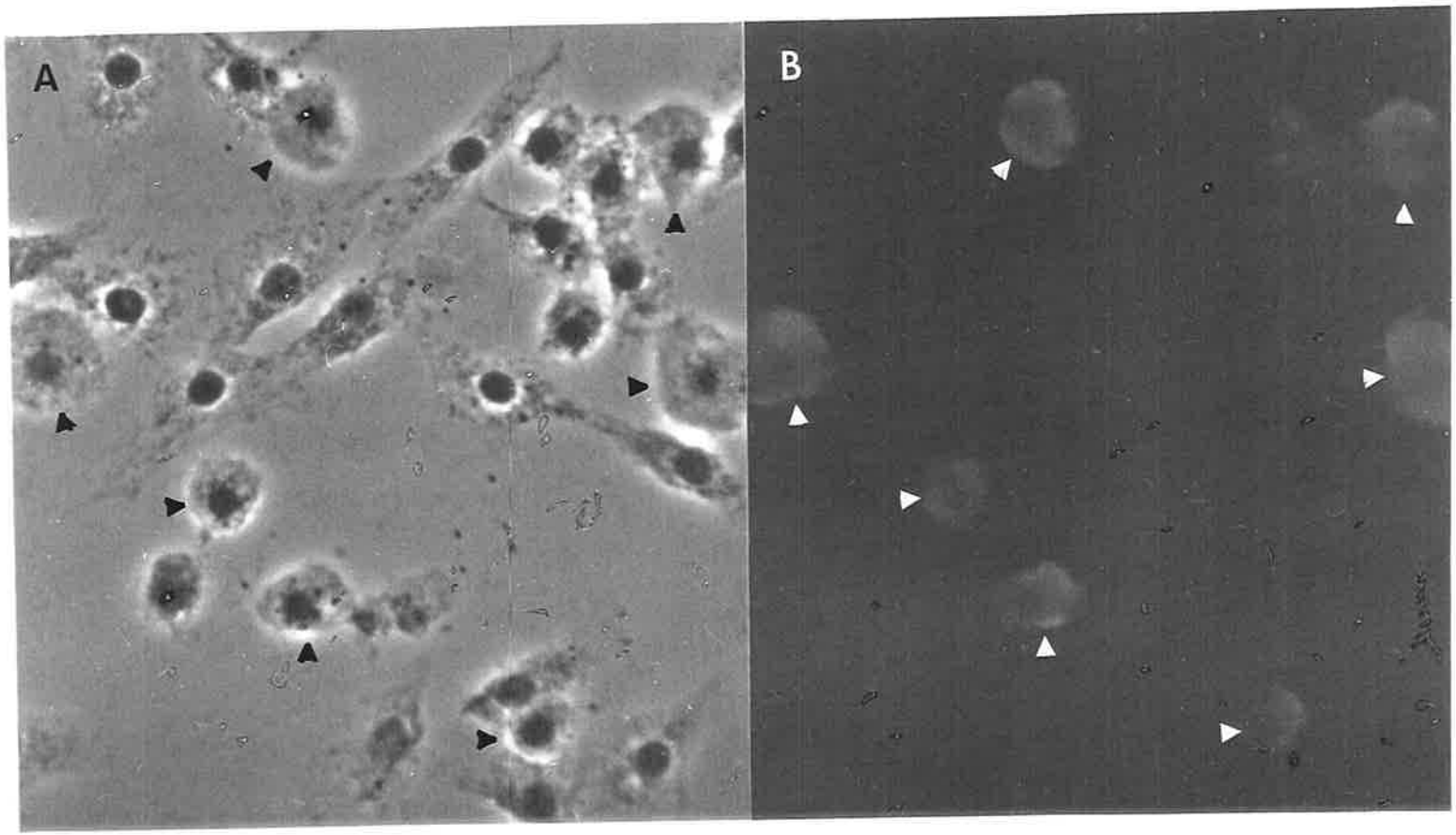

A
B

Fig. 33 Tissue sections of fourth instar *E. kühniella* caterpillars incubated with specific antibodies against the 42 kDa *E. kühniella* hemolymph protein. (B) Indirect immuno-fluorescence staining of FITC-conjugated secondary antibodies. The section shows distinct labelling of epidermal and muscle tissue surface over basement membranes that are exposed to the hemocoel (h). Other basement membranes, *eg.* between epidermis and muscle are not labelled (arrowheads). Cuticular structures (c) show auto-fluorescence that is distinct from FITC-related immuno-fluorescence at the lining. No labelling was observed with pre-serum. Apart from slight differences in background staining, extracellular structures labelled by specific antibodies against the 42 kDa protein proved to be virtually identical to larval tissue preparations treated with anti-VLP antibodies. (A) The same section under phase contrast.

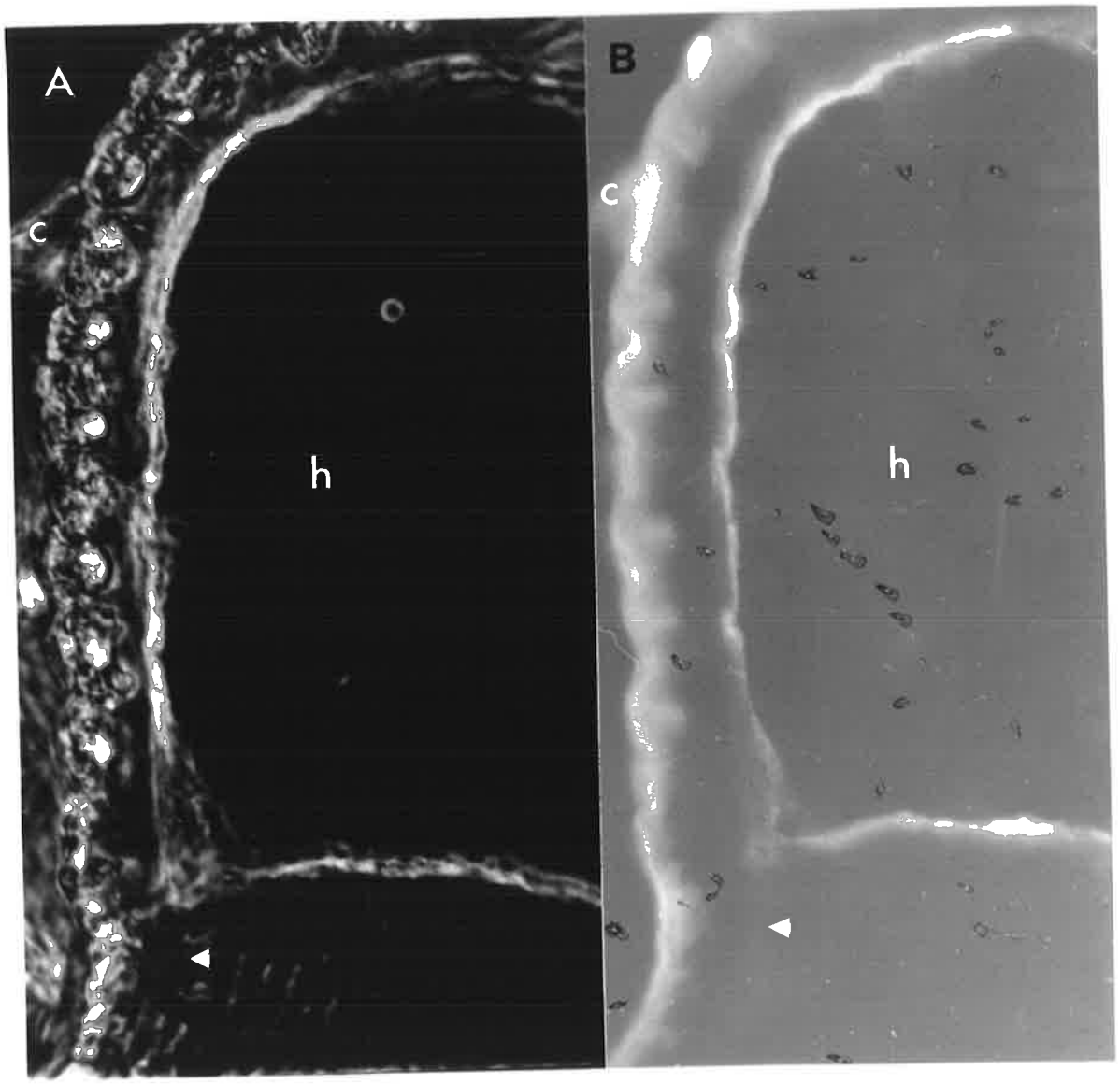

A
c
h
B
c
h

Fig. 34 Tissue sections of a fourth instar *E kühniella* caterpillars showing the gut (g). (B) Indirect immuno-fluorescence staining with FITC-conjugated secondary antibodies. Note that the lining of gut tissue (g) is only labelled towards the hemocoel (h) but not in the lumen (arrowheads). Labelling of cuticle (c) is due to auto-fluorescence. No immune-fluorescence labelling was observed with pre-serum. (A) The same section under phase contrast.

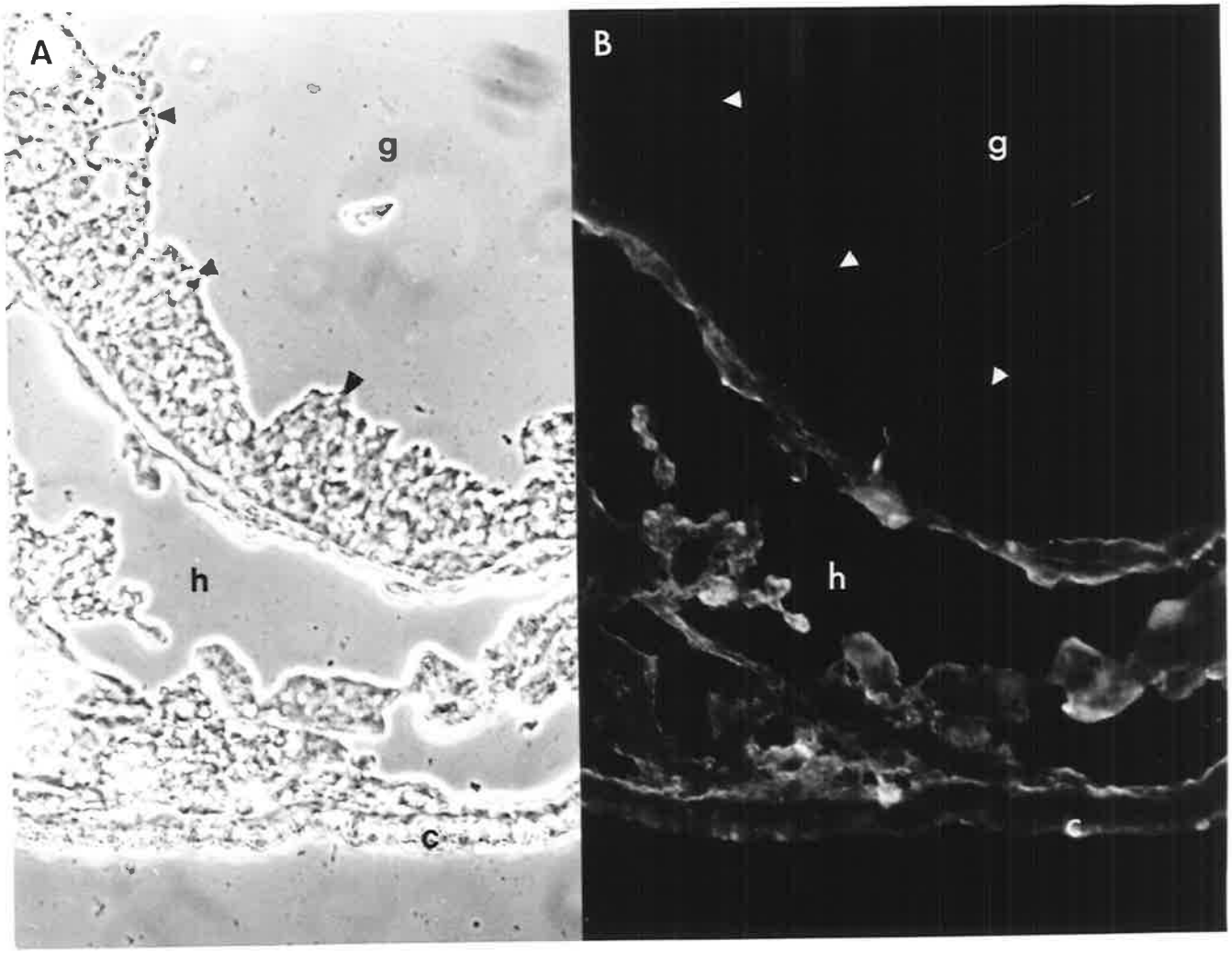

A
g
h
c
B
g
h
c

Fig. 35 *In vivo* binding of antibodies to the lining of *E. kühniella* caterpillars. Tissue sections of a fourth instar *E kühniella* caterpillars, injected with the specific antiserum against 42 kDa hemolymph protein, preserum and BSA. The caterpillars were frozen and sectioned and the location of antibodies visualised by treatment of tissue sections with FITC-conjugated secondary antibodies. Indirect immune-fluorescence staining with FITC-conjugated secondary antibodies of a caterpillar injected with antiserum (B). Caterpillar injected with pre-serum (C). Note conspicuous attachment of hemocytes to the lining of antiserum treated caterpillars (arrowheads) in (A) and (B) that was not observed in pre-serum or BSA treated caterpillars. In pre-serum (C) or BSA treated caterpillars no labelling was observed at the site of the basement membranes at the lining (arrowheads) (c, cuticle; h, hemocoel; m, muscle). (A) The same section under phase contrast.

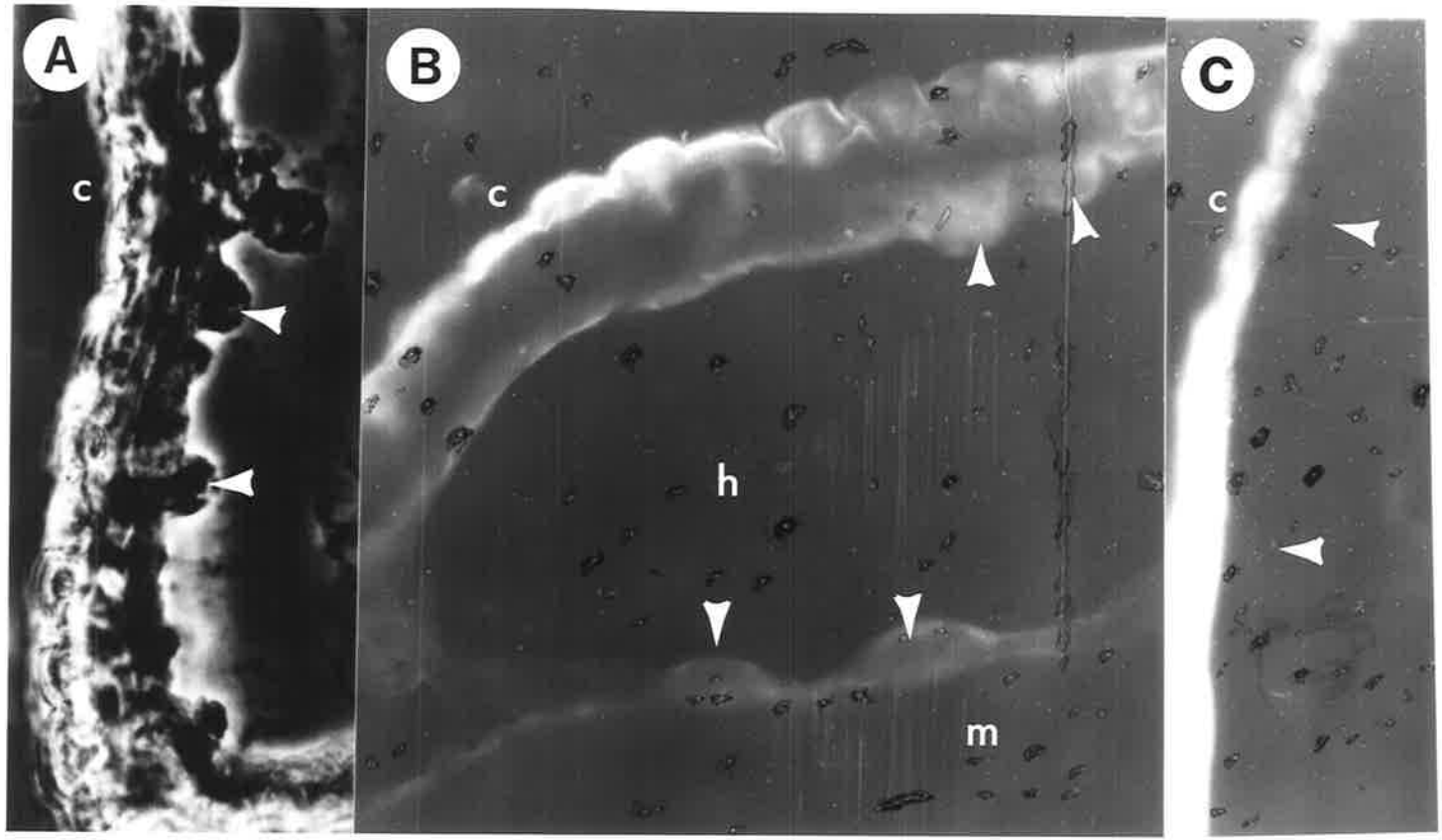

A
c
B
c
h
m
C
c

Fig. 36 Schematic representation of sites of the 42 kDa localisation in the open circulatory system of *E kühniella* caterpillars and its possible functions (see text).

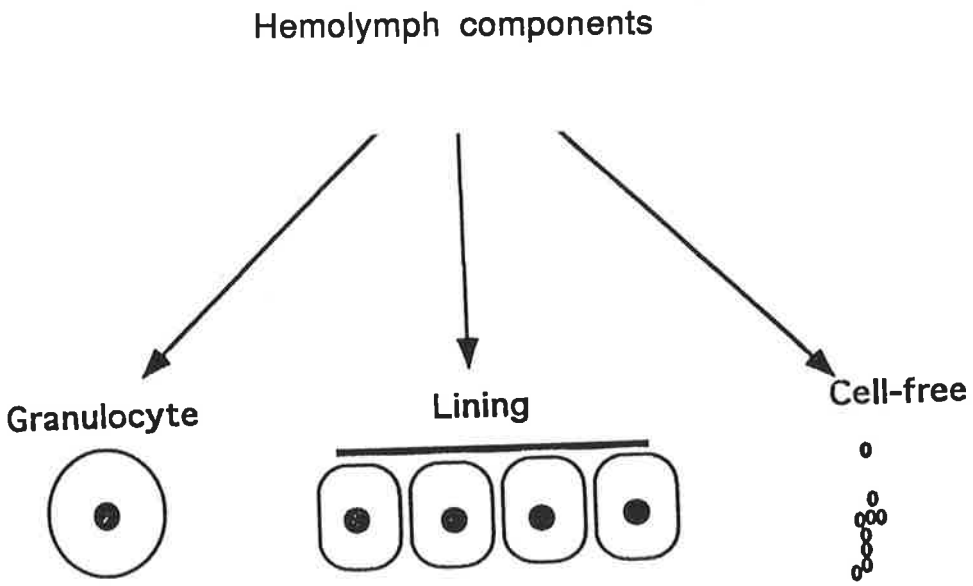

Hemolymph components
Granulocyte
Lining
Cell-free

Fig. 37 Tissue section of *V. canescens* ovarioles (posterior part of the ovary) incubated with FITC-conjugated *H. p.* lectin. The section shows labelled sheath surrounding the oocytes (arrow) as well as the follicle cells around the developing embryo (arrow head).

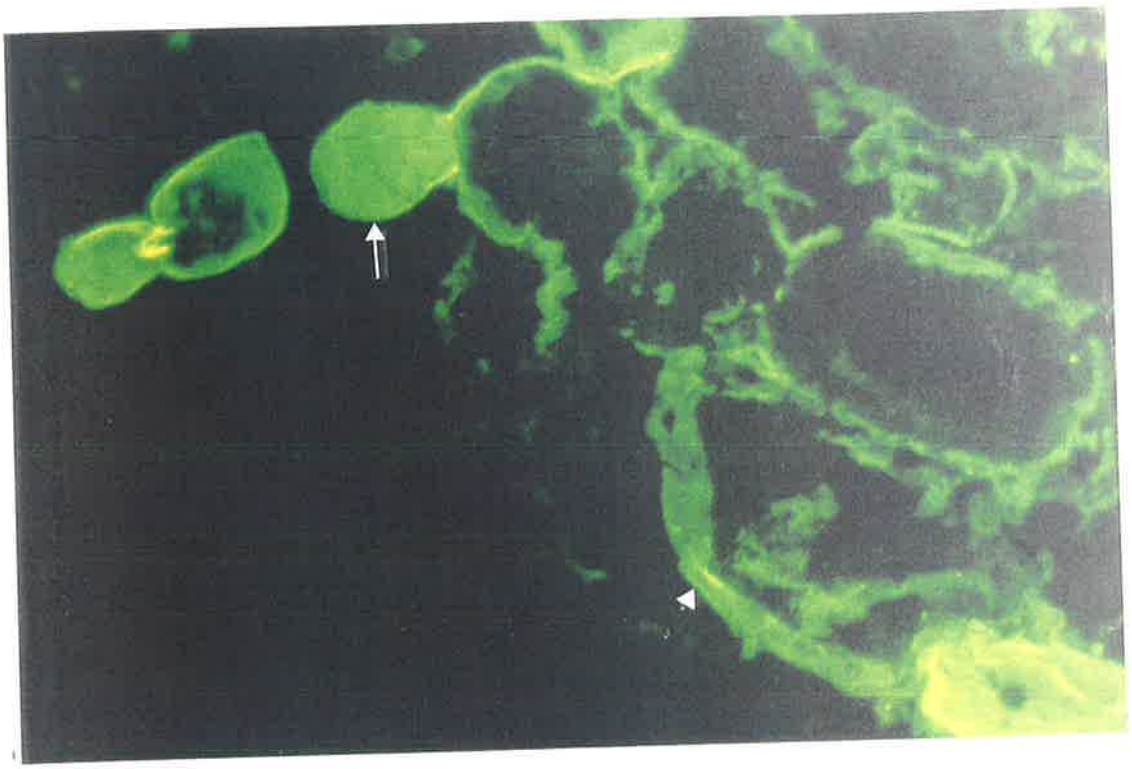


Fig. 38 Tissue section of *V. canescens* ovarioles (posterior part of the ovary) incubated with FITC-conjugated *H. p.* lectin. (A) The section shows an oocyte with the vitelline membrane labelled (arrow). (B) The same section under phase contrast.

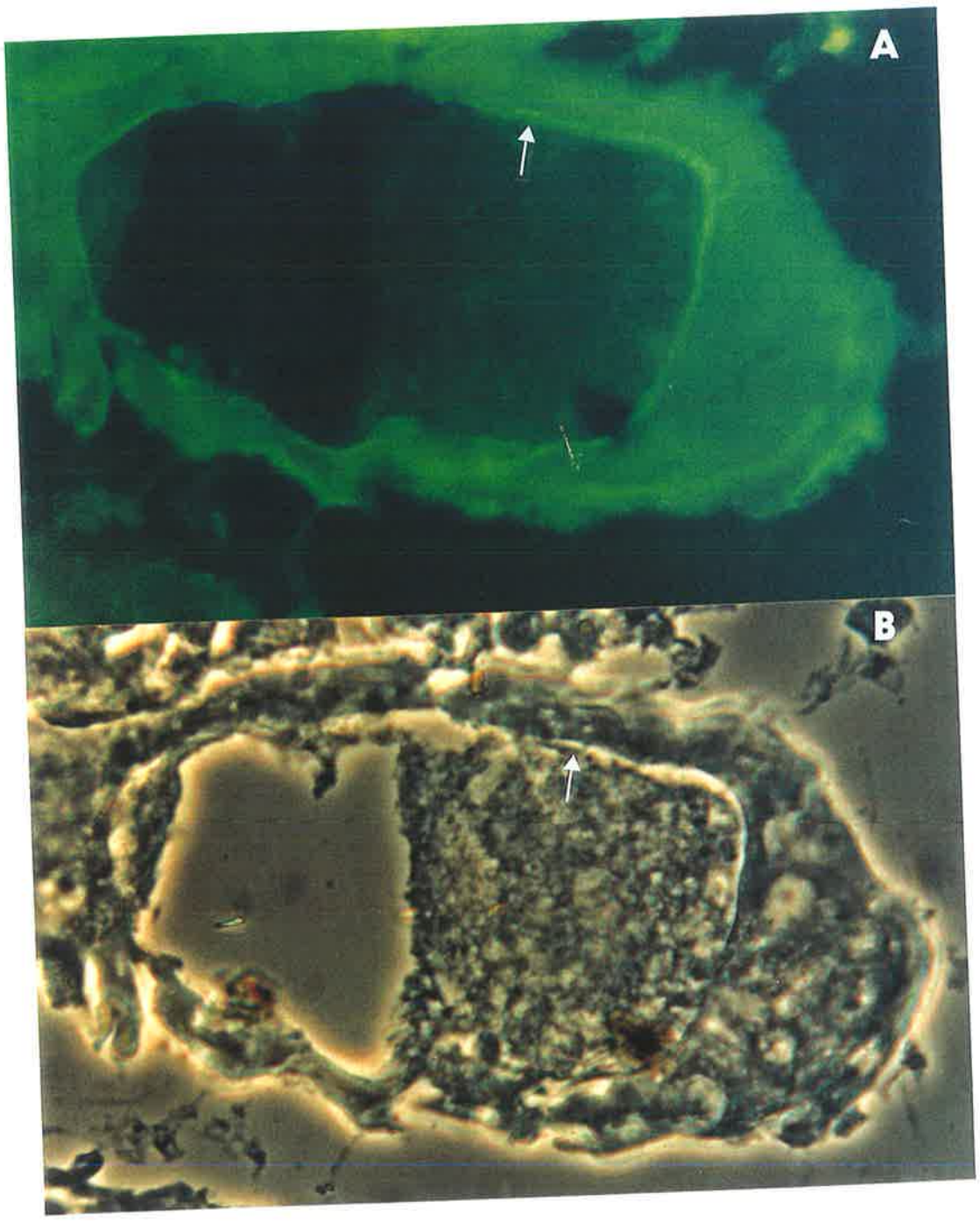

A
B

Fig. 39 Tissue section of *V. canescens* ovary incubated with FITC-conjugated *H. p.* lectin. The section shows the region where the ovarioles merge with the calyx gland. (A) The egg chorion in both the ovarioles and the calyx gland are labelled uniformly (arrows). (B) The same section under phase contrast.

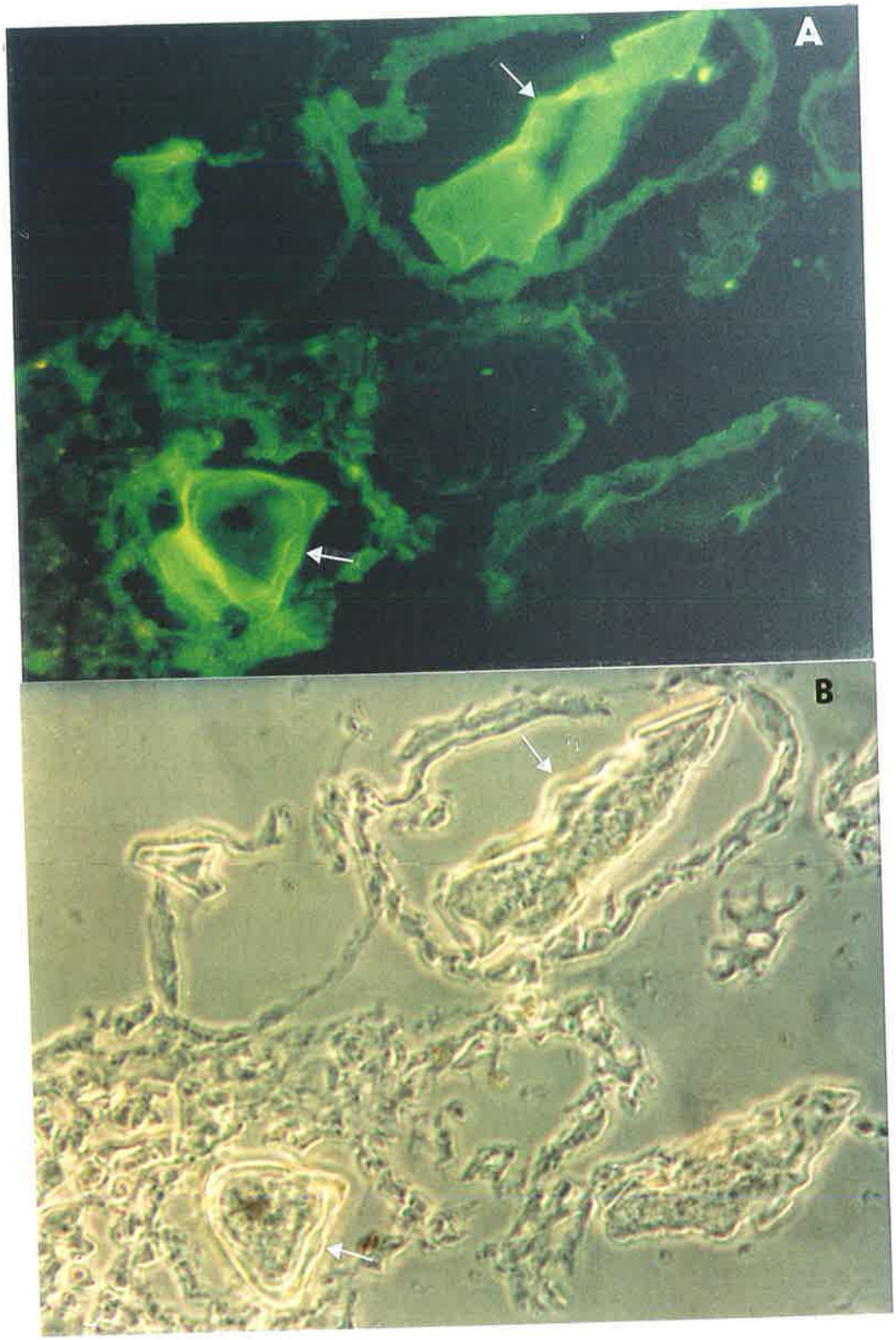

A
B

Fig. 40 Tissue section of *V. canescens* ovary incubated with FITC-conjugated *H. p.* lectin. (A) The section shows uniformly labelled calyx gland cells. The egg chorion inside the calyx gland is also labelled (arrow). (B) The same section under phase contrast.

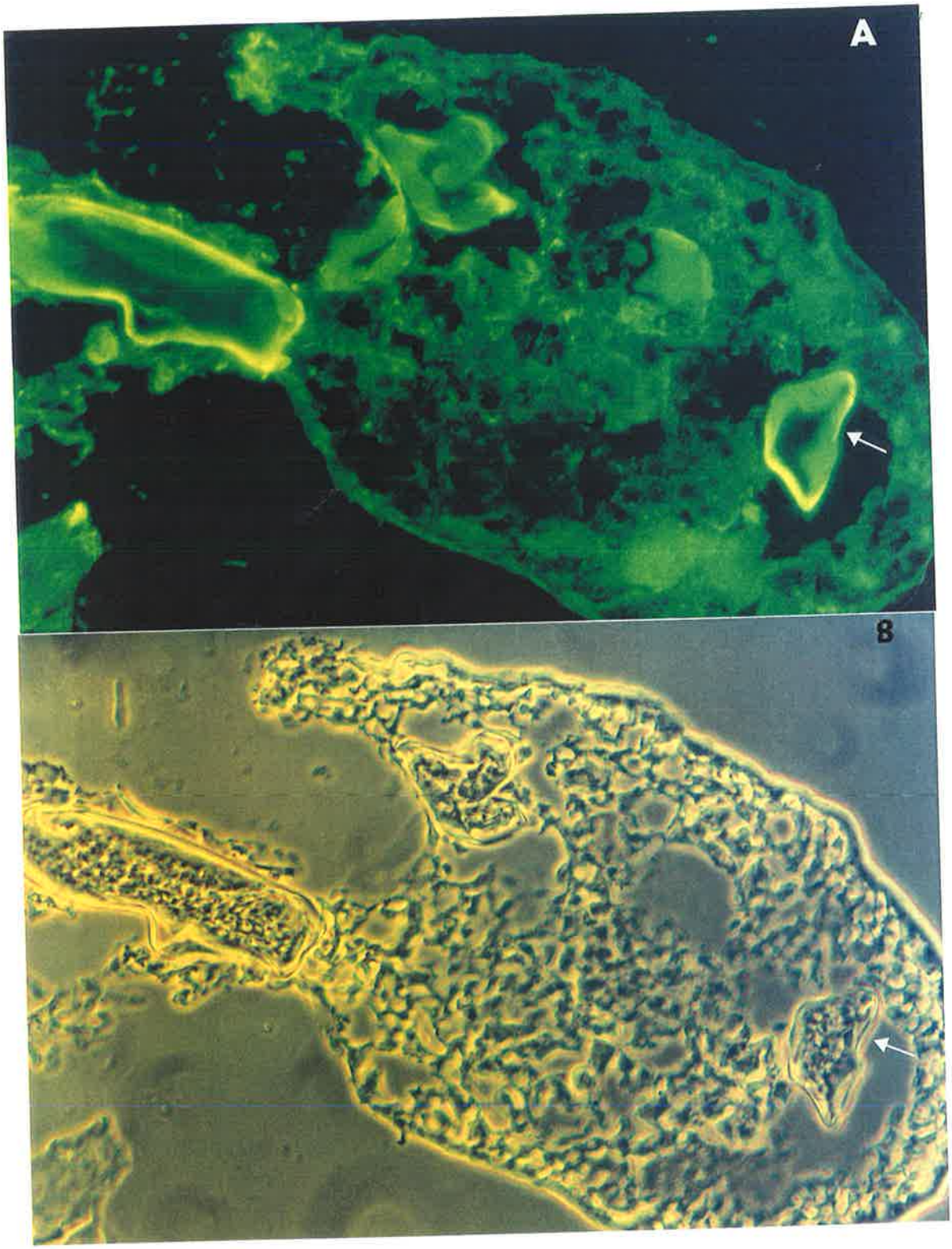

A
B

Fig. 41 Tissue section of *V. canescens* oviduct and part of calyx incubated with FITC-conjugated *H. p.* lectin. (A) Note the labelling of calyx fluid between the eggs (arrow). Similar labelling is seen in Fig. 10 A. (B) The same section under phase contrast.

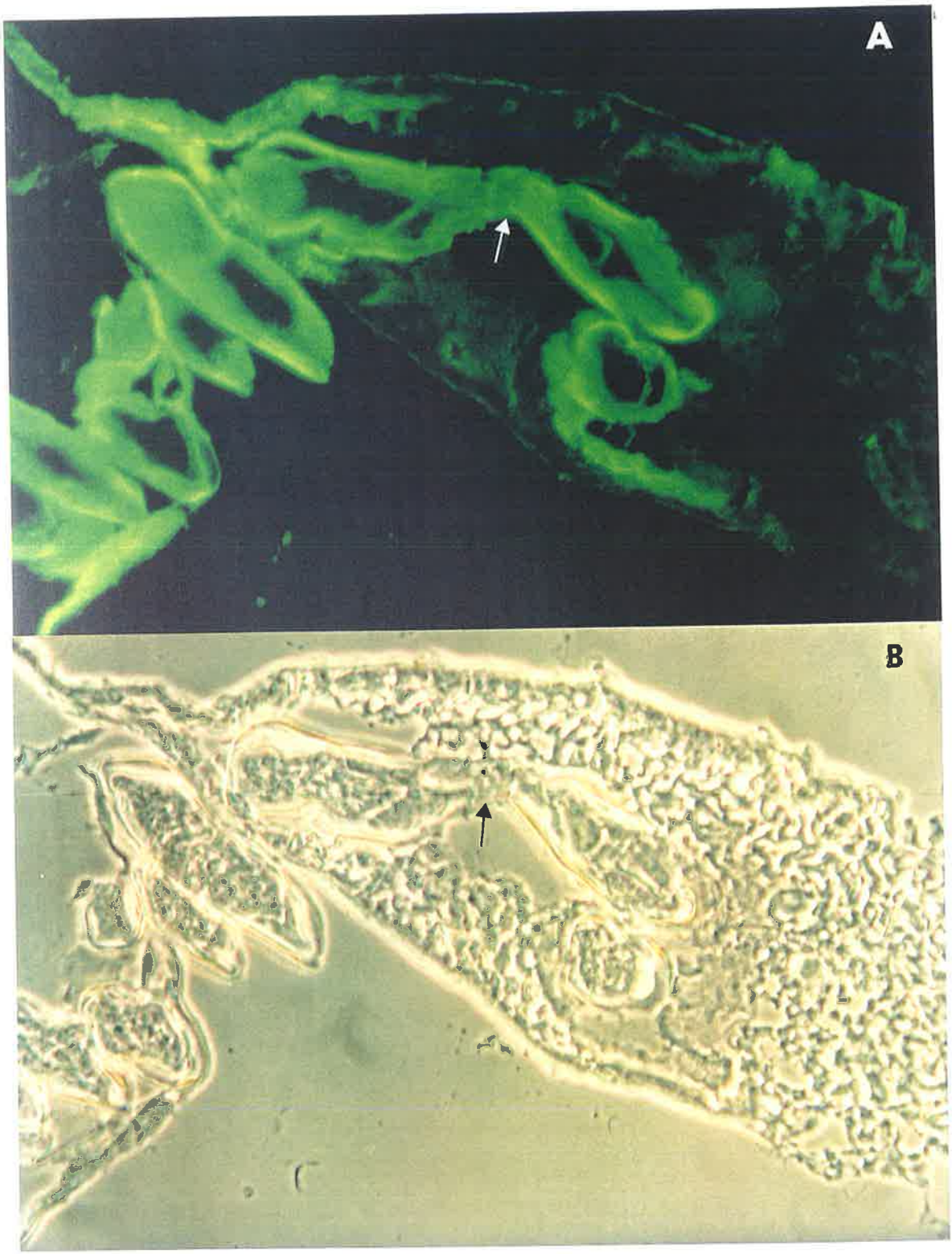

A
B

Fig. 42 Tissue section of *V. canescens* oviduct incubated with FITC-conjugated *H. p.* lectin. (A) Note the labelling of calyx fluid (arrow) and the egg chorion (compare with Fig. 10 A). The piece of tissue located next to the oviduct is a calyx gland section. Note the relative intensity of labelling which is clearly increased inside several openings of the calyx lumen. (B) The same section under phase contrast.

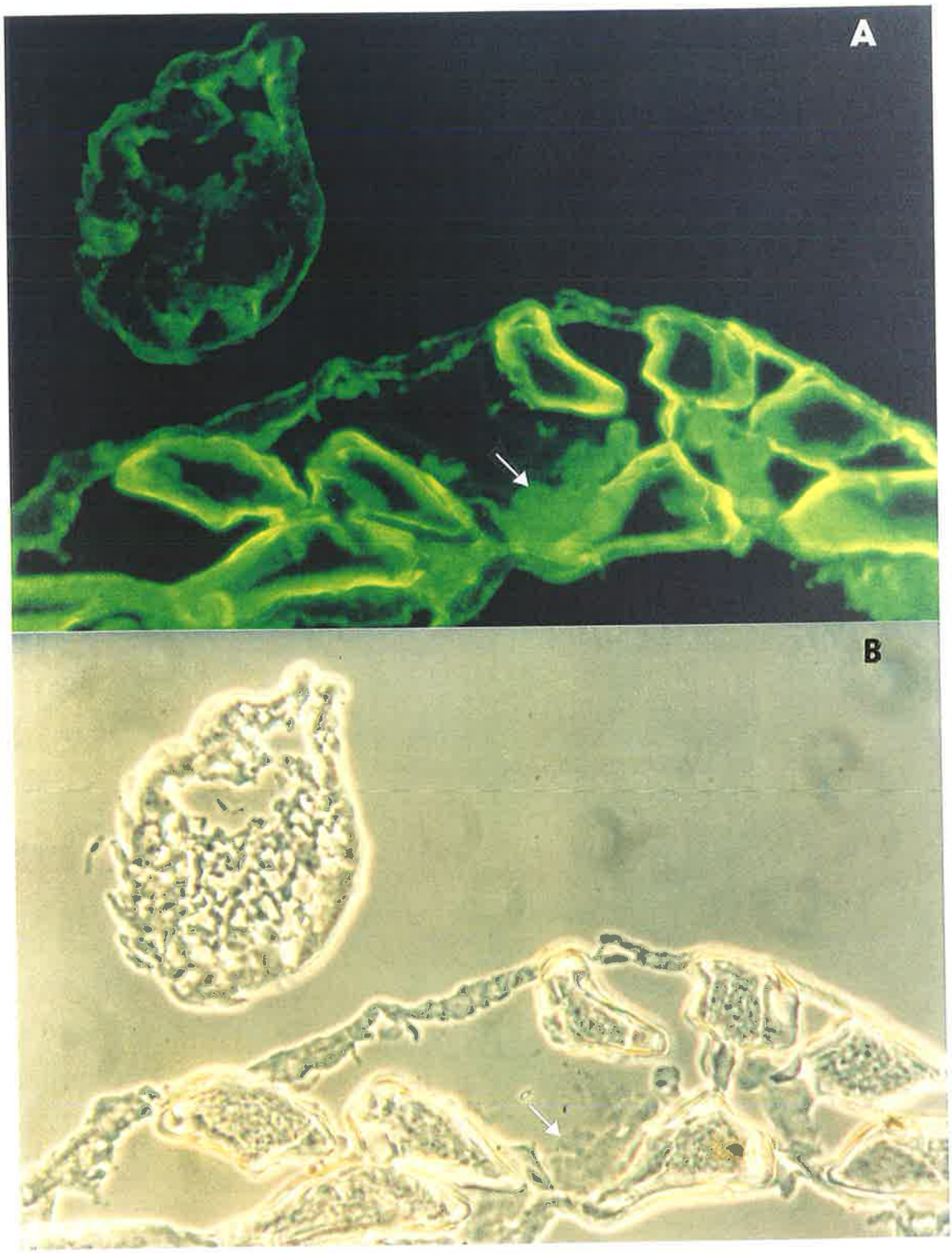

A
B

Fig. 43 *V. canescens* egg incubated with FITC-conjugated *H. p.* lectin. The egg is uniformly labelled with higher labelling visible at the polar ends of the egg.

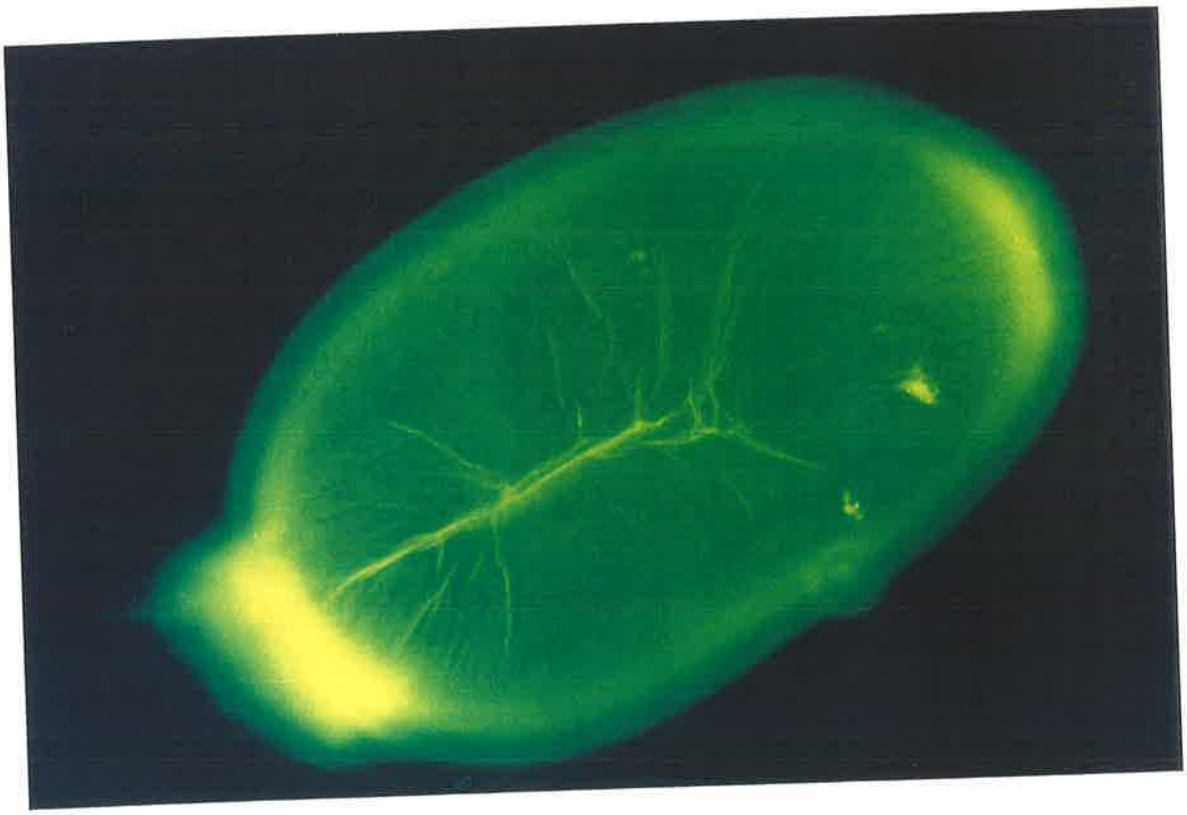


Fig. 44 *E. kühniella* hemocytes incubated with FITC-conjugated *H. p.* lectin. (A) The granulocytes are labelled (arrow head) while the flattened plasmatocytes are not (black arrow head). The glass surface around some cells is intensively labelled (arrow). The original cells from which the labelling may have been derived usually change morphologically or may have disintegrated. (B) The same specimen under phase contrast.

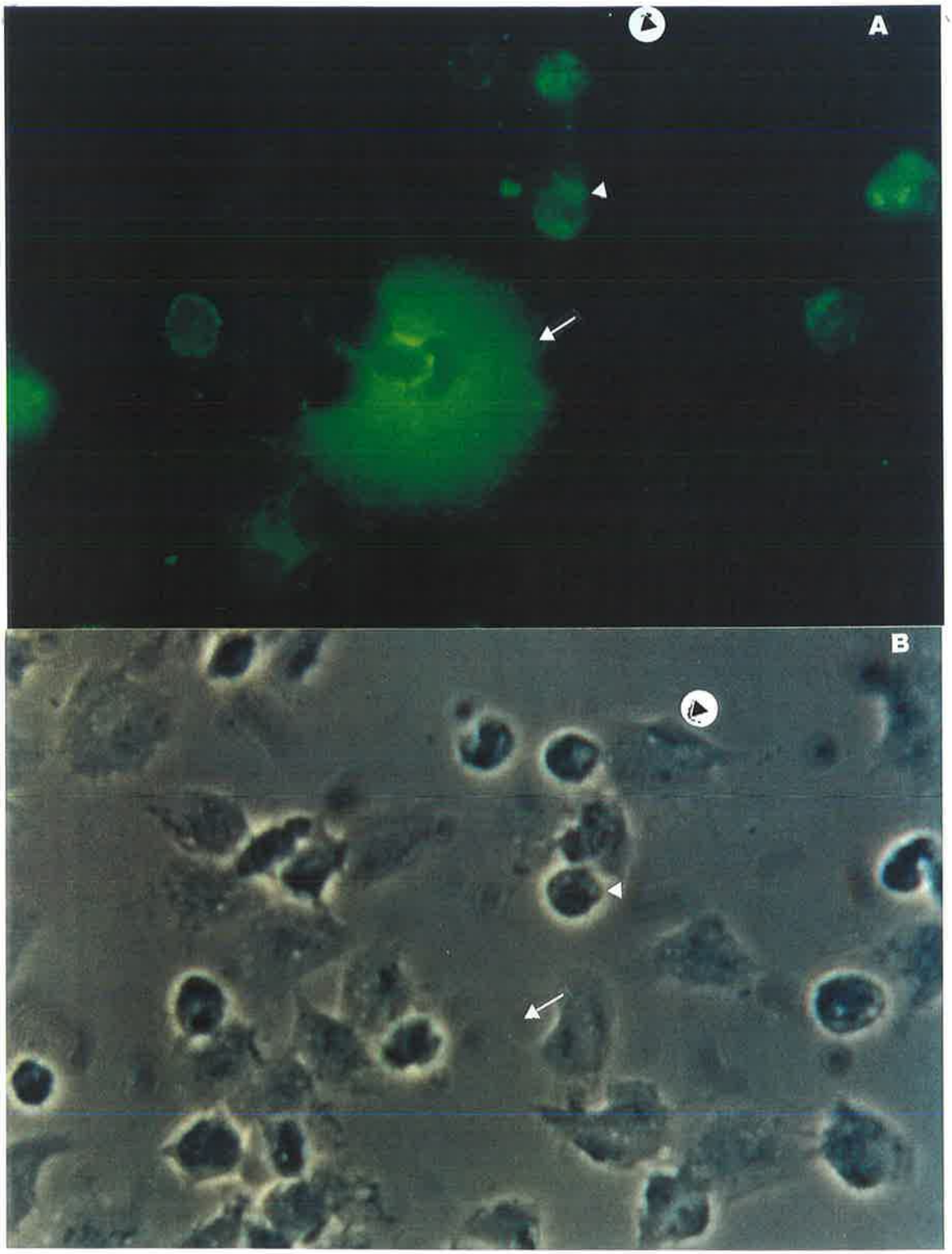

A
B

Fig. 45 *E. kühniella* hemocytes incubated with FITC-conjugated *H. p.* lectin. (A) The granulocytes are labelled while the flattened plasmatocytes are not. (B) The hemocytes in phase contrast under visible light. (B) The same specimen under phase contrast.

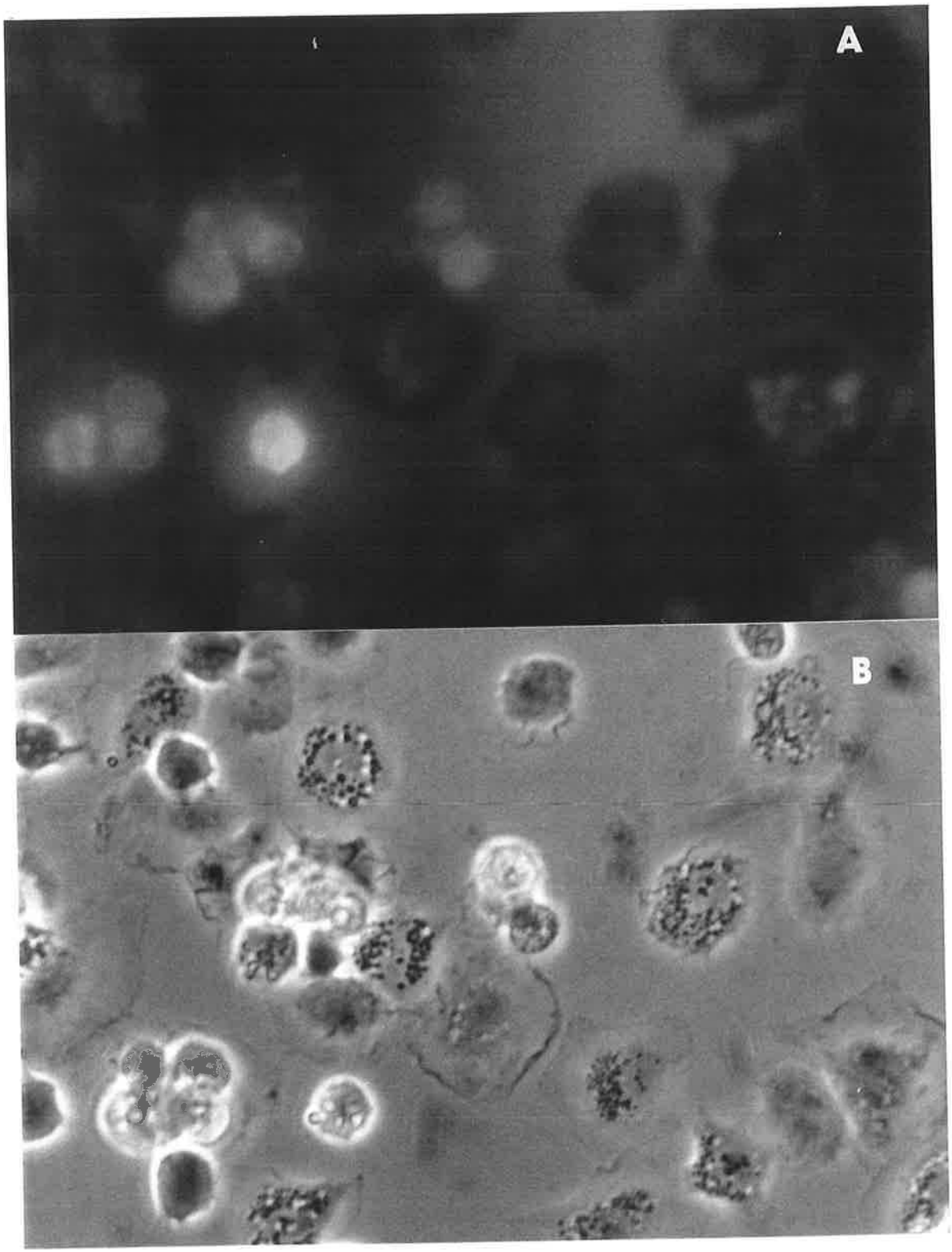

A
B

Fig. 46 Hemocytes from parasitised *E. kühniella* incubated with FITC-conjugated *H. p.* lectin. (A) The granulocytes are labelled (arrow head) while the flattened plasmatocytes are not (arrow). Note the tendency of the granulocytes to aggregate (black arrow). (B) The same specimen under phase contrast.

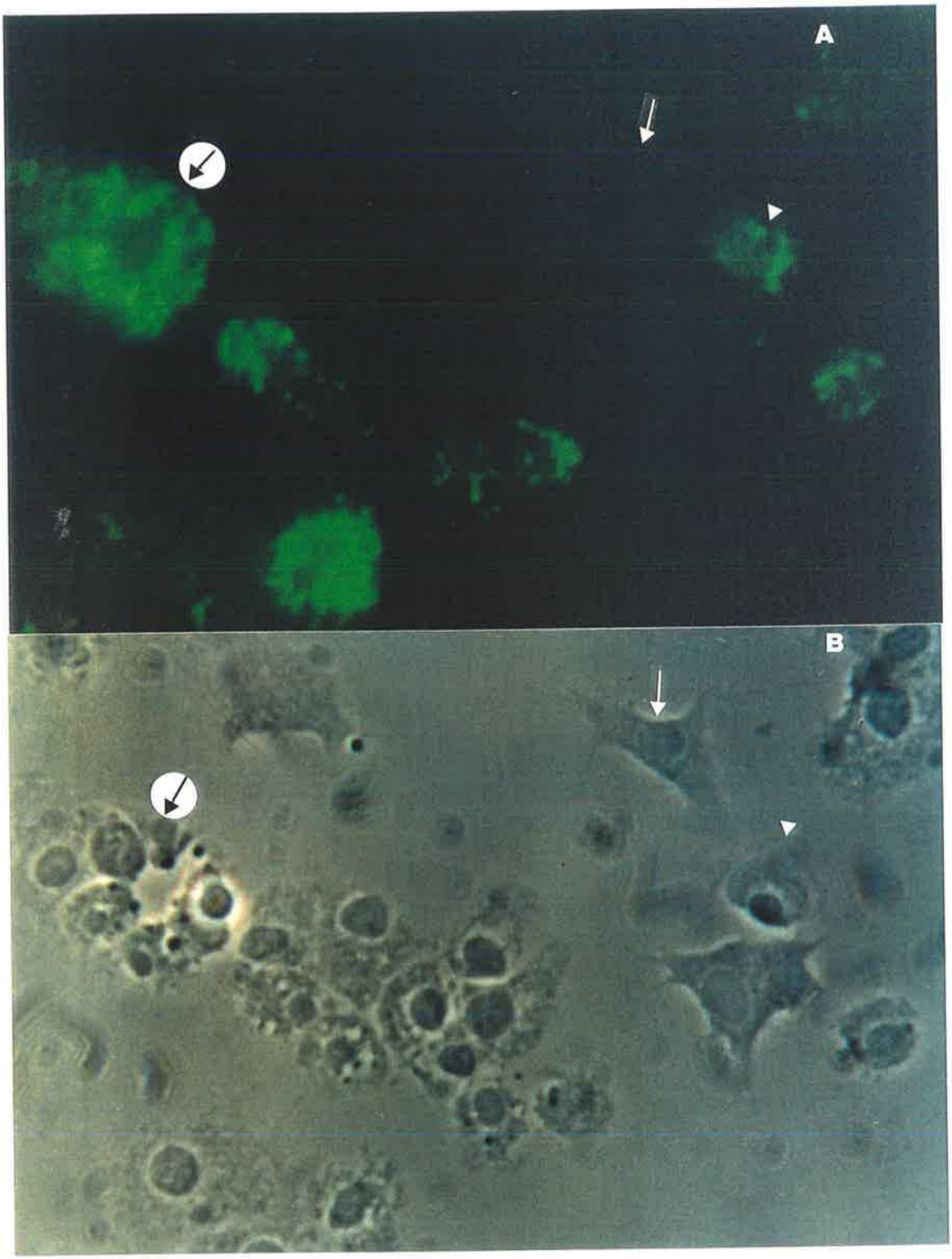

A
B

Fig. 47 Hemocytes from parasitised *E. kühniella* incubated with FITC-conjugated *H. p.* lectin. (A) The granulocytes are labelled (arrow head) while the flattened plasmatocytes are not (arrow). Note the granulocytes are labelled in a dot-like fashion. (B) The same specimen under phase contrast.

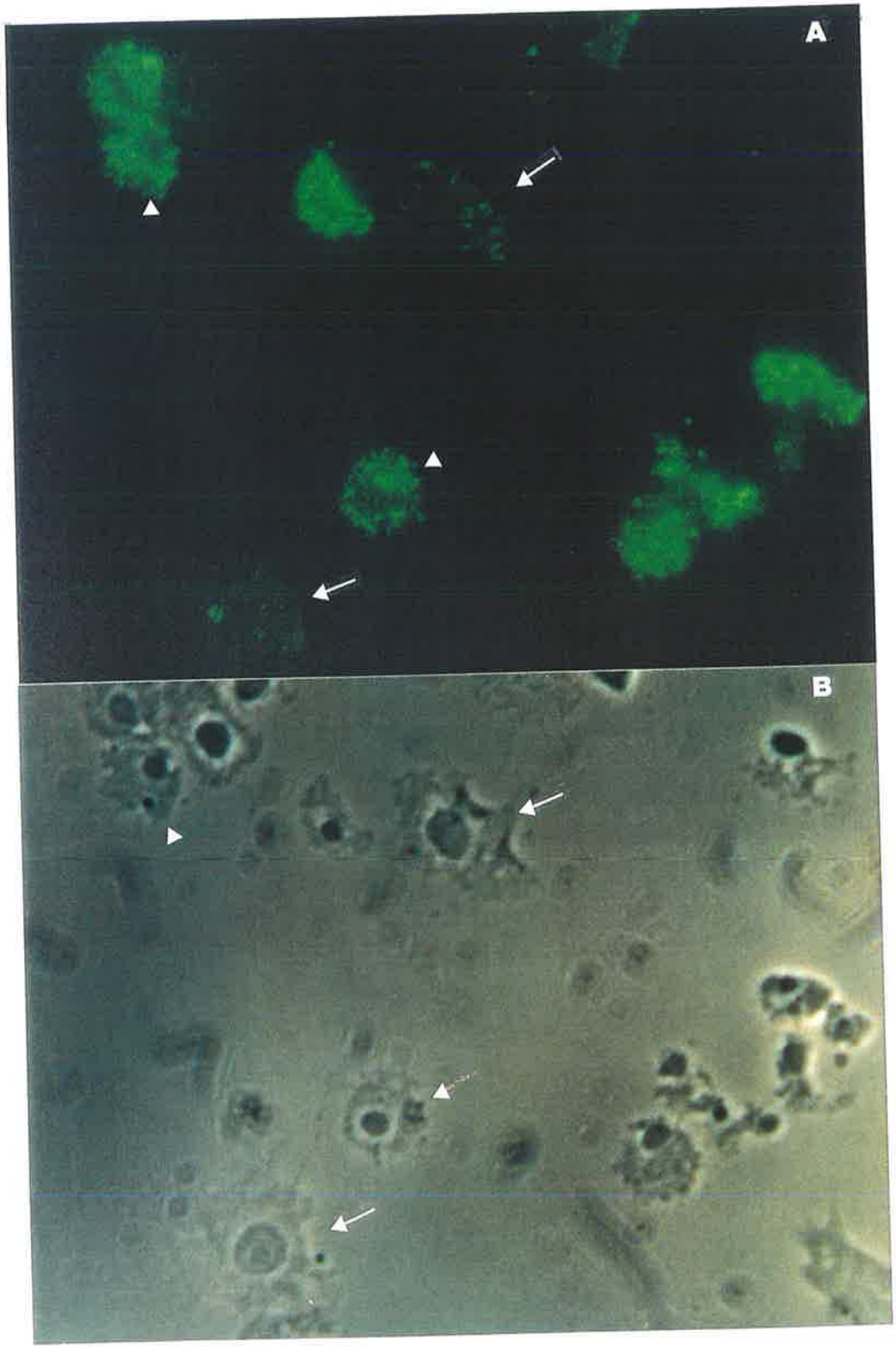

A
B

Fig. 48 *V. canescens* hemocytes incubated with FITC-conjugated *H. p.* lectin. (A) The hemocytes are labelled uniformly and form pseudopodia-like protrusions forming a star-like structure (arrow). (B) The same specimen under phase contrast.

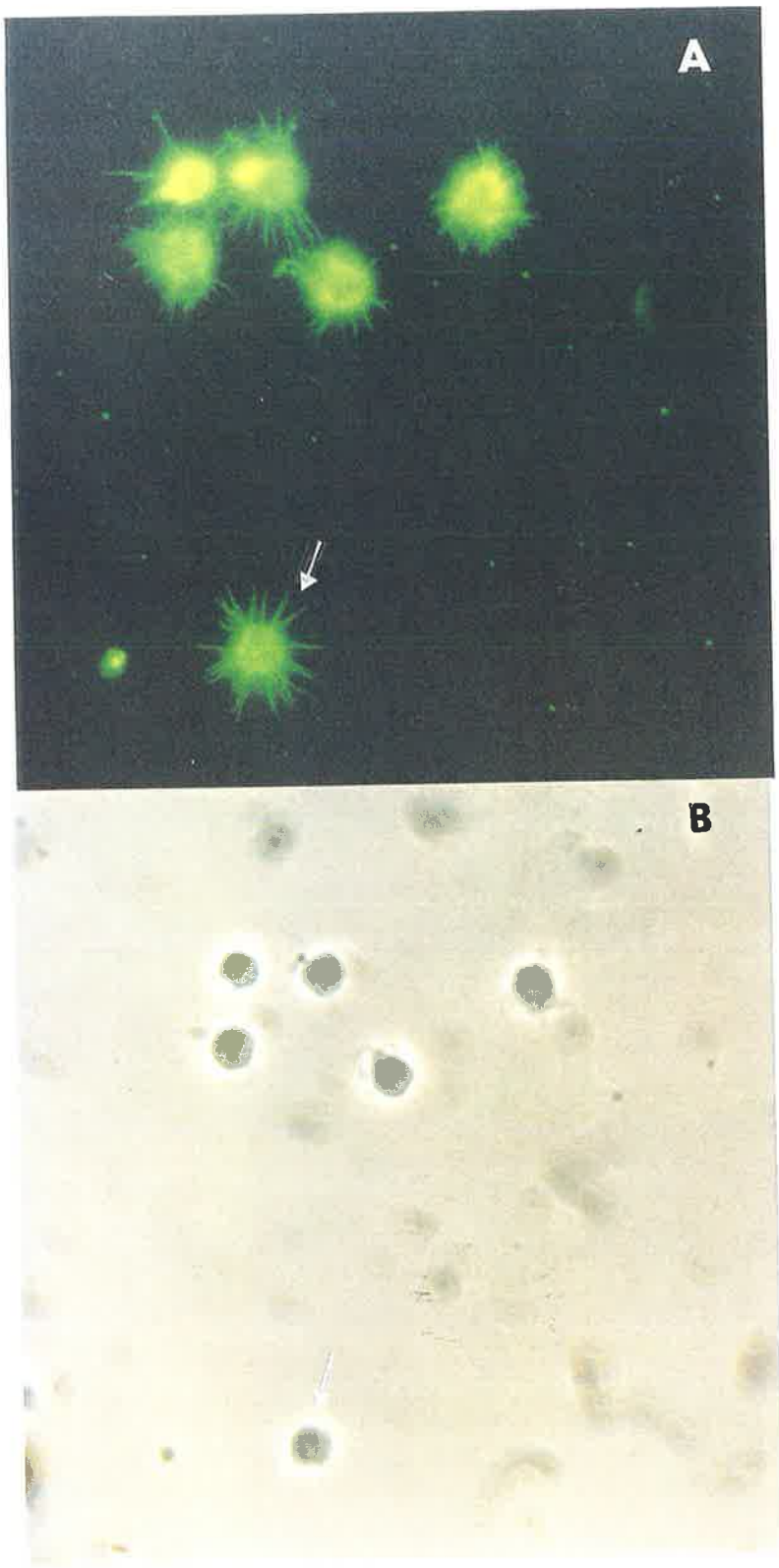

A
B

Fig. 49 *V. canescens* hemocytes incubated with FITC-conjugated *H. p.* lectin. The flattened plasmatocyte-like hemocytes show particles at the end of the pseudopodia-like protrusions (arrow). These particles are heavily labelled and are shed off by the hemocytes (arrow head).

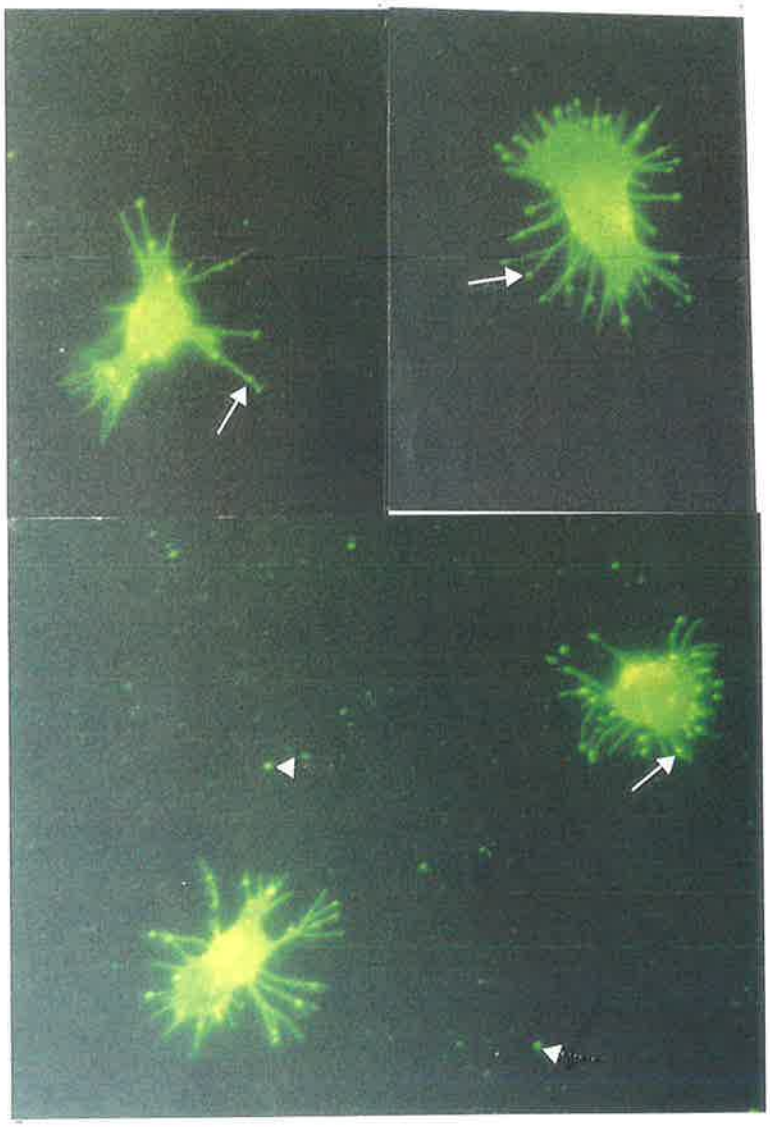


Fig. 50 *V. canescens* larva separated from the chorion and incubated with FITC-conjugated *H. p.* lectin (A) The labelling appears like rings around the larval epidermis which signify that the origin of the mucin-like components originate from segment-specific epidermal cells. (B) No segment anlagen are visible morphologically when the larva is inspected under phase contrast.

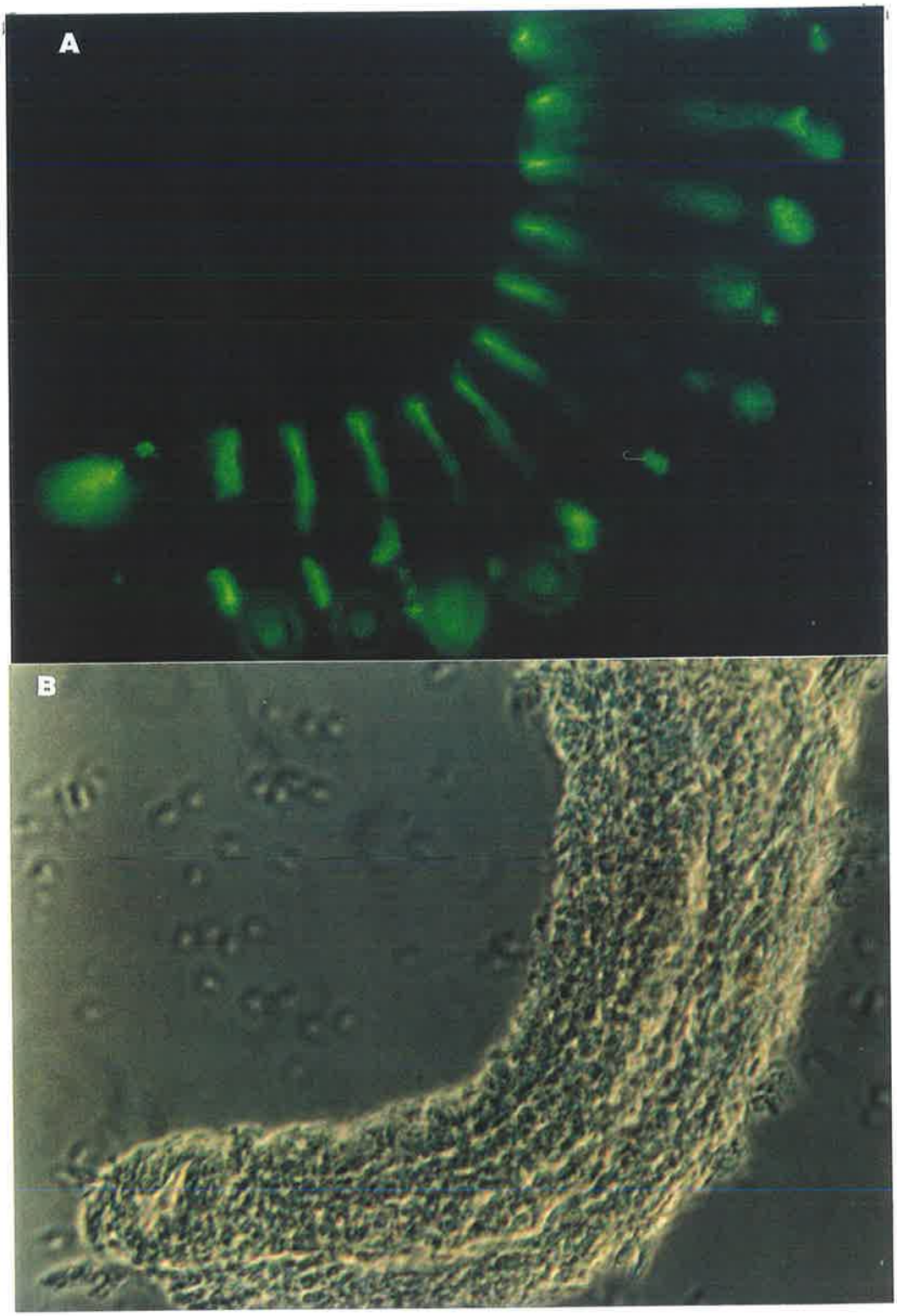

A
B

Fig. 51 (A) *V. canescens* larva teased from the chorion and incubated with FITC-conjugated *H. p.* lectin (see Fig. 50) at a larger magnification. (B) The same section under phase contrast.

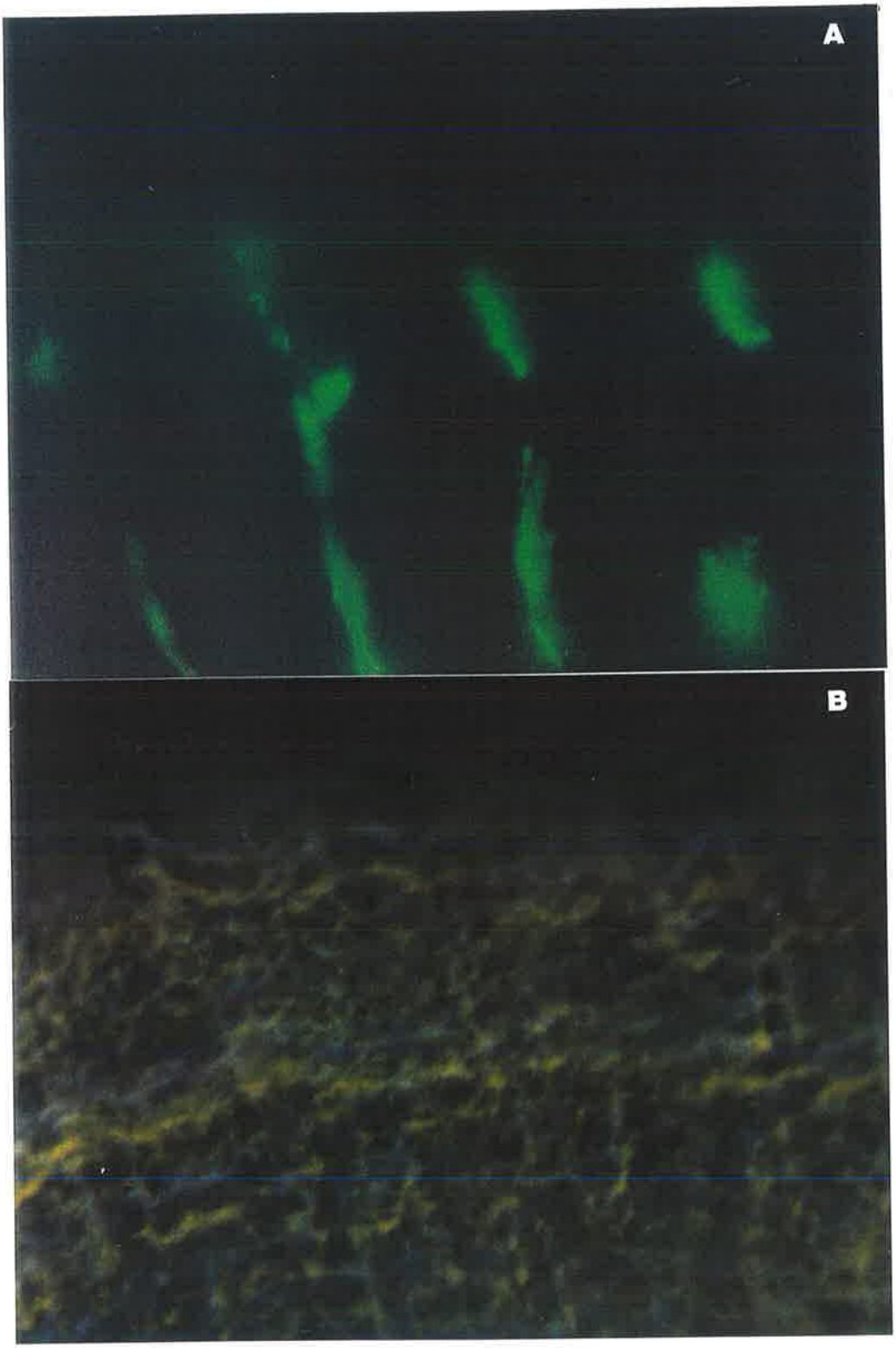

A
B

Fig. 52 Western blots of *E. kühniella* and *V. canescens* protein extracts reduced and analysed by gel electrophoresis. The Western blot was treated with phosphatase-conjugated *H. p* lectin. Extracts were from *E. kühniella* caterpillar hemocytes (a), hemolymph (b), silk gland (c), and from *V. canescens* ovary (d), larva (e), calyx gland (f). The hemomucin from mbn-2 *D. melanogaster* cell line (g) is shown for comparison. Note that *V. canescens* tissues, d, e and f have glycoprotein of a 100 kDa which is similar to the *D. melanogaster* hemomucin. *V. canescens* larva also have a light band at 200 kDa. See Blue™ molecular weight markers (m) in kDa were from Novex.

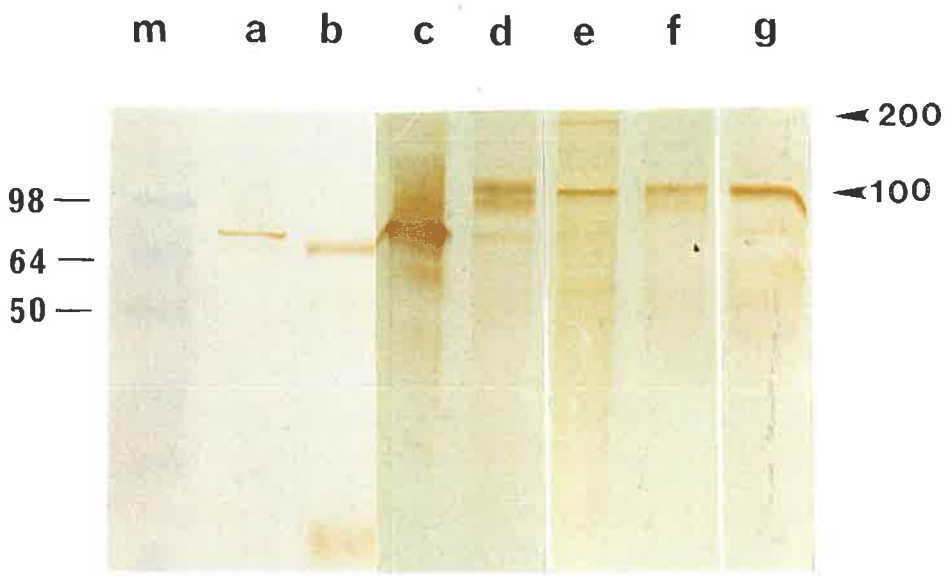
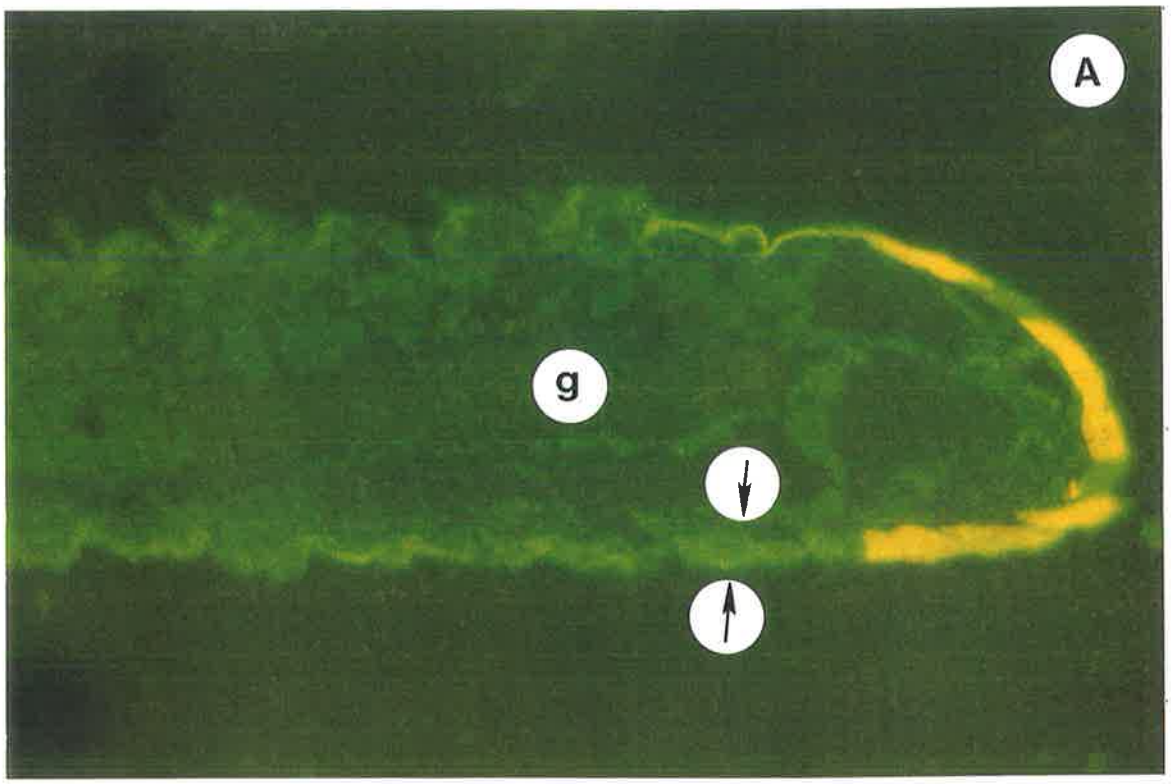

m
a
b
c
d
e
f
g
98
64
50
200
100

Fig. 53 *V. canescens* larval section incubated with anti-VLP antibodies. Bound antibodies were visualised with FITC conjugated secondary antibodies. (A) Antibody labelling is visible on various tissue structures inside the larva and importantly on the surface of the cuticle (arrows). Note that the gut (g) lining is also labelled. (B) The same section under phase contrast show. The gut lumen appears to be filled with spongy tissue structures.


A
g

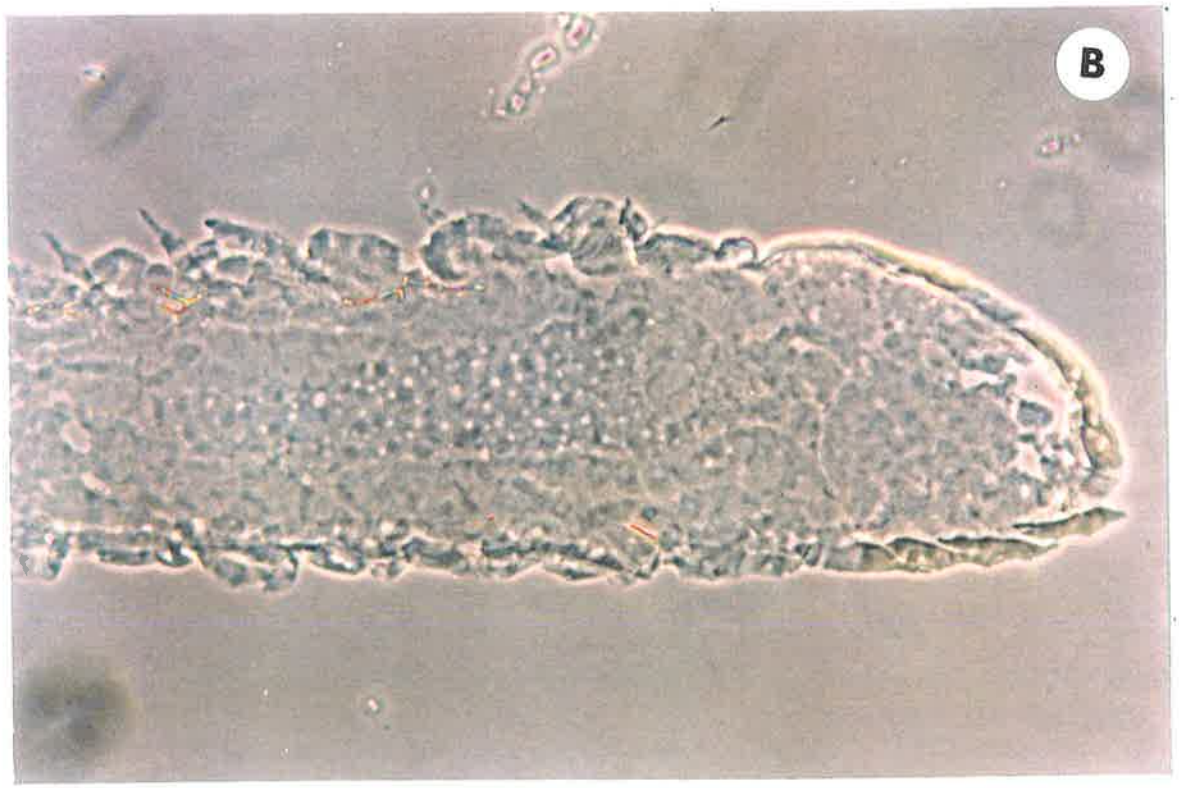
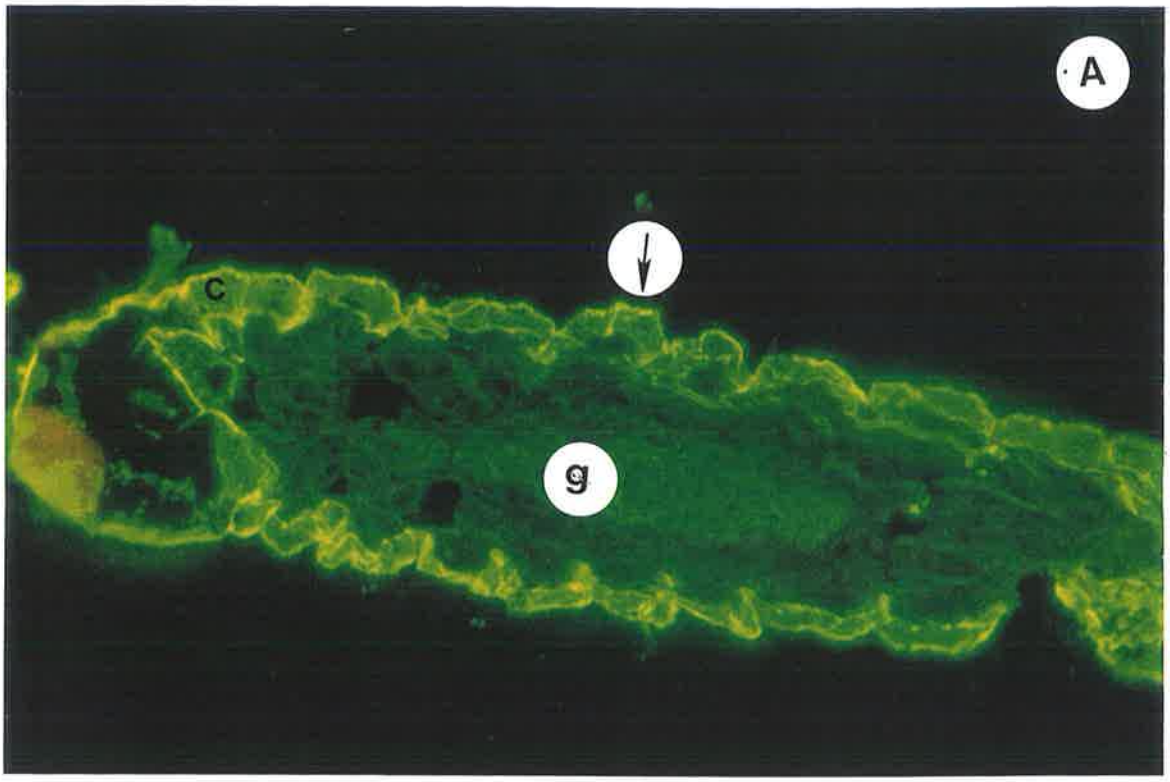

B

Fig. 54 Tissue section of *V. canescens* larva incubated with FITC-conjugated *H. p.* lectin. (A) Note, the surface of the larval cuticle (arrow) and at the head capsule is labelled but becomes obscured by auto-florescence of the sclerotised cuticle. The labelling in the gut is distinct above the spongy tissues inside the gut lumen. At later larval stages these cells melanise inside the gut. (B) The same section under phase contrast.


A
C
g

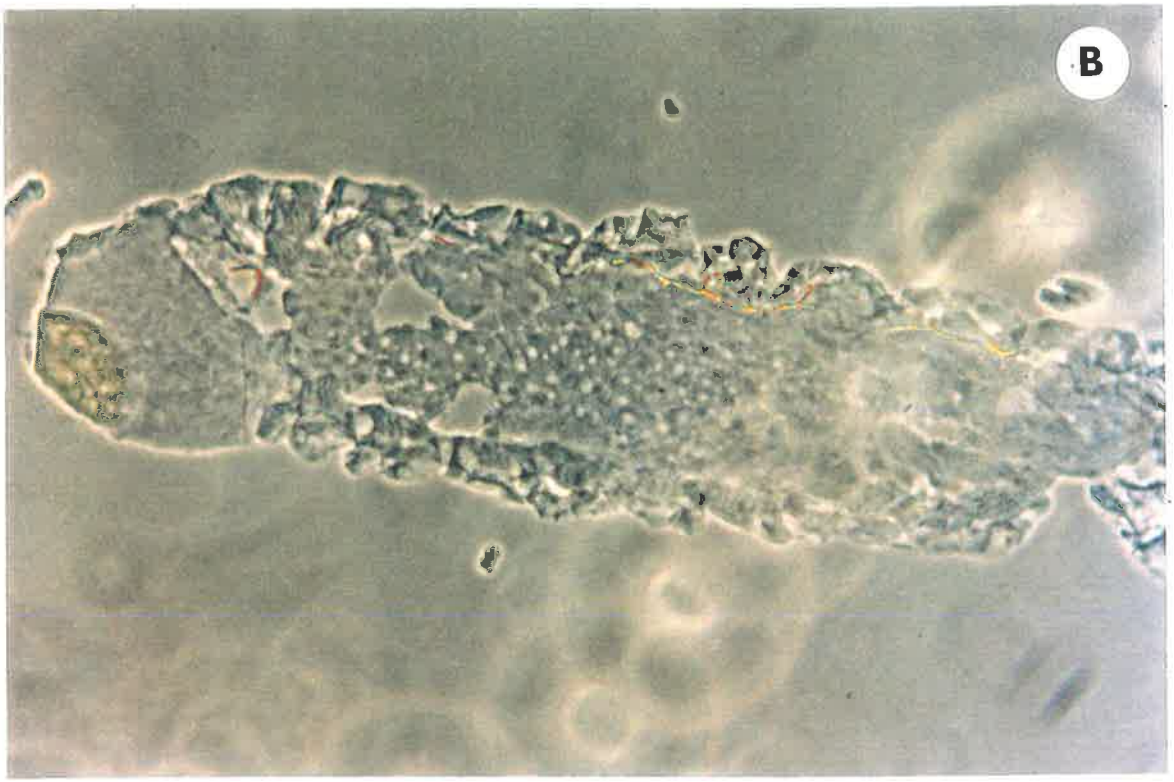
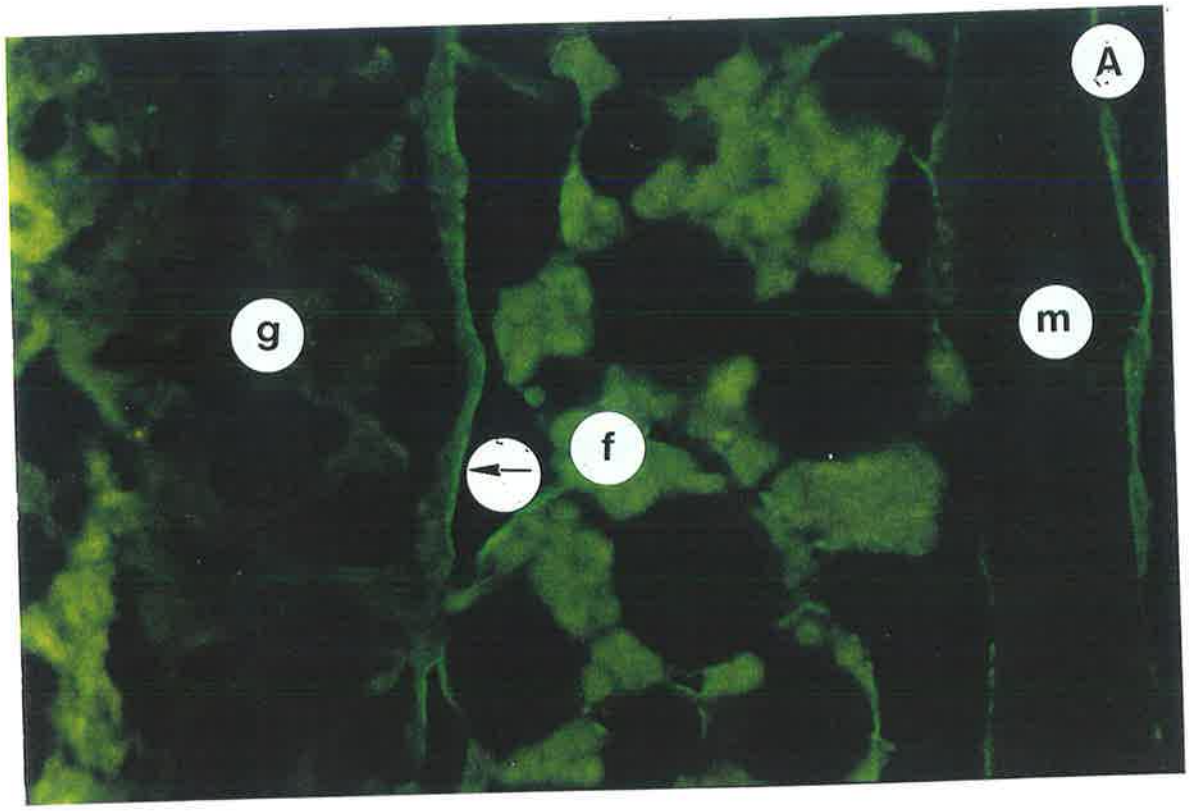

B

Fig. 55 Tissue section of a *V. canescens* larva shortly after leaving the host caterpillar. Sections were incubated with anti-VLP antibodies. Bound antibodies were visualised with FITC-conjugated secondary antibodies. (A) Section under indirect UV-light show labelling on hemolymph-exposed basement membranes of muscles (m), fat bodies (f) and gut (g). Note that the basement membrane of the gut is only labelled on the side which is exposed to the hemolymph (arrow). The gut lumen (g) has no FITC labelling. The yellow-green colour observed inside the gut lumen and the fat body cells are due to auto-fluorescence of unknown components. (B) The same section under phase contrast.


A
g
m
f

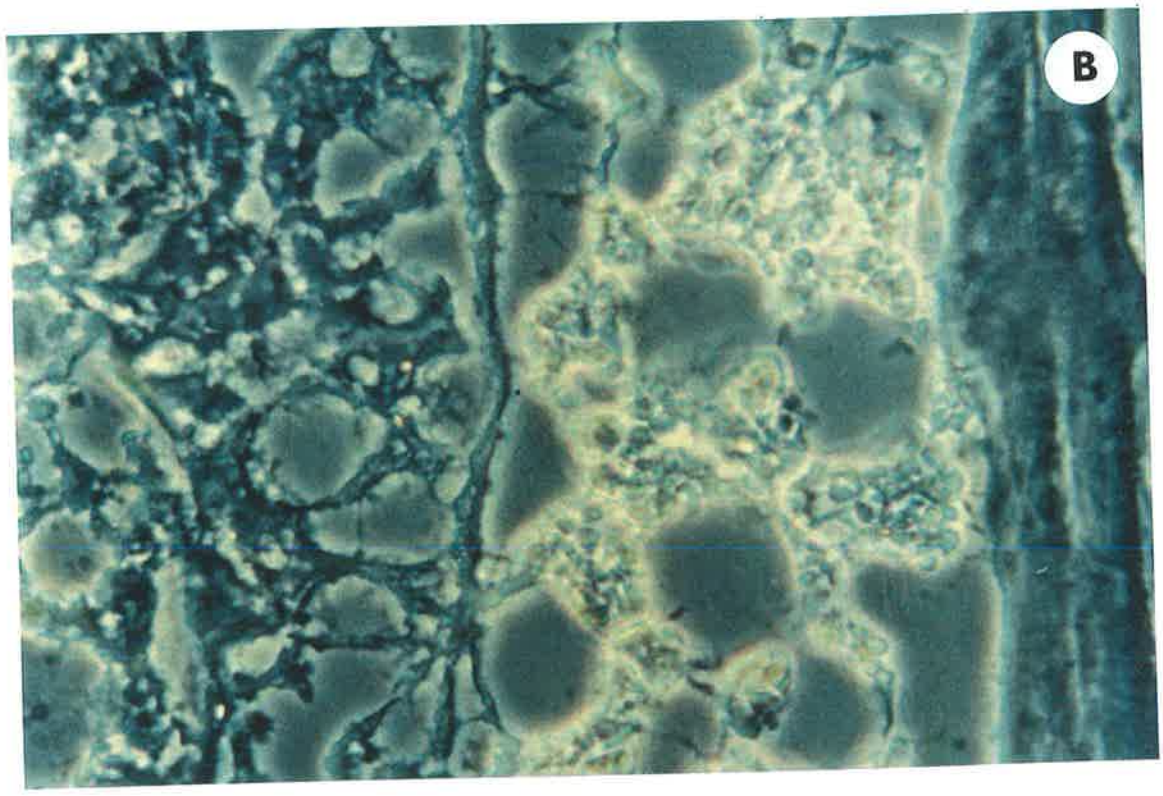
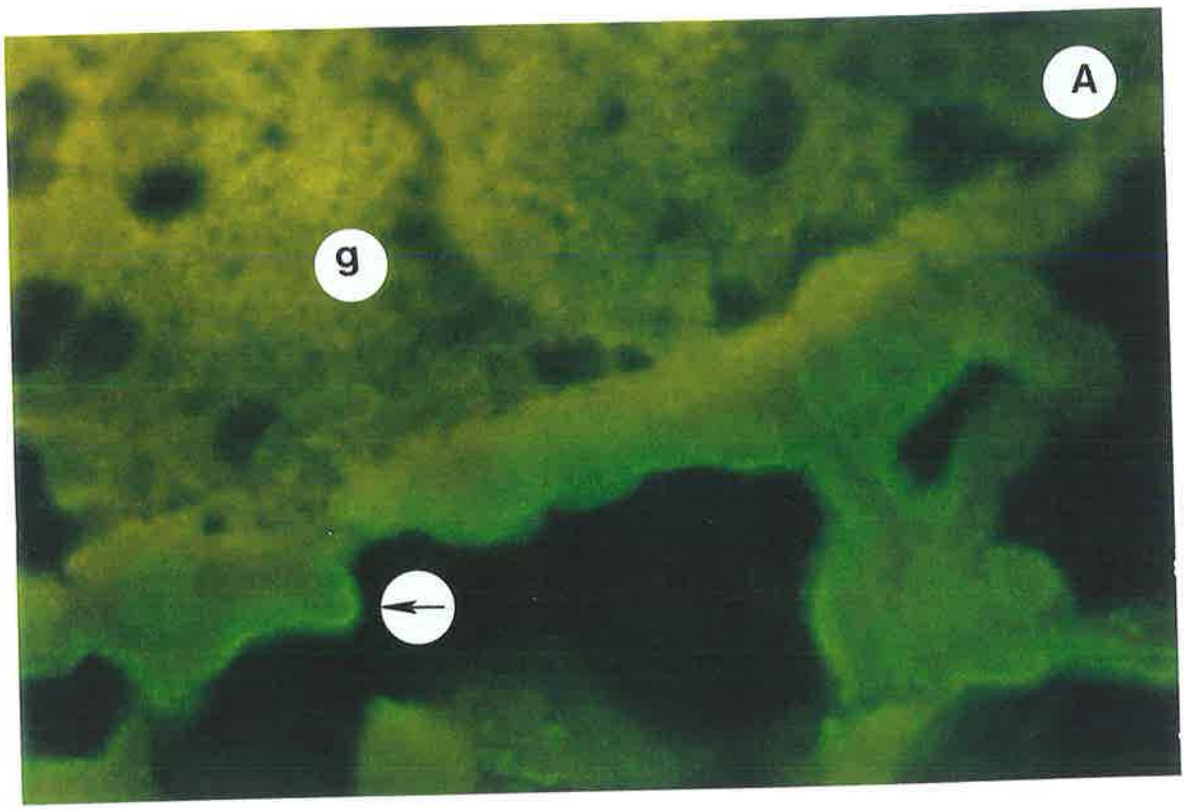

B

Fig. 56 Tissue section of a *V. canescens* larva after emergence (shortly before pupation). The section was incubated with anti-*E. kühniella* hemolymph antibodies. Bound antibodies were visualised with FITC conjugated secondary antibodies. (A) The labelling is only at basement membrane facing the hemocoel (arrow). Note that the melanised spongy tissues inside the gut lumen (g), showing yellow colour, is due to auto-florescence. In this late larval instar the gut lumen is full of cells at various stages of decay probably due to the change in feeding habit of the larva. (B) The same section under phase contrast.


A
g

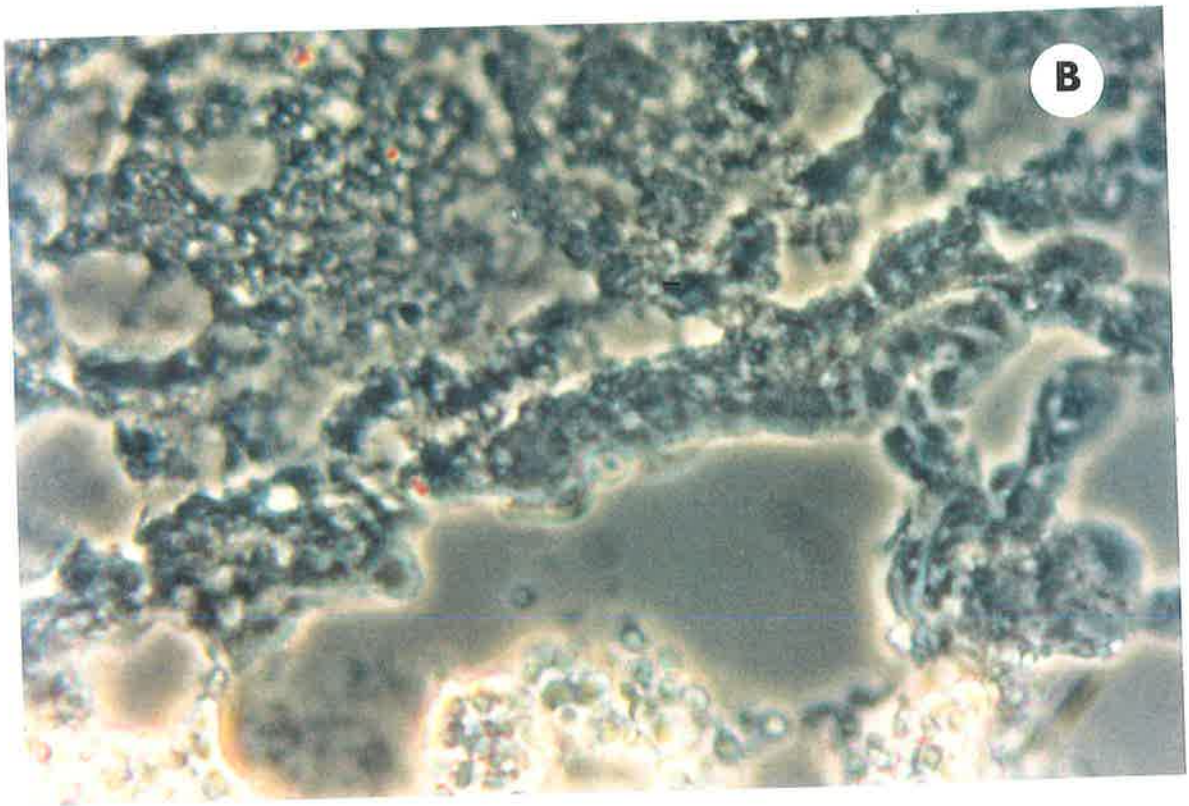

B

Fig. 57 Tissue section of *E. kühniella* late pupa incubated with FITC-conjugated *H. p.* lectin. (A) The epidermal cells (arrowhead) and the muscle surface (arrow) are covered with fat body cells or fat body remnants which are labelled by *H. p.* lectin. (B) The same section under phase contrast.

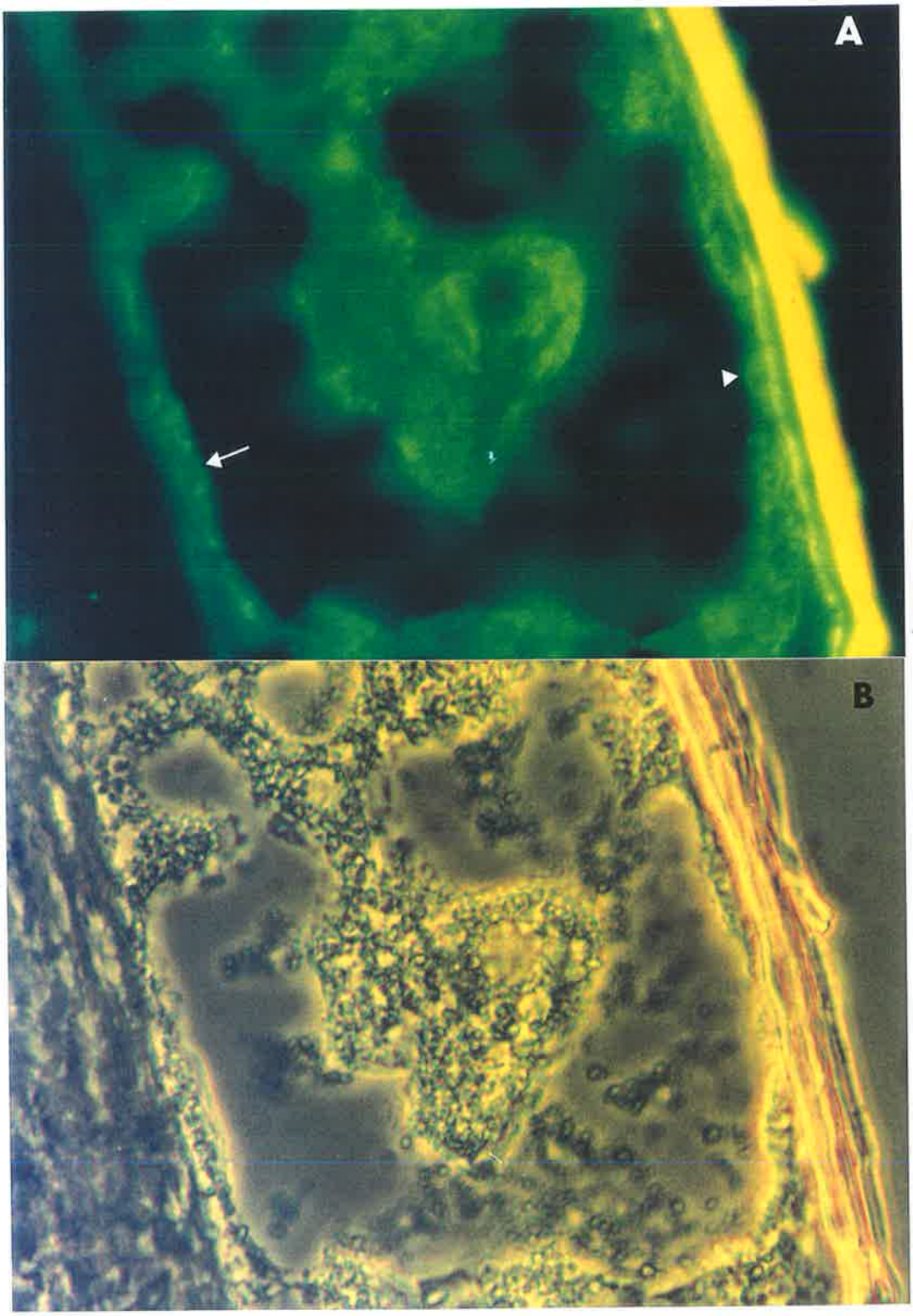

A
B

Fig. 58 Tissue section of *E. kühniella* late pupa incubated with FITC-conjugated *H. p.* lectin. (A) The fat body cells which have a crystal-like appearance (arrow) are relocated around the new adult tissues which are in the process of sclerotisation. The fat body cells are labelled. (B) Under phase contrast, the yellow colour of the sclerotised parts is due to auto-fluorescence.

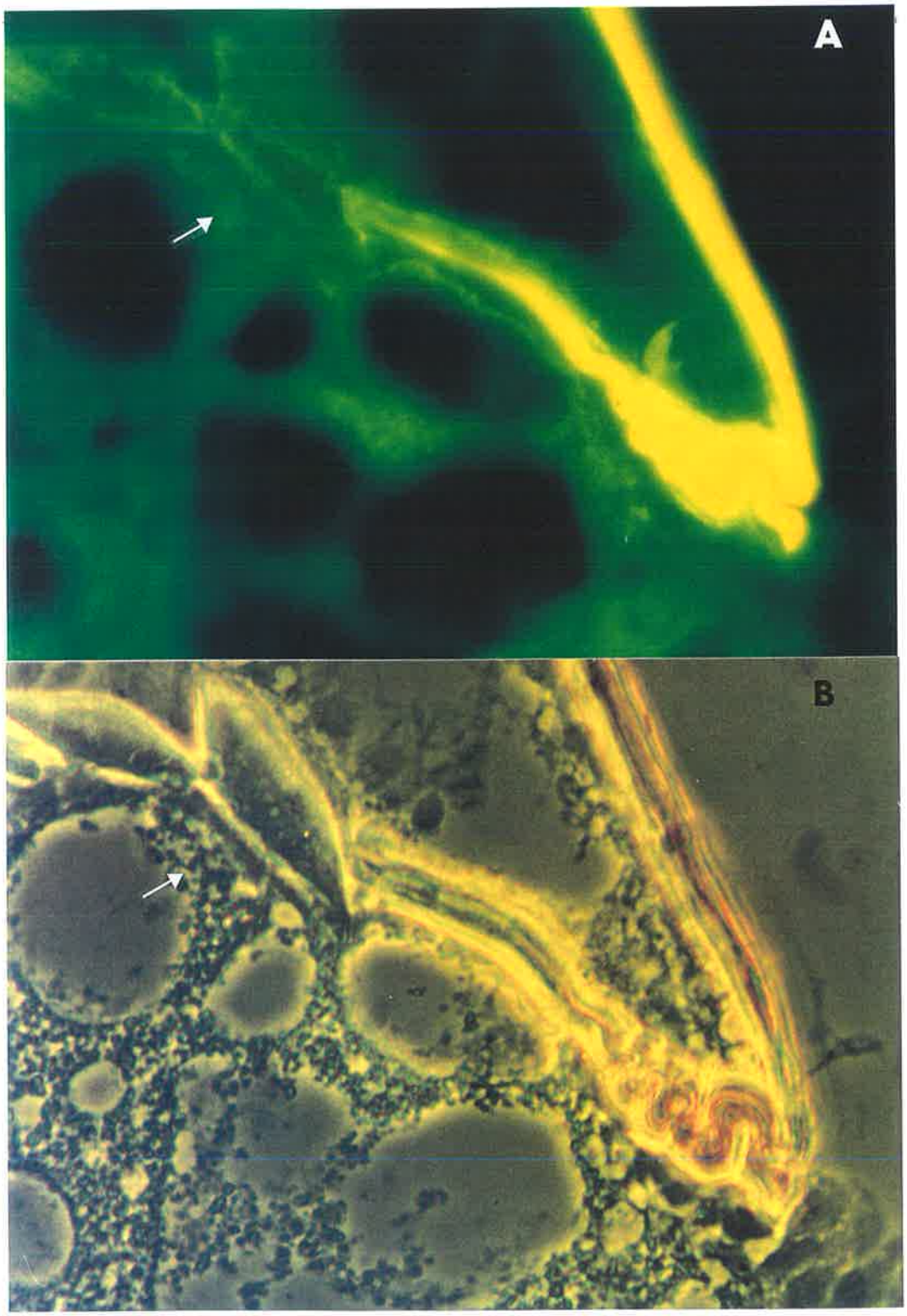

A
B

Fig. 59 Tissue section of an *E. kühniella* early pupa incubated with antibodies against a bacterial fusion protein encoded by the PHGPX-like domain of p40 (Hellers *et al.*, 1996). (A) Bound antibodies were visualised with FITC conjugated secondary antibodies. The section shows part of an epidermis (upper right) and fat body tissue (centre). The fat body is in a transition stage and the cells within the fat body begin to become labelled by the antibodies (arrowhead). The fat body tissue is still intact showing the labelling of the basement membrane (arrow). (B) The same section under phase contrast.

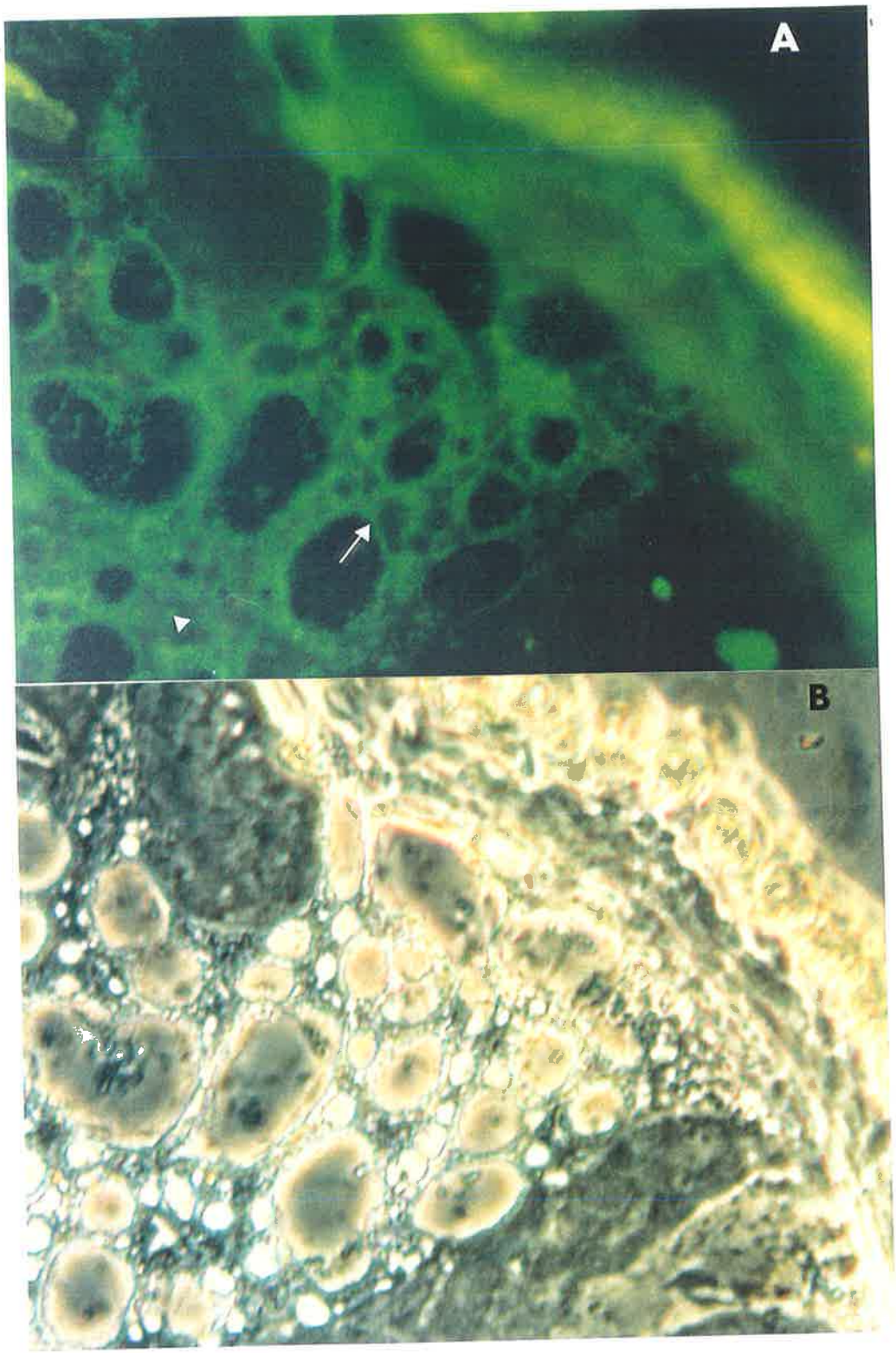

A
B

Fig. 60 Tissue section of *E. kühniella* pupa incubated with FITC-conjugated *H. p.* lectin. (A) The fat bodies, the membrane surrounding the trachea (t) and muscle (m) are labelled. (B) The same section under phase contrast.

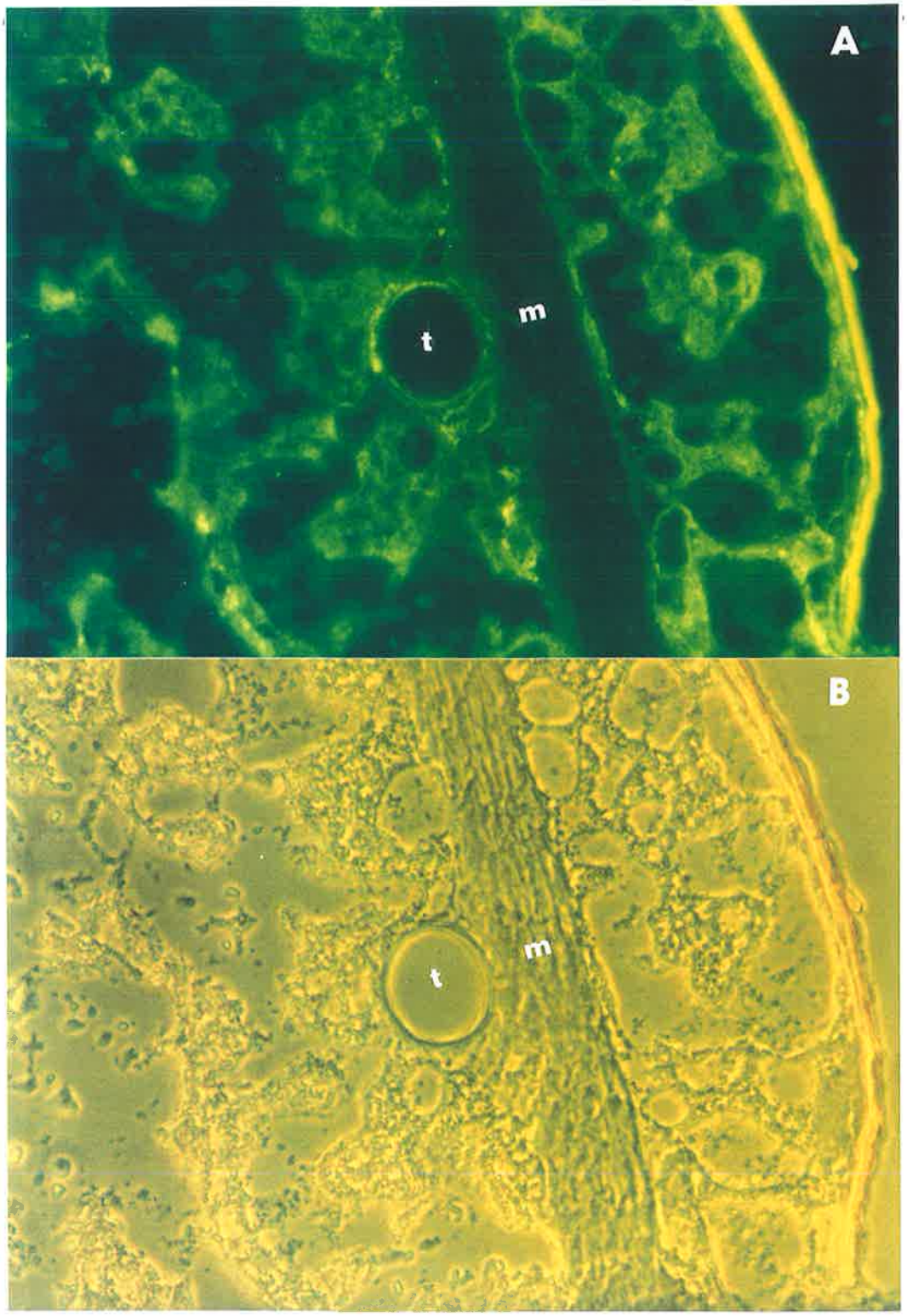

A
t
m
B
t
m

Fig. 61 Schematic representation of the location of hemomucin-like proteins in *V. canescens* and possible involvement in immune protection. The wasp ovaries on the left depict eggs in the process of passing through the calyx gland lumen and oviduct. The enlargement shows an egg chamber at late oogenesis, indicating the appearance of mucin-like glycoproteins on the chorion surface. A parasitised caterpillar depicting the developmental profile of the parasitoid is shown on the right. The egg increases in size due to host hemolymph uptake resulting in low VLPs density, relative to the chorion surface area. This reduction could be compensated by host hemolymph components. Similarly the larval mucin-like components on the cuticle could be covered by host hemolymph components. The emerged parasitoid larva at the posterior end of the caterpillar is covered by wasp-specific mucin-like glycoprotein and other components. These factors are probably derived from host hemolymph and show antigenic similarities to wasp VLP-components.

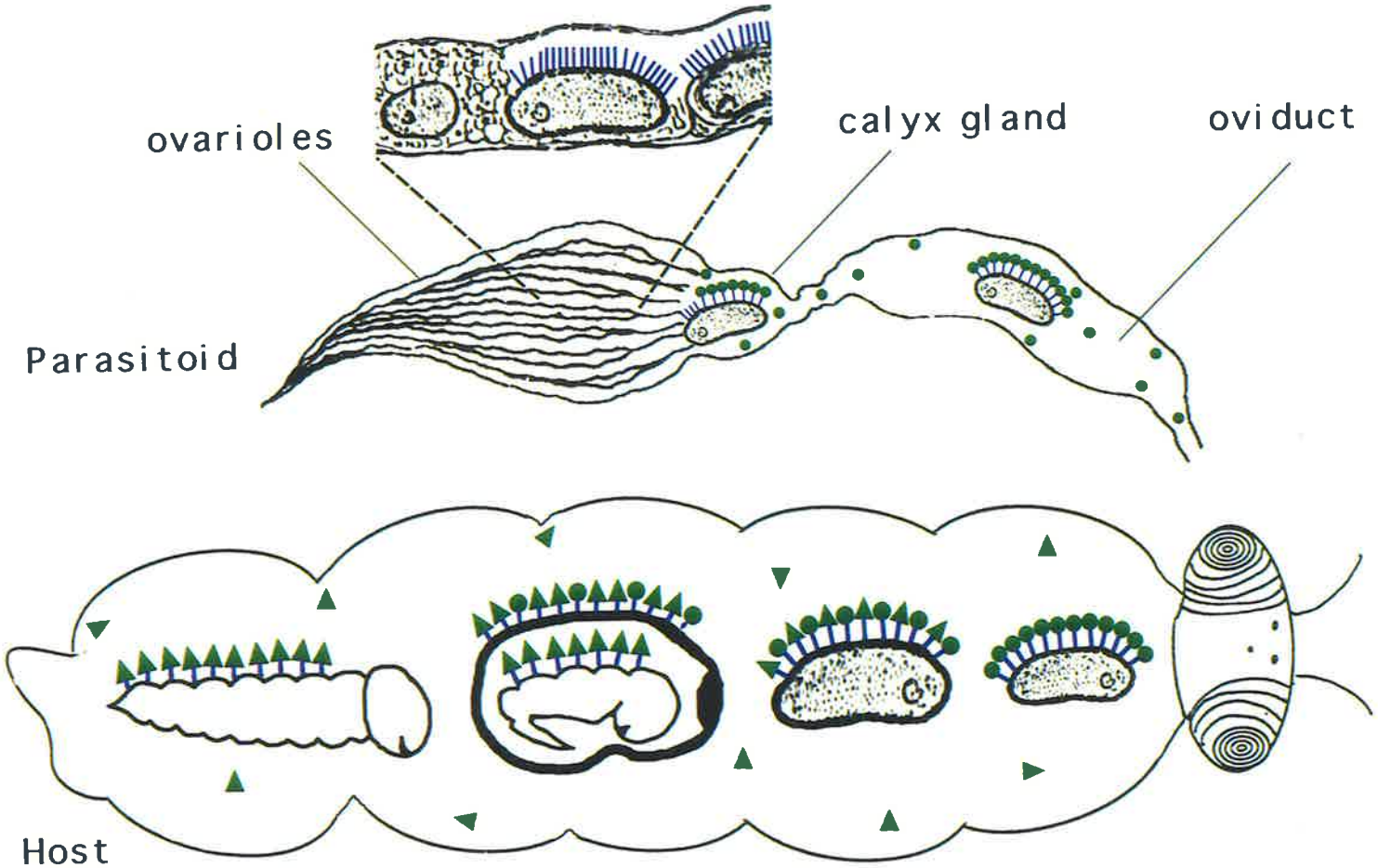

ovarioles
calyx gland
oviduct
Parasitoid
Host
Hemomucin-like components
Virus-like particles
Host components

Fig. 62 A Southern blot auto-radiograph of *D. melanogaster* (a-c) and *V. canescens* (d-f) genomic DNA. double digested with restriction enzymes, *Apa*I + *Sac*I, *Bam*HI + *Hin*dIII and *Eco*RI and *Pst*I, respectively. The Southern blot was hybridised to a *D. melanogaster* hemomucin fragment pDMu-105. Molecular size markers in kilobase pairs are shown on the left.

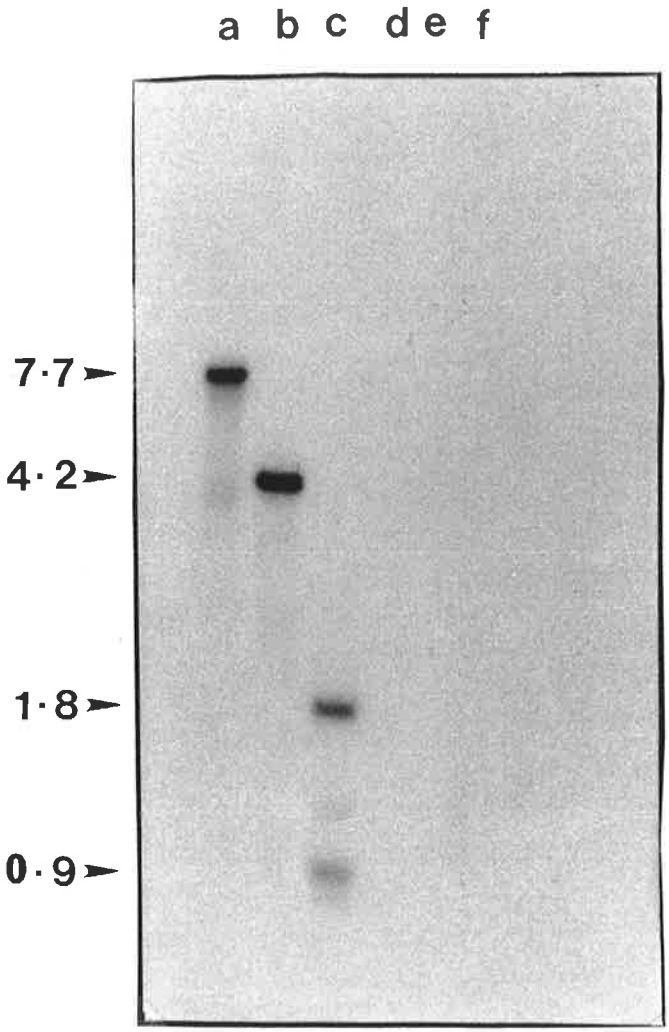

a b c d e f
7·7
4·2
1·8
0·9

Fig. 63 Schematic diagram of mucin primer positions and the *D. melanogaster* hemomucin fragment pDMu-105, used for hybridisation in relation to the *D. melanogaster* hemomucin.

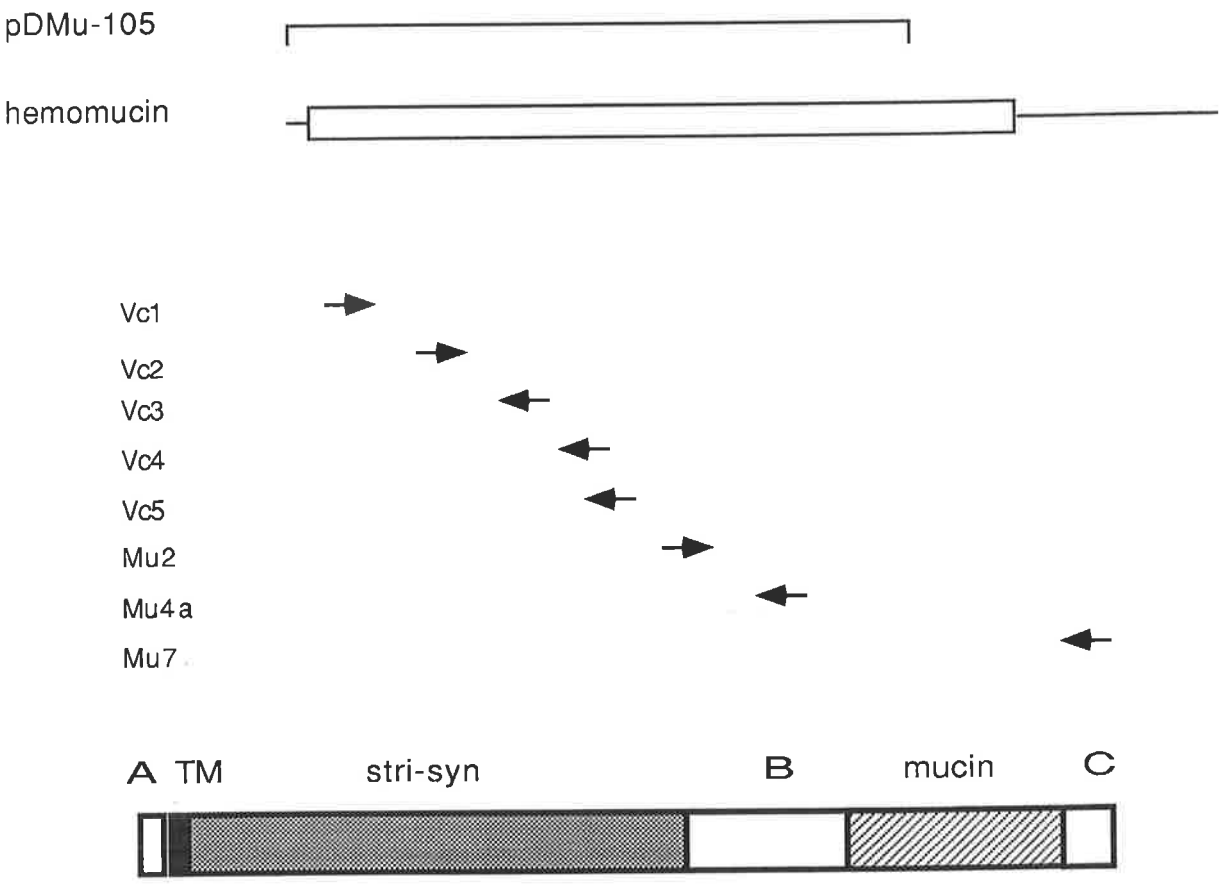

pDMu-105
hemomucin
Vc1
Vc2
Vc3
Vc4
Vc5
Mu2
Mu4a
Mu7
A TM
stri-syn
B
mucin
C

Fig. 64 Location of primers used on genomic DNA and cDNA of *V. canescens* and *D. melanogaster* genomic DNA in relation to hemomucin protein sequence.

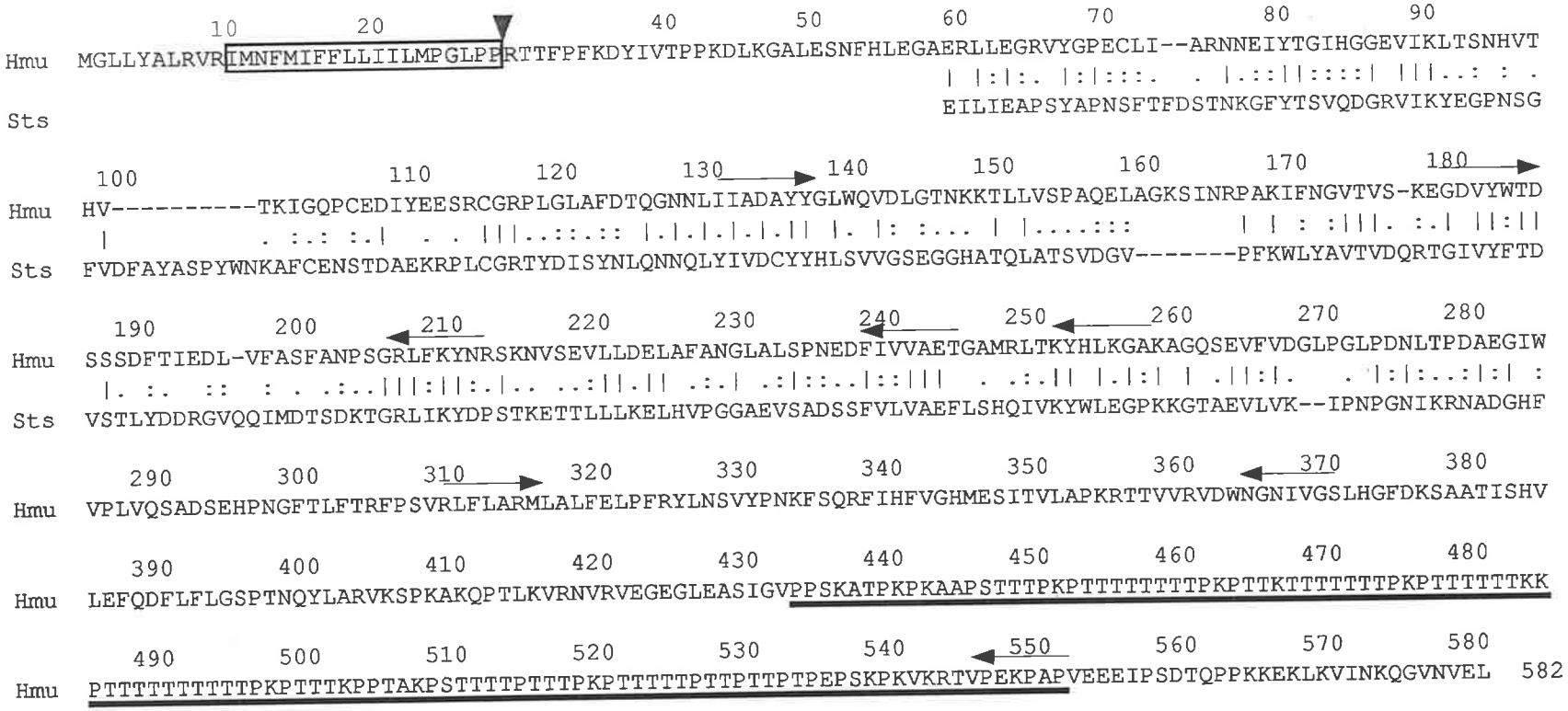

10 20 40 50 60 70 80 90
Hmu MGLLYALRVRIMNFMIFFLLIILMPGLPPRTTFPFKDYIVTPPKDLKGALESNFHLEGAERLLEGRVYGPECLI--ARNNEIYTGIHGGEVIKLTSNHVT
Sts EILIEAPSYAPNSFTFDSTNKGFYTSVQDGRVIKYEGPNSG
100 110 120 130 140 150 160 170 180
Hmu HV----------TKIGQPCEDIYEESRCGRPLGLAFDTQGNNLIIADAYYGLWQVDLGTNKKTLLVSPAQELAGKSINRPAKIFNGVTVS-KEGDVYWTD
Sts FVDFAYASPYWNKAFCENSTDAEKRPLCGRTYDISYNLQNNQLYIVDCYYHLSVVGSEGGHATQLATSVDGV-------PFKWLYAVTVDQRTGIVYFTD
190 200 210 220 230 240 250 260 270 280
Hmu SSSDFTIEDL-VFASFANPSGRLFKYNRSKNVSEVLLDELAFANGLALSPNEDFIVVAETGAMRLTKYHLKGAKAGQSEVFVDGLPGLPDNLTPDAEGIW
Sts VSTLYDDRGVQQIMDTSDKTGRLIKYDPSTKETTLLLKELHVPGGAEVSADSSFVLVAEFLSHQIVKYWLEGPKKGTAEVLVK--IPNPGNIKRNADGHF
290 300 310 320 330 340 350 360 370 380
Hmu VPLVQSADSEHPNGFTLFTRFPSVRLFLARMLALFELPFRYLNSVYPNKFSQRFIHFVGHMESITVLAPKRTTVVRVDWNGNIVGSLHGFDKSAATISHV
390 400 410 420 430 440 450 460 470 480
Hmu LEFQDFLFLGSPTNQYLARVKSPKAKQPTLKVRNVRVEGEGLEASIGVPPSKATPKPKAAPSTTTPKPTTTTTTTTPKPTTKTTTTTTTPKPTTTTTTKK
490 500 510 520 530 540 550 560 570 580
Hmu PTTTTTTTTTTPKPTTTKPPTAKPSTTTTPTTTPKPTTTTTPTTPTTPTTPTPEPSKPKVKRTVPEKPAPVEEEIPSDTQPPKKEKLKVINKQGVNVEL 582

Fig. 65 Southern blot auto-radiogram of PCR reaction of *V. canescens* DNA (lane a and c) and *D. melanogaster* (lane b and d) genomic DNA. The primers used were, forward primer Mu2, and reverse primers Mu4a (lane a and b) and Mu7 (lane c and d) designed from *D. melanogaster* hemomucin (see Figs. 63 and 64). Molecular size markers in kilobase pairs are shown on the left.

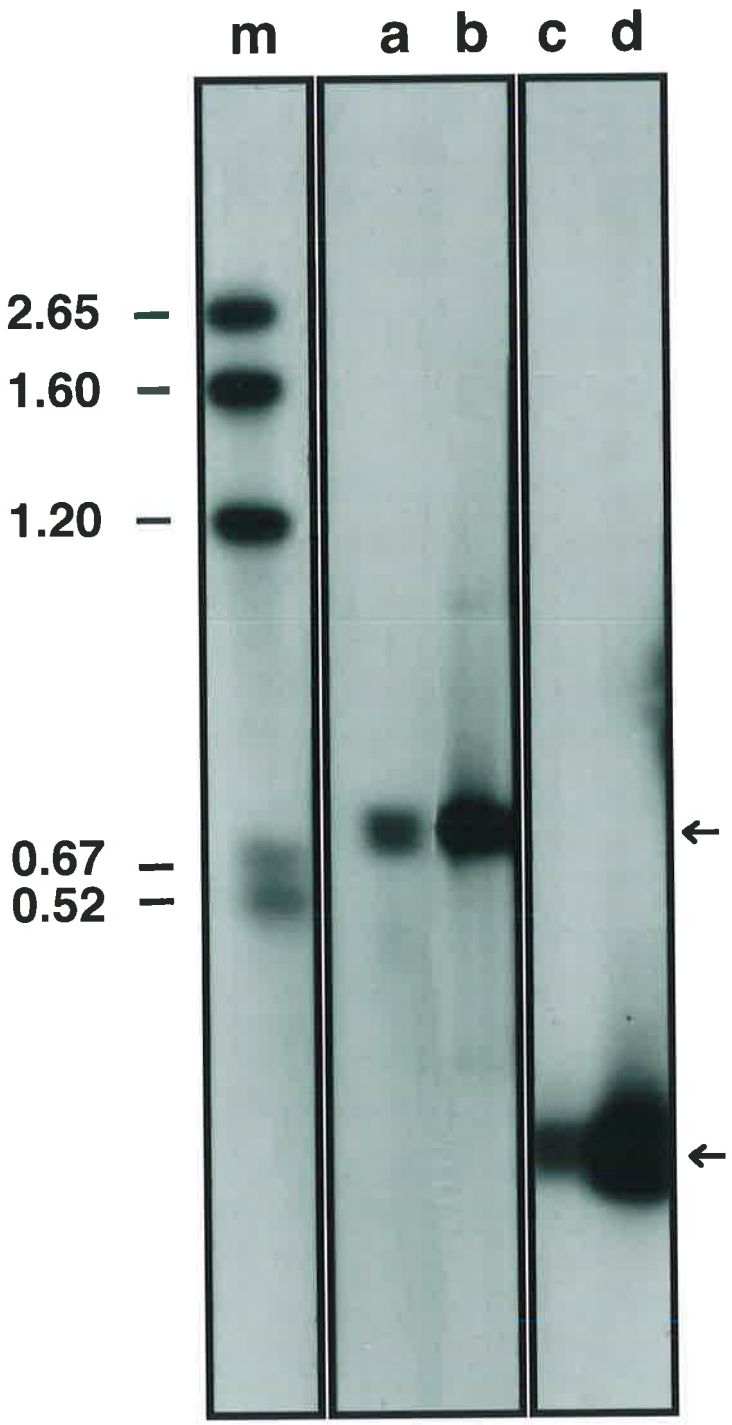

m
a
b
c
d
2.65
1.60
1.20
0.67
0.52

Fig. 66 The figure shows an agarose gel of the PCR reaction on *V. canescens* cDNA using the following primers: Forward Primer Vc1 and reverse primers Vc3 (a), Vc4 (b), Vc5 (c), mu4a (d) and mu7 (e). Forward primer Vc2 and reverse primers Vc3 (f), Vc4 (g), Vc5 (h), mu4a (i) and mu7 (j), (see Figs. 63 and 64) for primer positions. Molecular size markers in kilobase pairs are shown on the left.

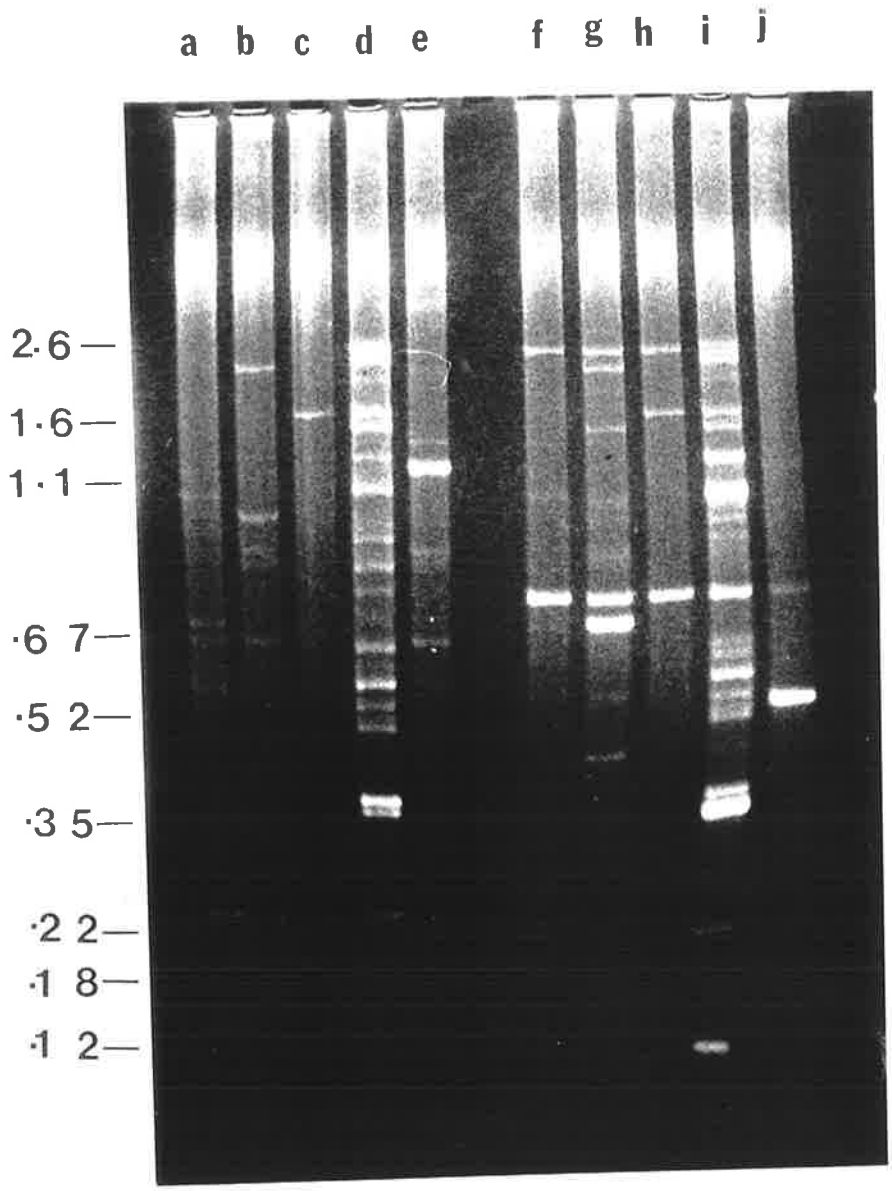
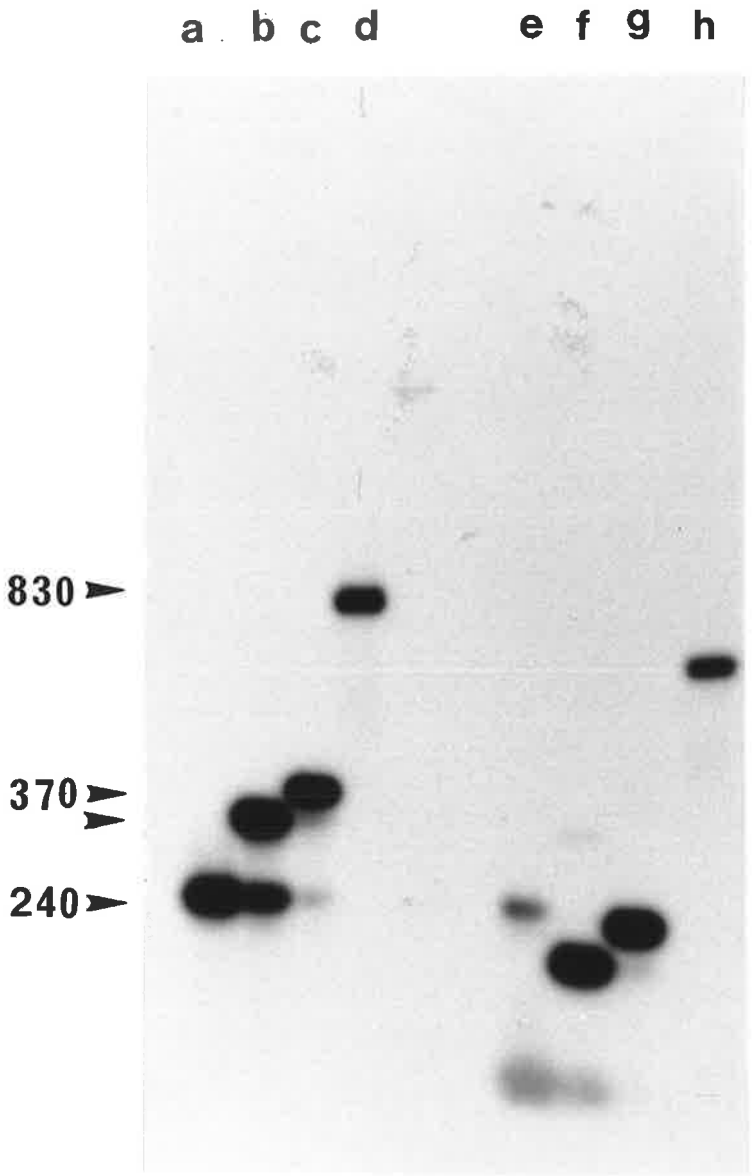

a b c d e f g h i j
2·6
1·6
1·1
·6 7
·5 2
·3 5
·2 2
·1 8
·1 2

Fig. 67 The figure shows a Southern blot of the gel in Fig. 66. The membrane was probed with hemomucin DNA fragment pDMu-105 tagged with ^{32}P. Molecular size markers in base pairs are shown on the left.


a b c d e f g h
830
370
240

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