THE EFFECT OF APHID LETHAL PARALYSIS VIRUS (ALPV) ON THE BIOLOGY OF GRAIN APHIDS IN THE WESTERN CAPE

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CERTIFICATION OF SUPERVISOR

In terms of paragraph GP 8 in "General rules for the Degree of Doctor of Philosophy (PhD)", I as supervisor of the candidate, Jacobus Martin Laubscher, certify that I approved of the incorporation in this dissertation of material that has already been submitted or accepted for publication.

Signed

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ABBREVIATIONS

A_{260}	Absorbance at 260 nm
ALPV	Aphid lethal paralysis virus
B. brassicae	*Brevicoryne brassicae*
BSA	bovine serum albumin (Fraction V)
cDNA	complementary DNA
CrPV	cricket paralysis virus
cv	cultivar
D. noxia	*Diuraphis noxia*
DAS-ELISA	double-antibody sandwich ELISA
DCV	*Drosophila* C virus
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein iso-thiocyanate
GAR	goat anti-rabbit
GLAD	gold labelled antibody decoration
hr	hour(s)
IgG	Gamma-globulin fraction of serum
ISEM(IEM)	Immuno(sorbent) electron microscopy
K	kilodalton
KBV	Kashmir bee virus
M	molar
m	meter
M. dirhodum	*Metopolophium dirhodum*
M. persicae	*Myzus persicae*
min	minute(s)
mM	millimolar
M_r	relative molecular mass
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PEG	polyethylene glycol
R. padi	*Rhopalosiphum padi*
RhPV	*Rhopalosiphum padi* virus
RNA	ribonucleic acid
rpm	revolutions per minute
S	Svedbergs
S. avenae	*Sitobion avenae*
S. graminum	*Schizaphis graminum*
SBV	sacbrood virus
SDS	sodium dodecyl sulphate
ss	single-stranded
TEM	Transmission electron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
TMV	tobacco mosaic virus
Tris	Tris (Hydroxymethyl)-aminomethane
μ	micron
uv	ultraviolet
VLP	Virus-like particle
v/v	volume per volume ratio
v/w	volume per weight ratio

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ABSTRACT

Aphid lethal paralysis virus (ALPV) could be detected by indirect immunofluorescent technique in dissected aphids. This technique was found to be more sensitive when compared to DAS-ELISA. The choice of a sensitive, low cost detection method was of importance to test for low levels of virus in infected aphid body tissues where inapparent infection could cause detection problems.

ALPV was visualized in ultrathin sections of diseased aphid body tissues by immunocytochemistry utilizing immunogold label. ALPV antigen was detected in the ovariole tissue, tracheocytes, symbionts of the mycetocytes, fat body cells, brain tissue, nerve tissue and stomach epithelial tissue. Virions were detected predominantly in the cytoplasm but were also found in the nucleus. ALPV antigen was not detected in muscle fibres or mitochondria.

ALPV and *Rhopalosiphum padi* virus (RhPV) are transmitted transovarially. Different incidences of transmissions of ALPV were obtained for *R. padi* (29%) and *Sitobion avenae* (16%) and ALPV infections dramatically reduced the longevity and fecundity of these aphids. Infected apterous *R. padi* aphids were more fecund than alate aphids of the same clone. The percentage of viral infections in different aphid species (*R. padi*, *S. avenae* and *Diuraphis noxia*) was positively associated with temperature; higher temperatures dramatically increased the incidence of ALPV and RhPV and *vice versa*.

The influence of ALPV on a natural *R. padi* aphid population was found to reduce the population size by 49%. This reduction coincided with a high death factor (70) of aphids per plant. A dramatic decline in *R. padi* aphid numbers and a high incidence of ALPV present in this aphid population was experienced. Parasitic fungal infections peaked at a later stage than ALPV, and a level of 21 parasitized aphids per plant was reached during this period. This appears to indicate that the presence of ALPV contributes to limit population development in *R. padi* aphids. Similar results were obtained with *S. avenae* aphids.

Based on this data, ALPV could be considered as a major growth limiting factor in the development of small grain aphid populations in the western Cape. If the presence of the virus is taken into consideration, it could influence pest management strategies directly.

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 HISTORICAL BACKGROUND

Aphids are undoubtedly the most important insects with pest status in agricultural crops of the temperate climatic zones (Dixon, 1985). They are economically important because of their ability to cause damage by withdrawing essential nutrients from phloem tissue, their ability to transmit plant viruses responsible for numerous serious crop diseases and for their phytotoxic effect.

The arrival of the Russian wheat aphid *Diuraphis noxia* in South Africa in 1978, highlighted aspects of aphid phytotoxicity (Aalbersberg, 1987) and aphid transmitted viruses in small grains (Von Wechmar & Rybicki, 1981; Rybicki & Von Wechmar, 1982; Von Wechmar & Rybicki, 1984). Wheat aphids, commonly found in association with small grain production in the western Cape and eastern and western Orange Free State, play an important role as vectors of plant viruses such as barley yellow dwarf virus (BYDV) (Von Wechmar, 1990a & b; Rybicki *et al.*, 1990). Essentially six aphid species, *Rhopalosiphum padi*, *R. maidis*, *Metopolophium dirhodum*, *Diuraphis noxia*, *Schizaphis graminum* and *Sitobion avenae*, form the most important group of economically important insects for small grain production in South Africa (Annecke & Moran, 1982).

Modern agricultural management practices alone are not sufficient to control aphid infestations without some loss in crop yield. For several decades agriculture relied heavily on harmfull chemical pesticides with little concern for the survival of other beneficial insects. The current trend to pay more attention to biological control agents such as native disease pathogens and natural predators, is environment friendly and needs to be explored for every insect with pest status.

Diseases of aphids have been recognized for more than a century (Latgé & Papierok, 1987). Different bacterial and protozoan pathogenic infections have not been conclusively demonstrated in aphids, but recently two picornaviruses were discovered and described. They are *Rhopalosiphum padi* virus (RhPV) which can be transmitted transovarially and decreases the longevity of the infected individuals (D'Arcy *et al.*, 198la & b; Williamson *et al.*, 1989) and aphid lethal paralysis virus (ALPV) which causes paralysis and death in *R. padi* and *D. noxia* colonies (Williamson *et al.*, 1988). Several other viruses have been noted in aphids, but none have been characterized in any detail (Parrish & Briggs, 1966; Kitajima, 1976;

Peters, 1965; Fraval & Lapierre, 1970; Allen & Ball, 1990). Aphid species found to be infected by RhPV in South Africa are *R. padi*, *S. graminum*, *M. dirhodum*, *R. maidis* and *D. noxia* (Von Wechmar & Rybicki, 1981; Rybicki & Von Wechmar, 1984) and by ALPV include *R. padi*, *M. dirhodum*, *S. graminum*, *D. noxia* and *S. avenae* (Williamson, 1988). ALPV and RhPV occur as either single- or double infections in aphids and have been detected in aphids collected from all the major small grain producing regions in South Africa (Rybicki, 1984; Williamson, 1988; Von Wechmar, unpublished results).

1.2 PROJECT OUTLINE

The current investigation was undertaken to obtain more information on the viral infection process of RhPV and ALPV in aphid tissues and to study the effect of virus infection under natural conditions.

For laboratory studies aphids were frequently introduced from the field and maintaining and containing rapidly multiplying aphid colonies was problematic, the most serious aspect being the fact that incorrect holding conditions of diseased aphids could lead to the rapid death of the host. This was overcome by holding small colonies at low temperature on plants in canning bottles fitted with gauze tops or self contained incubators. This procedure was found to be suitable for diseased aphids (Von Wechmar, 1990c). Attempts to grow ALPV and RhPV in tissue culture have been unsuccessful (Williamson, 1988).

On starting this project it was essential to establish the identity of the viruses isolated from laboratory established aphid colonies and from field collected aphids. In Appendix B, techniques utilized to purify, characterize and quantify ALPV and RhPV are described. Physical and serological characterization of several ALPV and RhPV isolates were done to positively identify the viruses used in this study and to detect the presence of ALPV and RhPV infection in aphid clones used in the subsequent experiments.

Insect viruses, in particular picornaviruses, are contagious and ubiquitous in nature but are often not detectable using standard serological screening methods (Scotti *et al.*, 1981; Williamson, 1988). To overcome this problem a more sensitive fluorescent antibody labelling system on dissected whole aphids (tissue non-destructive) was developed and was utilized to detect viral antigen in tissues of dissected aphids (Chapter 2). In chosing this option as apposed to more sensitive molecular biological techniques such as *in situ* cDNA hybridization, cost, time and the numbers that were processed, were a major consideration. Hatfill *et al.* (1990)

previously showed that cDNA hybridization with an ALPV probe could be applied successfully.

It was of interest to determine which organs and tissues of the aphid were affected most by the viruses and to identify possible sites of virus replication and accumulation. For this purpose the immunogold labelling technique was applied on ultrathin sections utilizing virus specific antiserum (Chapter 3).

Earlier observations of the effect of low temperatures on the survival of virus diseased aphids, initiated a study of the effect of temperature regimes on the biology of diseased aphids. For this purpose a study of the effect of different temperature regimes on known aphid populations under laboratory conditions was conducted. The level of viral infection in the progeny was also investigated (Chapter 4).

To determine whether aphid infecting viruses could play a role as natural biological control agents, an assessment was made of ALPV as a growth limiting factor of natural aphid populations in the presence of other natural enemies and pathogens (Chapter 5).

1.3 LITERATURE REVIEW

1.3.1 Introduction to the anatomy and reproductive physiology of the Aphidoidea

Aphids or Aphididae belong to the large order of hemimetabolous insects (that is, insects with an incomplete metamorphosis) known as the Hemiptera, or bugs. They are of the sub-order Homoptera, which includes scale insects, frog-hoppers, leaf-hoppers and white-flies. One distinct feature of this group is their polymorphism. This means that within one single species several distinctly different forms of individuals are produced. These various forms are called morphs. Another aspect of aphid polymorphism is that adult aphids can be winged (alate) or wingless (apterous), depending on environmental conditions (Lees, 1967). In the winged forms, their wings are membranous.

The known fossil aphids are largely from the Tertiary. There are suggestions that the main stock may have arisen in Permian or Carboniferous times (Heie, 1987). Only 125 fossil species have been described. This is a small number if one compares this with the well over 4000 aphid species described, and if one takes into account the time elapsed since the Triassic, when the oldest aphid wing known, petrified.

Some aphid species have asexual morphs, alternating with sexual morphs. Aphids, especially those in the tropics or in the countries with mild winters, seem to have discontinued the sexual phase of reproduction (Dixon, 1987a). Parthenogenetic reproduction started in aphids in the Permian, and has been important in determining their population structure and high rates of increase. This lead to the clonal polymorphism and telescoping of generations i.e. even before a young aphid is born it already has the embryos of the next generation (Dixon, 1985). This enables aphids to achieve extremely high reproduction rates (Dixon, 1987a).

1.3.1.1 General morphology of the body

The aphid body is comprised of a head, thorax and abdomen. Aphids have a simple internal morphology which is dominated by the digestive system and the reproductive system with embryos in various stages of development (Fig. 1.1).

Dominant exterior features of the head are antennae, eyes and a proboscis. The proboscis is comprised of the labium which contains the stylets, the most anterior

part of the alimentary tract.

The sucking pump occupies most of the antero-dorsal region of the head. The brain or protocerebrum occupies the dorsal region of the head and is comprised of two optic lobes. The brain extends into the thoracic region in the form of the suboesophageal ganglion which is connected to the brain by means of the thoracic ganglion mass (Hardie, 1987a). Inside this region there are also the salivary glands, the oesophagus and the anterior region of the stomach (Ponsen, 1972; 1987).

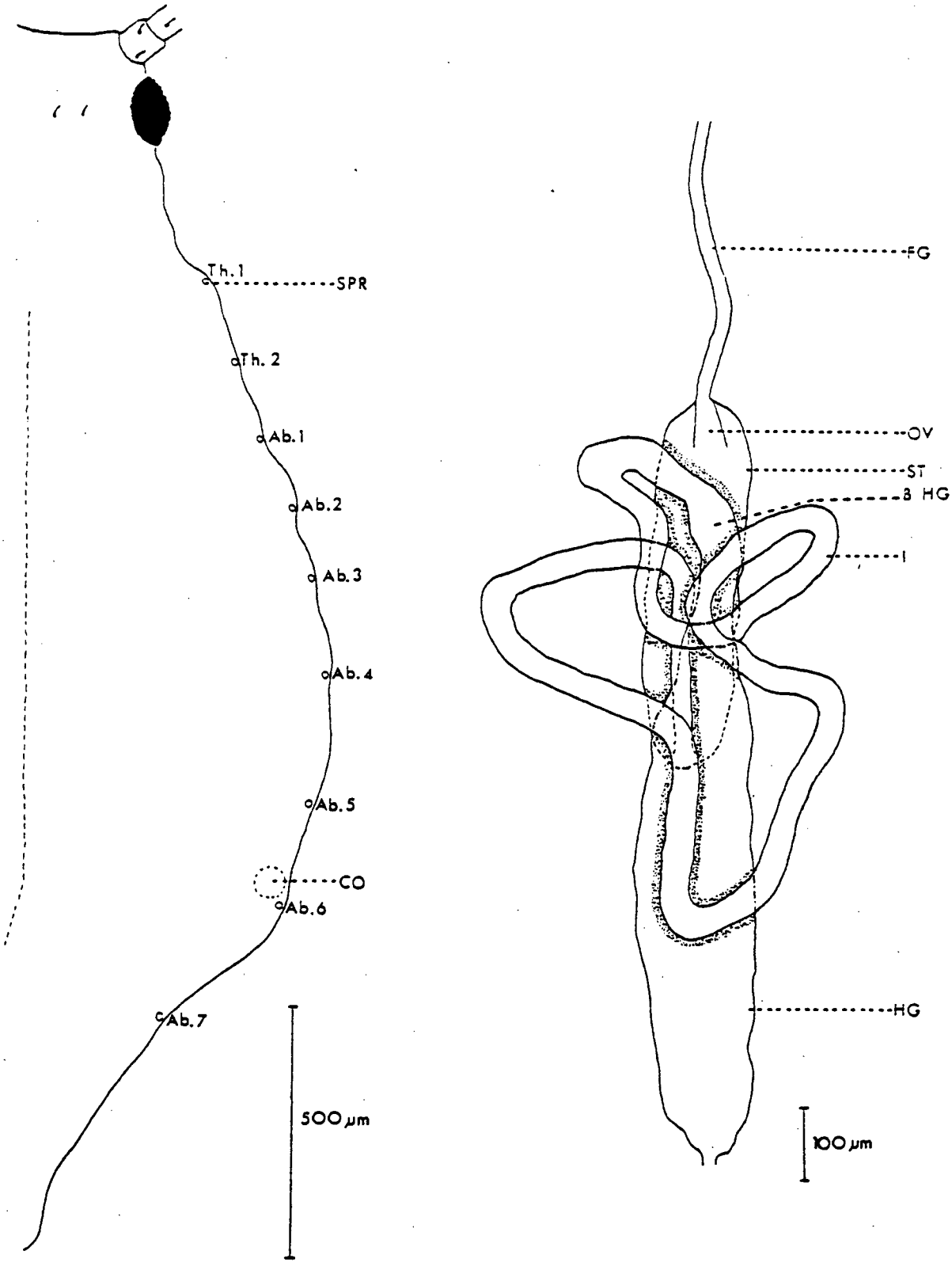


Fig.1.1. Graphical reconstuction of alimentary canal of *S. avenae* (ventral view). Ab, abdominal spiricle; B HG, beginning of hindgut; CO, cornicle; FG, foregut; HG, hindgut; I, intestine; OV, oesophageal valve; SPR, spiracle; ST, stomach; Th., thoracic spiracle (from Laubscher, 1985).

1.3.1.2 **Mouthparts**

The mouthparts of aphids consist of two pairs of flexible stylets, a labium, and labrum. The labrum is short and triangular. The labium arises postero-ventrally and when not in the feeding position, extends posteriad along the thoracic sternum between the coxae (Forbes, 1977).

The stylet bundle is comprised of an inner maxillary pair and an outer mandibular pair. The opposing grooves of the stylets form minute food and salivary canals. The stylet bundle lies in a groove along the anterior surface of the labium when at rest. The labium telescopes within the wide basal segment when the aphid is feeding, allowing the stylets to emerge from the tip to penetrate the plant tissue (Forbes, 1977). After penetration of the epidermis, the stylets penetrate different cell layers in the leaf until they reach the phloem tissue or sieve tube. The maxillary stylet is inserted through these cell walls. The saliva, which passes through the salivary canal to the tip of the stylet bundle, assists penetration. An enzyme, pectin polygalacturonase, helps the stylets to penetrate different plant cells (Pollard, 1977). Stylets also play an important role in virus transmission.

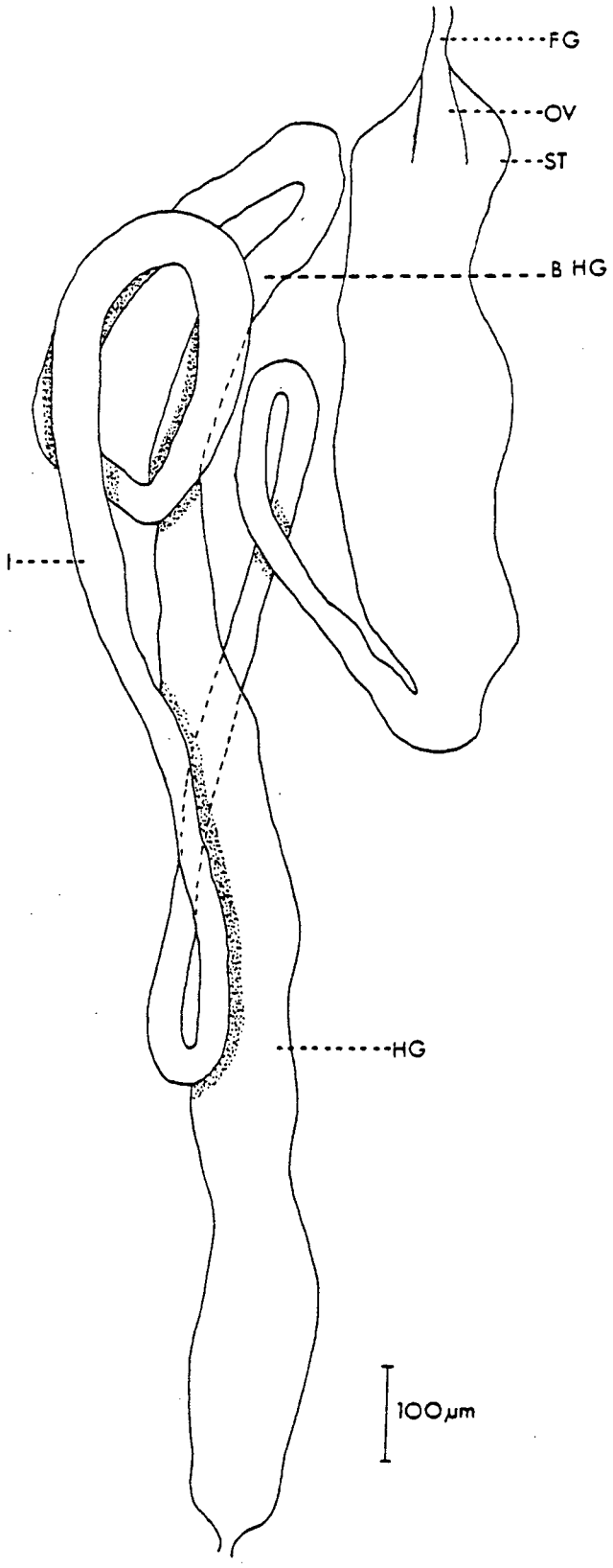


Fig. 1.2. Exploded view of graphic reconstruction of alimentary canal of *S. avenae*. FG, foregut; OV, oesophageal valve; ST, stomach; B HG beginning of hindgut; I, intestine; HG, hindgut (from Laubscher, 1985).

1.3.1.3 Morphology of the foregut and gut

Morphologically the alimentary tract consists of three parts; the oesophagus, midgut and hindgut (Fig. 1.2). The oesophagus is slender and thin-walled. The junction of the oesophagus and the midgut is marked by the oespahageal valve internally. The midgut can be divided into two distinct regions, the stomach and the intestine.

The stomach is mostly centrally to ventrally situated in the body. Its position can vary considerably depending on the state of development of the embryos.

The intestine leaves the stomach to form various loops in the abdominal cavity. The change from the stomach to the intestine is not clearly marked externally. The position of the intestine tends to vary relative to the state of development of the reproductive tracts.

The transition from the intestine to the hindgut is marked by the gradual increase in the diameter of the lumen. This proceeds towards the anus where it narrows (Ponsen, 1987).

1.3.1.4 Histology of the foregut and gut

The feeding channel within the stylets opens into the lumen of the tubular mouth which opens into the lumen of the pump chamber of the sucking pump (Fig. 3). The sucking pump is controlled anterior by two pairs of dilator muscles which are attached by tendons to the anterior surface of the pump (Fig. 1.3).

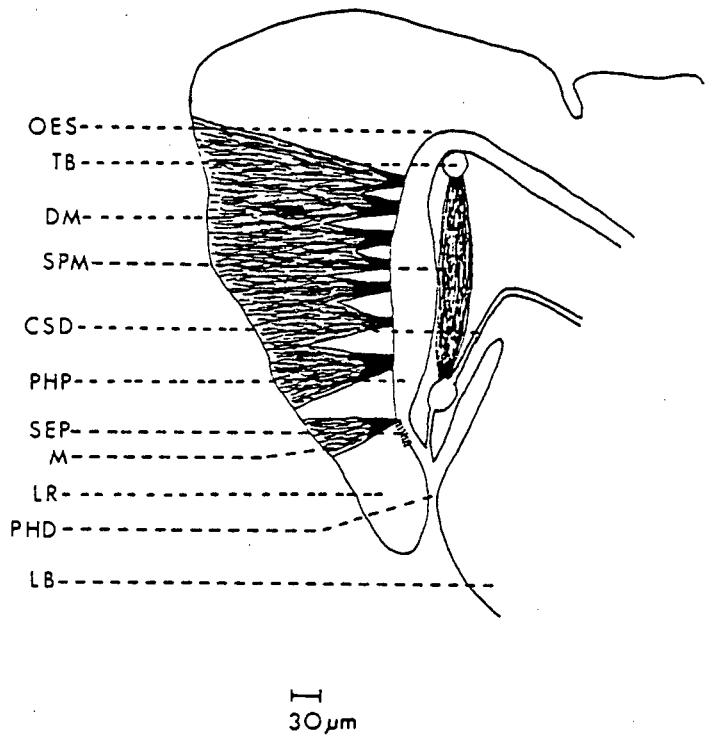


Fig. 1.3. Diagrammatic drawing of a saggital section through midline of the head of *S. avenae*. CSD, common salivary duct; DM, dilator muscle; LB, labium; LR, labrum; M, muscle; OES, oesophagus; PHD, pharyngeal duct; PHP, pharyngeal pump; SEP, sensillum pore; SPM, salivary pump muscle; TB, tentorial bar (from Laubscher, 1985).

The oesophagus wall consists of a single layer of cuboidal epithelial cells with indistinct borders and centrally situated nuclei which occupy a large part of the cell. The anterior region of the oesophagus has a smooth lumen. Posterior to the region of the eye, the lumen loses its smooth-walled appearance and becomes uneven in outline and the epithelial cells project into the lumen to form a series of longitudinal ridges. Anterior to the oesophageal valve, the lumen of the oesophagus loses its uneven outline. Externally the oesophagus epithelium is partitioned by a tunica propria, and internally it is lined by a longitudinally folded intima. Muscle fibers are also visible on the oesophagus and are enveloped by the tunica propria.

Wigglesworth (1956) pointed out that the basement membrane or tunica propria of the midgut is comprised of different layers, namely the basement membrane of the epithelial cells, the basement membrane of the muscle fibres, the basement membrane of the tracheoblasts, as well as other connective tissue. Other authors use this term for the rather diffused osmiophilic layer which occurs next to the basal cell membranes of the epithelial cells (Forbes, 1964).

The outer layer of the oesophageal valve is comprised of slightly flattened cuboidal cells. The inner layer is comprised of slightly flattened epithelial cells.

The stomach epithelium consists of a single layer of cells which rests upon a tunica propria. The tunica propria of the oesophagus and the stomach is continuous, but that of the stomach is markedly thicker. Circular muscle fibres surround the stomach and are closely connected to the tunica propria.

The stomach epithelium can be divided into three distinctive regions which are histologically well-defined. These are referred to as regions A to C (Fig.1.4). Region A refers to the cells that lie next to the oesophageal valve. These have a columnar appearance with oval nuclei and striated borders (Fig.1.4b). Region B (middle region) encompasses nearly half of the epithelial lining of the stomach with large columnar cells, their striated borders extending into the lumen and their oval nuclei are centrally situated (Fig. 1.4c). Region C cells are flat, cuboidal in appearance with striated borders hardly extending into the lumen and the nuclei are smaller and oval. These occupy most of the anterior region of the stomach (Fig.1.4d) (Laubscher, 1985).

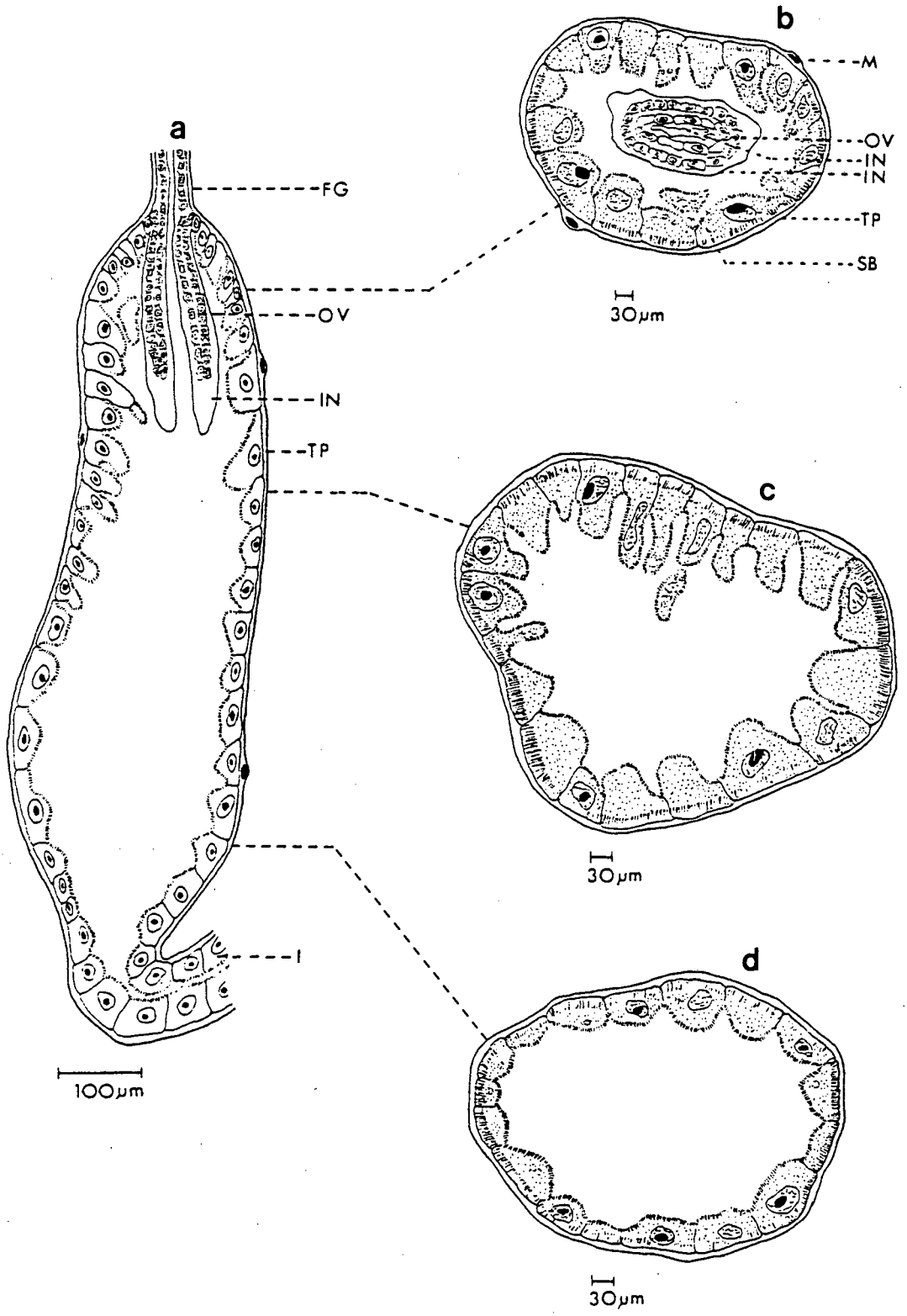


Fig. 1.4. a) Saggital section through midline of stomach, b) transverse section through anterior region of stomach, c) transverse section through mid region of stomach, and d) transverse section through posterior region of stomach of *S. avenae*. FG, foregut; I, intestine; IN, intima; M, muscle; OV, oesophageal valve; SB, striated border; TP, tunica propria (from Laubscher, 1985).

Morphologically the digestive cells of the stomach can undergo different structural changes. These structural changes of the cells are directly influenced by the state of digestion and are clearly visible in the middle region (region B) of the stomach. During digestion parts of these cells are pushed into the stomach lumen, after which they start to dissolve. During this process the cells become swollen and numerous vacuoles and granules appear in the apical part of the cells in the cytoplasm. This secretive cycle repeats itself during the adult stage without complete degeneration of the cells (Ponsen, 1987).

Histologically the intestine comprised of two distinctive regions joined by a transitional region. The anterior region is comprised of a thin tube (Fig. 1). The anterior region of the intestine is comprised of more or less five triangular shaped cells, with their narrow apices projecting into the lumen (Fig. 1.5a). This gives an irregular, narrow shape to the lumen of the intestine. The nuclei appear to be spherical to ovoid and the cells contain an extremely granular cytoplasm.

The lumen of the intestine posterior to the transitional region, is barely visible, and becomes gradually narrower. Beyond this first part, the lumen is more marked, and reaches its maximum width in the posterior region of the intestine, immediately before the transition to the hindgut (Fig. 1.5b and c)) (Laubscher, 1985).

The lumen of the hindgut widens. An extremely delicate intima lines the lumen of the hindgut. The lumen of the anterior region is comprised of columnar cells with irregular borders (Fig. 1.5d and e). These cells have very thin striated borders. The nuclei are ovoid to spherical. The lumen of the hindgut has a strongly folded and contracted appearance.

Posteriorly the hindgut gradually narrows towards the anus. The anal opening is controlled by muscles. The epithelium of the hindgut also rests on a tunica propria, similar to that of the stomach (Fig. 1.5f) (Laubscher, 1985).

1.3.2 **Nutritional physiology**

Once the aphid settles on its food source, it starts to collect information by physical and chemical stimuli. After penetrating the epidermis, aphids normally feed on phloem sap (Van Emden *et al*, 1969; Pollard, 1973). The early instar, however, may feed on the mesophyll tissue (Miles, 1987).

Original work by Mittler (1957) showed that aphids depend almost entirely on the

natural turgor pressure of their hosts. Mittler and Dadd (1962), however, showed that turgor pressure is not entirely responsible for the uptake of sap. According to Saxena and Chada (1971) the small diameter of the oesophagus is partly responsible for maintaining the continuous inflow of the plant sap by means of capillary forces after the reduction of pressure inside the sucking pump chamber. This pump functions by means of active swallowing.

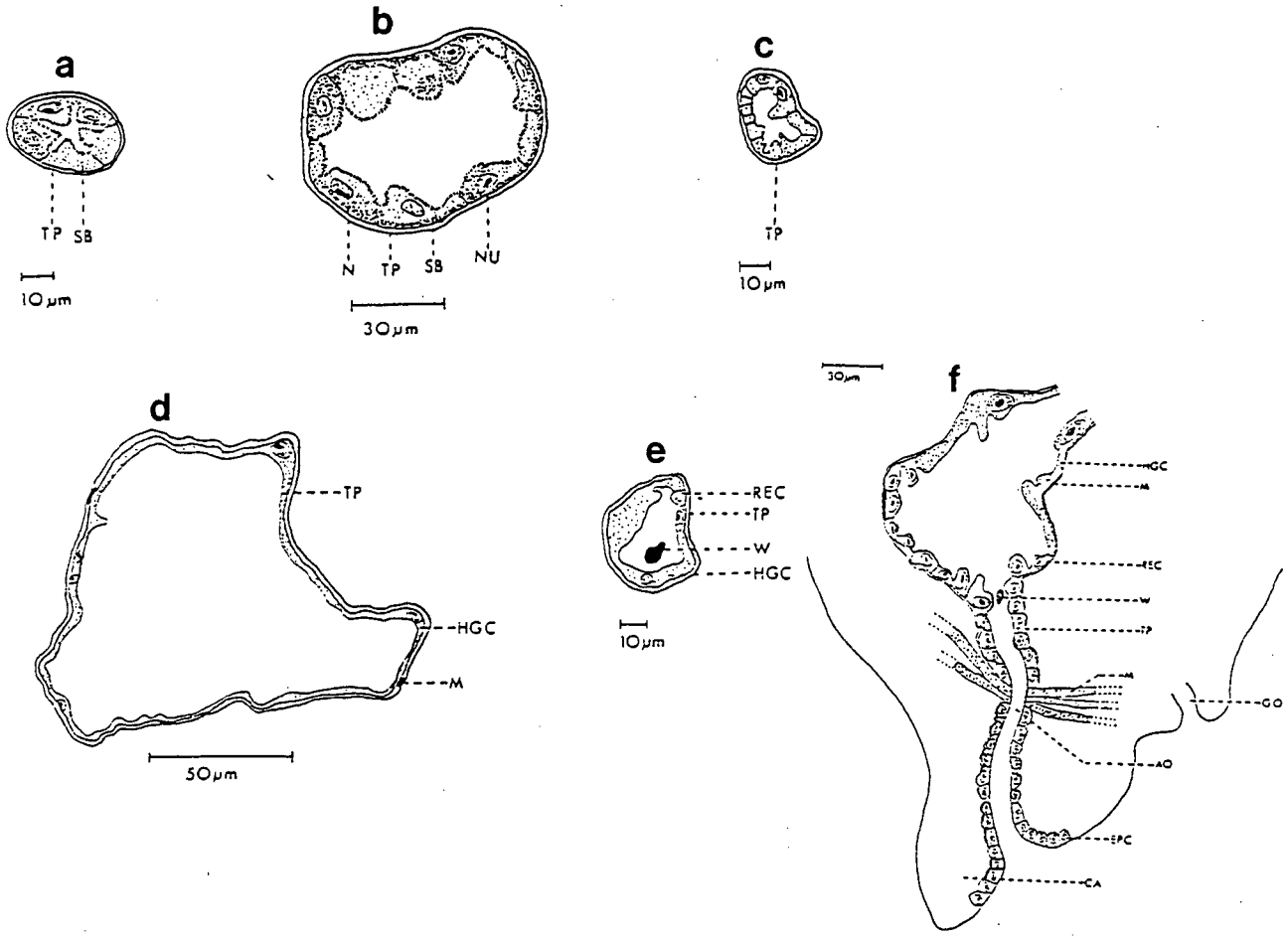


Fig. 1.5. a) Transverse section through anterior region of intestine, b) mid region of intestine, c) anterior region of hindgut, d) mid region of hindgut, e) anal region of hindgut, and f) saggital section through rectal region of hindgut of *S. avenae*. AO, anal opening; CA, cauda; EPC, epidermal cell; GO, gonopore; HGC, hindgut cell; N, nucleus; NU, nucleolus; M, muscle; REC, rectal cell; SB, striated border; TP, tunica propria; W, waxy droplet (from Laubscher, 1985).

Results obtained from chemical analyses of phloem sap of many hosts indicated that it is a complete diet (Srivastava, 1987). It is thought that aphids change or alternate host plants in order to change the level of nitrogenous substances or to obtain essential amino-acids (Blackman, 1974).

The ingested phloem sap is moved through the alimentary tract of the aphid by means of peristaltic action of muscles. Food absorption takes place in the stomach and intestine. The hindgut serves as a storage chamber from where excretion takes place in the form of honeydew. The pyloric valve and Malphigian tubules are absent in aphids (Forbes, 1964).

1.3.2.1 **Symbionts**

All aphids have an obligatory, intimate association with microorganisms, either yeast or bacteria (Houk & Griffith, 1980). This association has evolved to be symbiotic; a living together for mutual benefit and these microorganisms are referred to as symbionts. They are found within cells termed mycetocytes; an aggregate of mycetocytes is referred to as a mycetome.

While mycetocytes contain a single morphological structure of symbionts, more than one population of mycetocytes may comprise a mycetome.

The mycetomes are most often found just beneath the digestive tract within the abdomen of the adult aphid and circumintestinal in developing embryos (McLean & Houk, 1973). A few mycetocytes, singly or in small groups of two or three, can be found scattered about in the haemocoel. The mycetocyte contains the normal complement of cellular organelles. In addition to the symbiotic microorganisms distributed throughout the cytoplasm, a number of ultra-structural features have been described (Griffiths & Beck, 1974).

The symbionts are typically Eubacteriales in their ultrastructure and chemistry (Houk & Griffiths, 1980) and like other symbionts, are gram negative. Morphologically they can vary from coccoid to filamentous. They contain visible strands of DNA, low in percentage of guanin (G) and cytocin (C) and nucleotides typical of procaryotes adapted to intra-cellular habitat (Houk & Griffith, 1980; Houk *et al.*, 1980). The ribosomes are the appropriate size for procaryotes (i.e. 14 - 16 nm) and have been shown to be distinct from those of the aphid host (Ishikawa, 1977; 1978). Recent work has shown that the symbionts are probably specialized descendants of an ancestral *Pseudomonas* species and that the symbiont genome

(14×10^{10} daltons) is almost entirely repressed *in vivo* (Ishikawa, 1982; 1984). Only one symbiont protein has been demonstrated (symbionin) and this protein is encoded by the mycetocyte genome, and is synthesized by the prokaryotic machinery of the symbiont (Ishikawa, 1982; 1984).

A number of functions have been hypothesized for symbiotic microorganisms : biosynthesis (i.e., amino acids, sterols, vitamins and various polysaccharides), energy production, nitrogen fixation and osmoregulation (Buchner, 1965: Houk & Griffiths, 1980). The role of symbionts in the production of sterols has not been elucidated fully (Houk, 1987). A more acceptable function of symbionts is the synthesis of vitamins, specifically the vitamins of the B-complex (Houk & Griffith, 1980). There is no direct evidence for vitamin synthesis by symbionts, only implications from aphid survival on holidic diets devoid of an exogenous vitamin source (Ehrhardt, 1968; Houk & Griffith, 1980). An interesting new hypothesis suggests the involvement of symbionts in the biosynthesis of cell wall and middle lamellar matrix degrading polysaccharases (e.g., pectinases, cellulases, and hemicellulases) (Campbell & Dreyer, 1985).

The mycetome is composed of two longitudinal masses of cells or mycetocytes linked together dorsally of the hindgut. The cytoplasm of the mycetocytes is filled with symbionts. The mycetome is enclosed by a nucleated sheath and grows slowly by enlargement of the mycetocytes during larval life to disintegrate into groups of one or more mycetocytes. In adult aphids the mycetocytes are present as single cells in the body cavity, whereas in aphid embryos inside the adult, the mycetocytes still form a mycetome (Ponsen, 1977). The mycetocytes and their symbionts begin to degenerate soon after the adult stage is reached. At a later stage of adult life, the mycetocytes also start to dissolve after which the degenerating symbionts are liberated into the haemolymph. The destruction of the symbionts in their mycetocytes appear to be brought about by lysosomes (Hinde,1971; Griffith & Beck, 1973). In the body cavity the symbionts are dissolved by oenocytes or are engulfed and digested by phagocytic, spindle-shaped haemocytes (Toth, 1938; Hinde, 1971).

The mycetome is ventrally situated to the paired ovaries (Ponsen, 1977). During the development of the parthenogenetic embryo, symbionts enter it through a rather extensive opening of the blastoderm at the posterior pole. According to Couchman and King (1980) there is no ovarian follicle around the embryo, and the symbionts pass directly through the temporarily disrupted area of the ovariole sheath. This description is in accordance with work done by Hinde (1971).

The invagination of the germ band starts before invasion by symbionts is completed. There is a difference in the period of invasion by the symbionts between aphid species (Toth, 1933; Brusle, 1962). During blastokinesis (a series of complex movements within the developing embryo) the mycetome takes up its final position in the body cavity. In the parthenogenetic embryo, this movement takes place in almost total absence of yolk (Blackman, 1987.).

1.3.3 Circulatory and respiratory system

The blood or haemolymph of the aphid is contained in the general body cavity, where it bathes the various internal organs and also enters the appendages and the tubular cavities of the wing-veins (Richards & Davis, 1977). Blood is the only extracellular fluid in the insect body and makes up 15 - 75 percent of the volume of the insect. The amount and composition vary with species. The haemolymph circulates through the body via a simple segmented heart called the dorsal vessel. It extends from near the caudal end of the body, through the thorax and terminates in the head. It lies along a dorsal median line. It is a continuous tube, closed posteriorly, and open at its cephalic extremity. The dorsal vessel is comprised of two regions, the heart or pumping organ and a conducting vessel or aorta. The heart is maintained in position within the pericardial sinus by suspensory filaments. The haemolymph is collected through the pericardial sinus and is pumped out through the dorsal aorta in the head from where the haemolymph then flows back into the abdomen. Ponsen (1977) stated that the haemolymph of *Myzus persicae* is characterized by the absence of circulating haemocytes. Hinde (1971), on the other hand, stated the presence of phagocytic, spindle-shaped haemocytes, responsible for the destruction of free symbionts in the haemolymph. These haemocytes are relatively small, with large nuclei.

The tracheal system of aphids is responsible for the transportation of oxygen in the aphid's body and the trachea are connected to openings in the exoskeleton of the aphid, called spiracles. The spiracle is opened by contraction of a muscle (Ponsen, 1977).

1.3.4 Nervous system

The aphid nervous system is comprised of four main structural parts (Fig. 1.6):
i) brain or supraoesophageal ganglion
ii) suboesophageal ganglion
iii) thoracic ganglion mass
iv) ganglia and nerves.

The brain is divided into three main regions:
a) The protocerebrum is the largest and most dorsal portion. It is formed by the fusion of two ganglia and forms laterally the optic lobes. On the outside these lobes are connected to the eye and are totally devoted to vision.
b) The deutocerebrum is comprised of the fusion of the ganglion pair of the antennal segments.
c) The tritocerebrum comprises the most ventral parts of the brain and consists of two conical lobes. The latter structure has a chemosensory function.

Neurosecretory cells are present in the protocerebrum, deutocerebrum, tritocerebrum, suboesophageal ganglion and the thoracic ganglion mass (Amiressami, 1973; Steel, 1977). There are five major groups of neurosecretory cells (Group I, II, III, IV and V). Parthenogenesis and reproduction are controlled by a substance which is secreted by the neurosecretory cells (Steel, 1977).

The endocrine gland, corpus allatum, produces juvenile hormone which regulates metamorphosis, body shape in many polymorphic insects, reproduction and behaviour (Hardie, 1987b).

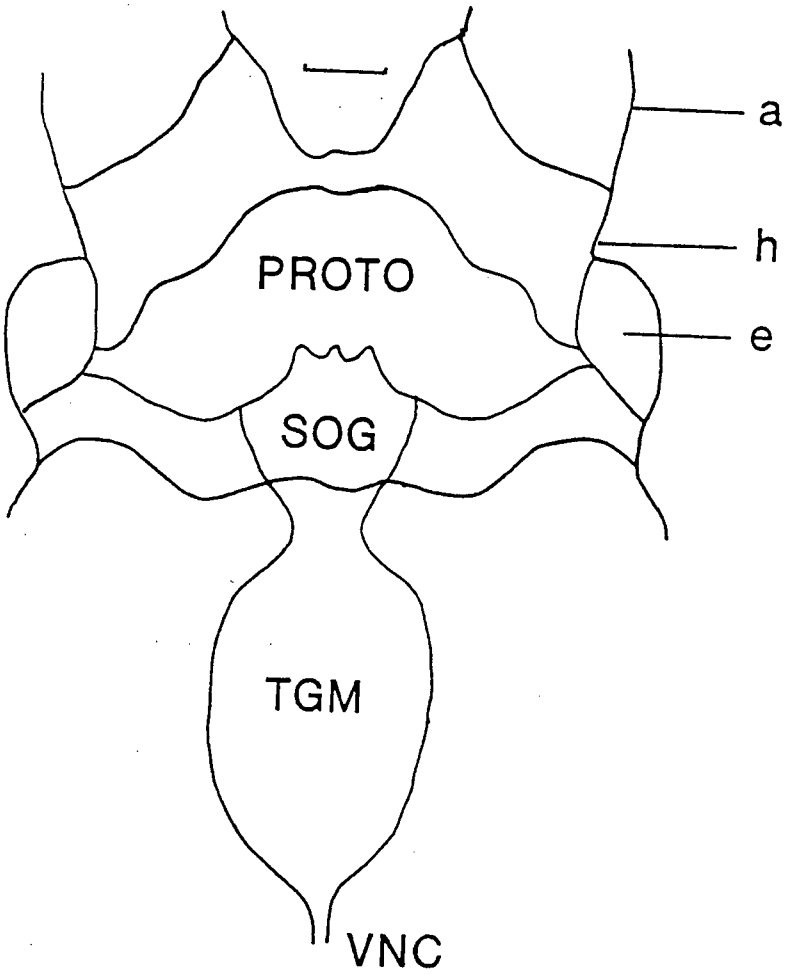


Fig. 1.6. A diagrammatic drawing of the central nervous system of an adult aphid. a, antenna; e, eye; h, outline of head; PROTO, protocerebrum; SOG, suboesophageal ganglion; TGM, thoracic ganglion mass; VNC, ventral nerve cord. Bar marker presents 100 μm. (Modified from Steel (1977).

1.3.5 Reproductive system

According to Ponsen (1977), the ovaries are paired organs situated dorsally of the mycetome. Each ovary consists of a few telotrophic ovarioles grouped at the end of one of the paired oviducts.

The embryonic development of the female reproductive system initially follows the same course in both sexual and parthenogenetic females. The course of further development of the female reproductive system depends on whether the embryo is destined to become oviparae (sexual) or viviparae (parthenogenetic). Determination of the reproductive category may be genetically controlled, but is commonly a response to certain environmental stimuli, of which photoperiod is usually the most critical. Viviparae normally develop when females are maintained at a long photoperiod, while oviparae develop in response to a short photoperiod.

The production of males by parthenogenetic females is a form of arrhenotoky. This term is used for the production of haploid males. Aphid males are XO i.e. they have two sets of autosomes, but only one X-chromosome or a set of X-chromosomes. The production of male individuals is initiated in autumn as soon as the night period (scotophase) has increased above a certain critical length (Blackman, 1987). This phenomenon can also be influenced by temperature. The photoperiodic effect is presumably mediated by the neuroendocrine system through virginopara-promoting neuro-hormone activity. This activity lies in the Group I neurosecretory cells. When Group I cells were destroyed in long-day reared virginoparae (parthenogenetic females), there was a spontaneous switch to the production of oviparae (Steel & Lees, 1977). Group II cells have a separate function in that they produce a prothoracicotropic hormone, and cessation of this hormone during the third instar prevents the final molt (Steel, 1978). Prothoracicotropic hormone reaches its target cells via the circulatory system.

In general the male reproductive system is comprised of two multifolicular testes which are connected to a short ejaculatory duct by a paired ductus differens. The number, size and shape of the follicles in the testis is species specific (Blackman, 1987).

1.3.6 Aphid biology

The coexistence with each species of the Aphidoidea of sexual reproduction and a successful mechanism for parthenogenetic reproduction, indicates that there must

be a very strong selective advantage for a sexual phase in order to overcome the reproductive disadvantage. Continuous parthenogenesis in aphids is rare and is associated with agricultural ecosystems that are far less heterogeneous than natural ecosystems, and in which the host plant tends to be genetically uniform (Dixon, 1987a).

Parthenogenesis and feeding on phloem sap, which both developed early in the evolution of aphids, have been major factors shaping the ecology of the group. This resulted in aphids being able to survive only for short periods off their host plants, in host specificity, and in random location of host plants. The capture of a new host plant may be a random event. It is thought that initially the host range was likely to be fairly wide, and become restricted with specialization and speciation (Dixon, 1987b). Host alteration has enabled some aphids to extend the period in which they can grow and reproduce. This has lead to a further speciation, with the development of species that are secondarily monoecious on either the primary or secondary host. Aphids can be divided into different groups with respect to their life cycle : the non-host-alternating (monoecious) species which feed on the same host plant, and the host-alternating (heteroecious) species which migrate between their primary hosts for instance *R. padi*, moving from *Prunus* spp. to Graminae. Polyphagy is rare in aphids and commoner in the tropics than in temperate regions (Dixon, 1987b).

1.3.6.1 **Polymorphism**

From the agricultural standpoint, the polymorphism of aphids is troublesome, because the alatae widely disseminate various plant virus diseases (Kawada, 1987).

Many probable causes have been proposed and studied in an effort to explain the appearance of alatae. It is however, not simple to identify which factor plays the major role. Temperature may play a role, but the results obtained have been very inconsistent. Hales (1976) showed with *Brevicoryne brassicae* that apterous individuals kept at 10°C have less active corpus allata than individuals kept at 25°C. This suggested that the reproduction of apterous morphs is related to hyperactivity of the corpus allata at high temperatures. In some cases, higher temperatures have inhibited the production of alate forms (Lamb & White, 1966). In this study daylength did not appear to influence wing determination.

It is well known that the rate of appearance of alatae increases with a higher number of aphids per host plant together with the increase in crowding density.

One of the reasons for this phenomenon appeared to be the change in the nutritional condition of the host plant, but it was found that nutritional conditions were not related to wing development, but that crowding was (Lees, 1967). This contact stimulation in a crowding population is interpreted as a signal for overpopulation via the aphid's appendages, e.g. antennae, legs and bristles. A few aphid species are also influenced by food with its gustatory and nutritional aspects, and is an important factor in wing formation (Harrewijn, 1978).

In Aphididae maternal crowding effects prevail, but both prenatal and postnatal effects are present. In older groups nutritional effects are also found. These effects have not been documented well enough to be certain whether there is a preference for the maternal or larval stages (Harrewijn, 1978).

1.3.6.2 **Rate of population increase in aphids**

Parthenogenetic reproduction in aphids evolved 200 million years ago in the Permian, and has been of paramount importance in determining their population structure and high rates of increase. It led to the evolution of the clonal polymorphism and the telescoping of generations, so characteristic of aphids (Dixon, 1987a).

An aphid passes through four instars (stadia) from birth to adult. The length of time required for this is variable and dependent on two extrinsic factors, food quality and temperature; and two intrinsic factors, birth weight and whether the morph is alatae or apterae. Food quality and temperature can also effect birth weight either directly or indirectly, and morph determination. Increase in both food quality and temperature generally increases the developmental rate. For each species there is a range of temperatures over which the developmental rate increases linearly. Outside this range, the relationship is curvilinear, and aphids are adversely effected with an increase in mortality (Dixon & Wratten, 1971).

1.3.6.3 **Population dynamics**

The objective of population dynamics is to identify the causes of numerical change in the population studied, and to explain how these causes act and interact to produce the observed pattern and numbers (Hughes, 1972). Two factors complicating this study are polymorphism and the overlap of generations.

Aphid species often occur as pests of more than one host plant and usually have to migrate aerially between hosts, sometimes having to enter a resting stage to pass

an adverse climatic period. The various morphological and physiological forms of the aphid appear facultatively in successive generations of the same genetic strain, and the ecological properties of these forms can be as diverse as their appearance (Hughes, 1972).

Within the polymorphic system of an aphid species, there has usually developed a super-parasitic form. This is the apterous viviparous parthenogenetic female, which allows the species to exploit any favourable host plant at the most favourable season of the year and has a high rate of reproduction associated with the telescoping of generations (Hughes, 1972).

In sub-populations of aphids where generations overlap, all the life history stages occur together so that all relationships may be operating concurrently. This situation is analogous to that in human populations for which the technique of time-specific life tables have been developed. Time-specific life tables describe numerical changes in terms of what has happened to individuals of a population (grouped into age-classes) during a given short time interval. In human populations the time-specific life table does not change over several years, but with aphids the environment and consequently the whole pattern of relationships can change very rapidly. Thus several life tables are needed to describe systematically the events of a single population cycle (Hughes, 1972).

1.3.7 Small RNA viruses of insects

Most of the small RNA viruses described, belong to, or are possible members of picornavirus, *Nodamura* virus and *Nudaurelia* ß virus (NßV) groups.

The *Nodamura* virus is the type member of the Nodaviridae and was isolated from mosquitoes (*Culex tritaeniothyncus*) in Japan (Sherer & Hurlbut, 1967; Sherer *et al.*, 1968). This virus contains two pieces of ssRNA (Newman & Brown, 1973) and can replicate in mosquito and baby hamster kidney (BHK) cells and is lethal to suckling mice (Baily & Scott, 1973; Bailey et al., 1975; Moore et al., 1985).

A second member of this group, the black beetle virus (BBV), was isolated from *Heteronychus arator* in New Zealand (Longworth & Archibald, 1975; Longworth & Carey, 1976). BBV does not replicate in BHK cells or kill suckling mice, however it does replicate in *Drosophila melanogaster* cells (Friesen *et al.*, 1980).

The type member of the *Nudaurelia capensis* ß virus (NßV) group was isolated from the pine emperor moth, *Nudaurelia cytherea capensis*, which causes extensive

Teleogryllus oceanicus and *T. commodus*. Early instar nymphs showed apparent paralysis of their hind legs before dying (Reinganum *et al.*, 1970). CrPV has been detected in several insect species and has a wide experimental host range (Scotti *et al.*, 1981: Plus *et al.*, 1978: Anderson & Gibbs, 1988). A virus similar to CrPV, $CrPV_{ark}$, was isolated from *Pseudoplusia includens* (Chao *et al.*, 1986) . CrPV neutralizing antibodies have been detected in mammalian species, but there is no evidence that CrPV replicated in mammalian hosts (Scotti & Longworth, 1980; Moore *et al.*, 1987) . A relationship between encephalomyocarditis virus (EMCV) and CrPV has been reported (Tinsley *et al.*, 1984).

Many insect viruses have been tentatively placed in the Picornavirus Group. Matthews (1982) listed them as "unclassified small RNA viruses of invertebrates" within the picornaviruses. Among them are the bee acute paralyses (BAPV), bee slow paralyses, bee virus X, *Drosophila* P and A and sacbrood viruses (Moore *et al.*, 1985). Further examples include infectious flacherie virus isolated from the silkworm, *Bombyx mori* (Hashimoto & Kawase, 1983); *R. padi* virus (RhPV) isolated from the oat bird cherry aphid, *R. padi* (D'Arcy *et al.*, 1981b; Von Wechmar & Rybicki, 1981; Williamson *et al.*, 1989); aphid lethal paralysis virus (ALPV) also isolated from *R. padi* (Williamson *et al.*, 1988); Kawino virus from the mosquito *Mansonia uniformis* (Pudney *et al.*, 1978); *Lymantria ninayi* virus from the Tussock moth larvae (Pullin *et al.*, 1984); and the *Triatoma* virus (TrV) from *Triatoma infestans* (Muscio *et al.*, 1988). Bailey (1976) described several bee viruses which include the black queen cell virus (BQCV), Berley bee virus (BBV), Egypt bee virus, two isolates of sacbrood virus (SBV), Kashmir bee virus (KBV) and Arkansas bee virus (Bailey, 1976; Lommel *et al.*, 1985).

1.3.7.2 **Viral infection of aphids**

Parrish and Briggs (1966) described isometric particles in labial cells of *Rhopalosiphum maidis*. These virus-like particles (VLP's) were 24 - 27 nm in diameter, occurred as a crystalline array which were part of an inclusion body. Fraval and Lapierre (1970) reported the isolation of a virus from *R. padi*. Peters (1965) isolated VLP's from *Myzus persicae*. The literature on *M. persicae* histology does not report such isometric particles in nonviruliferous individuals (Forbes, 1964; Ponsen, 1972).

Kitajima (1978) reported the detection of rod-like particles in two aphid species. These particles are found in cells of the fat body and less frequently in adjacent muscle cells and occurred mostly within the nucleus. In a few cases they were detected in the cytoplasm within membrane bound cavities.

The transmission of VLP's to the ovarial or developing embryo, either directly or indirectly inside the mycetome, was suggested and has been demonstrated for rice dwarf virus (Nasu, 1965)

1.3.7.3 Characterization of aphid infecting viruses

Only three aphid viruses have been characterized in detail; *Rhopalosiphum padi* virus (RhPV), aphid lethal paralysis virus (ALPV) and *Sitobion avenae* virus (SAV).

1.3.7.3.1 *Rhopalosiphum padi* virus (RhPV)

D'Arcy *et al.* (1981b) reported the purification and characterization of a 27 nm icosahedral virus from *Rhopalosiphum padi* aphids and called it *Rhopalosiphum padi* virus (RhPV). In a separate independant study conducted in South Africa, Von Wechmar and Rybicki (1981) isolated a similar virus along with barley yellow dwarf virus (BYDV) and brome mosaic virus (BMV) from wheat infested with *D. noxia* and *R. padi.*

Further characterization showed that this isolate and the isolate from Illinois were serologically closely related but were physically distinct (Rybicki & Von Wechmar, 1982; Williamson *et al.*, 1989). Although isolated from plants, there was no evidence of RhPV multiplying outside the aphid (Williamson, 1988; Rybicki, 1984). This was supported by work done on BYDV and RhPV where dsRNA analysis of oats and barley, failed to detect evidence of RhPV replication in plants (Rybicki, 1984; Gildow & D'Arcy, 1988; Williamson, 1988). Based on the physical characteristics of RhPV, Williamson *et al.* (1989) proposed that RhPV should be classified as a picornavirus (Table 1.1).

1.3.7.3.2 Aphid lethal paralysis virus (ALPV)

Aphid lethal paralysis virus (ALPV), a second aphid pathogenic virus, was isolated from *R. padi* in 1987 (Williamson, 1988; Williamson *et al.*, 1988). The virus particles are isometric with a diameter of 26 nm. ALPV is physically and serologically distinct from RhPV, but is serologically related to an insect picornavirus, cricket paralysis virus (Williamson *et al.*, 1988). cDNA derived from ALPV ssRNA did not hybridize with CrPV RNA which indicated that there is no direct sequence homology between ALPV and CrPV, and that ALPV may be classified as a distinct virus (Williamson et al., 1988). The virions contain three major proteins of M_r 34 400 ± 500 daltons, 32 000 ± 800 daltons and 31 200 ± 800

daltons, and a smaller amount of M_r 40 000 ± 500 daltons protein.

ALPV occurs in several aphid populations in the major grain producing areas of the western Cape and the eastern and western Orange Free State. ALPV often occurs as a mixed infection with RhPV (Williamson *et al.*, 1989)). Laboratory studies with surface contaminated plants showed that the aphid can acquire ALPV from such plants. This manner of contamination has obvious epidemiological implications and could facilitate virus spread. Retrospective analysis of field collected plants infested with aphids, kept frozen since 1978 and 1979, and antisera prepared from aphid extracts in 1979 (Von Wechmar, unpublished results), showed that the virus had been present in natural aphid populations since 1979 (Williamson, 1988). Williamson *et al.* (1988) speculated that the higher M_r protein found in ALPV could be analogous to the VPO found in CrPV, DCV, IFV and mammalian picornaviruses. These physical and serological properties indicated that ALPV should be classified in the family Picornaviridae (Table 1.1).

Williamson *et al.* (1988) and Von Wechmar (unpublished results) found ALPV in aphids collected in geographically different regions and showed that this virus was not an adventitious pathogen of laboratory aphid colonies. Amongst these field collected aphids there were individuals that were positive for ALPV, but negative for RhPV which indicated that ALPV was not dependent on RhPV for replication in aphids.

Table 1.1. Physical properties of aphid picornaviruses compared with other picornaviruses[a]

VIRUS PROPERTY	ALPV[1]	RhPV[2]	CrPV[3]	TrV[4]
Diameter (nm)	26-28	27	27	30
RNA ($M_r \times 10^{-6}$)	3.3	2.7	2.9	3
Capsid protein ($M^r \times 10\text{-}3$)	34, 32 31, 40.8*	31, 30 28	35, 34, 30 43.0*	39, 37, 33, 45*
Buoyant density in CsCl (g/ml)	1.34	1.37	1.37	1.39
Sedimentation coefficient (S)	164	165	167	165
Polyadenylated RNA tail (3')	+	ND	+	ND

a adapted from Williamson (1988)

* Minor capsid protein, assumed to correspond to VPO of mammalian picornavirus.

ND not determined

1. aphid lethal paralysis virus
2. *Rhopalosiphum padi* virus
3. cricket paralysis virus (Moore *et al.*, 1985; Williamson *et al.*, 1988)
4. *Triatoma* virus (Muscio *et al.*, 1988)

1.3.7.3.3 *Sitobion avenae* virus (SAV)

Allen and Ball (1990) isolated a new aphid infecting virus called *Sitobion avenae* virus (SAV) from the *S. avenae* and *M. dirhodum* aphids under laboratory conditions. The virus particles are isometric with a diameter of 30 nm. SAV is serologically unrelated to RhPV and ALPV (Williamson *et al.*, 1989) and did not multiply in grain plants or in pupae of the honey bee, *Apis mellifera*. It was not detected in field-collected aphids. SAV can be transmitted to healthy aphids by contaminated honeydew from infected aphids (Allen & Ball, 1990).

1.3.7.4 Site of insect virus multiplication

Kitajima (1976) studied VLP's within *Myzus persicae* and showed that they contained VLP's in the stomach and hindgut, nerve ganglion complex, and salivary gland cells, but not in other organs. These isometric VLP's had a diameter of 20 - 25 nm. VLP's were more conspicuous in the intestine and less frequently seen in the stomach and hindgut. The particles in the intestinal lumen appeared as aggregates, either randomly arranged or in a crystalline array, usually between the microvilli. Many epithelial cells of the intestine showed areas rich in vesicles. VLP's were also detected in the basal membrane and also between the invaginated plasmalemma. VLP's were present in the nerve ganglionar complex, protocerebrum and the cytoplasm of the peripheral neurons.

In addition, VLP's were found in both the accessory and principal salivary glands. Examination of *M. persicae* suggested a spread of VLP within the body cavity of the aphid. During the initial phase of infection, VLP appeared in the midgut epithelial cells. Cell changes indicated possible multiplication, and showed increased vacuolization interspersed with electron-dense amorphous material (Esau & Hoeffert, 1971; 1972). Some evidence also exists for transovarial passage.

Nerve ganglia and the salivary gland seems to act as preferential sites for VLP accumulation after their release from the stomach. In *Aphis* and *Pentalonia* spp., VLP's were confined to the fat body, muscle and occasionally in the mycetome (Kitajima, 1976).

The pathology of insect picornaviral infection has not been studied fully. Most of the detailed studies on infections have been done on KBV, and SBV of the honey bee, *Apis mellifera* and $CrPV_{ark}$ (Mussen & Furgala, 1977; Dall, 1987; Chao *et al.*, 1986).

Insect picornavirus multiplication take place in the cytoplasm as is the case of mammalian picornaviruses. Similar vesicular structures, have been observed in $CrPV_{ark}$ infection of *P. includens* (Chao *et al.*, 1986) and *Gonometa* virus infection of *G. podocarpi* (Longworth *et al.*, 1973). In virus-infected cells, cytoplasmic microtubules have been demonstrated to be involved in intra- as well as intercellular transport of virions (Mayhew & Carrol, 1974; Chao *et al.*, 1986; Satake & Luftig, 1982). Thick bundles of these microtubules were first reported by Chao *et al.* (1986). *Gonometa* virus accumulated in columnar cells, and the presence of this virus was associated with degeneration of mitochondria and cell membranes. The virus was also observed in the midgut (Longworth *et al.*, 1973).

$CrPV_{ark}$ particles and associated cytopathic effects were evident in the epidermis, haemocytes, fat body and midgut epithelium. The cytopathic effect on the different tissues varied; epithelial cells showed no degeneration, whereas fat body cells contained disrupted organelles and/or disorganized cytoplasm. The increase in the number of microtubules and associated membranous fibril-containing versicles was the most consistent and common effect in infected cells of all different tissues. This was mostly observed in cells of epidermis and of fat body (Chao *et al.*, 1986). Particles of CrPV were observed in the gut cells and nerve ganglia cells. Crystalline arrays and scattered particles of CrPV were observed in large numbers in the epidermal cells (Reinganum *et al.*, 1970).

SBV is more infectious to bee larvae than to the adult honey bee. In larvae, SBV seemed to have accumulated in the gut lumen adjacent to the peritrophic membrane. The perineurium, glial cells, neurons and axons or larval nervous tissue and the haemocytes were all infected. In adults, viral particles were observed in tracheocytes of the air sacs, around the brain, the hypopharyngeal glands, and the abdominal ganglia (Mussen & Furgala, 1977).

Vesicular membrane-bound structures associated with KBV were observed in infected honeybee larvae (Dall, 1987). KBV containing vesicles were noticed in the epithelial tissue of the foregut, hindgut, alimentary canal musculature, epidermis, tracheal epithelium, haemocytes, oenocytes and tracheal end cells. Although degeneration of glial cells were apparent, multiplication of particles in the nervous system was not noticed. Cytoplasmic organelles lost their integrity, and condensation of chromatin and sloughing of tubules of the cytoplasm was observed. There was a significant rise in the haemolymph osmolarity, but this alone was not responsible for glial cell degeneration (Dall, 1987).

Two VLP's have been noticed in the cytoplasm and nucleus of *Drosophila* tissue

and cell lines. Virus particles were noticed in the cells of tissue from the midgut, trachea, connective tissue, paragonia and nerve cells, fat body cells, oenocytes, melenotic masses, central nervous tissue, gut cells and larval imaginal discs (Rae & Green, 1967, Akai *et al.*, 1967; Perotti & Bairati, 1968; Philpott *et al.*, 1969; Wehman & Brager, 1971). DCV occurs in the tracheal cells and in cells surrounding the cerebral ganglia of infected *D. melanogaster*. In *Ceratitis capitata* the virus evidently multiplies in the intestinal cells and in the Malpighian tubule cells (Jousset *et al.*, 1977; Scotti *et al.*, 1981). With *Drosophila* P virus infection appears to take place in the ovaries and Malpighian tubules of *D. melanogaster*. Virus particles were also noticed in the male accessory glands (Teninges & Plus, 1972).

1.3.7.5 **Plant viruses transmitted by aphids**

In addition to aphid infecting viruses, plant pathogenic viruses may also be detected in aphids. Aphids are important vectors of plant infecting virus diseases (phytopathogenic). These viruses can be separated into two groups:
a) non-circulative (stylet-borne)
b) circulative.

Non-circulative or stylet-borne viruses are carried on the tip of the stylets, where they are held in extremely fine grooves (Forbes, 1969; 1977). Most of the viruses transmitted by aphids are stylet-borne (Harris, 1980). The alate aphid's habit of probing plants, often taking off from several different plants before settling down to feed, provides the virus with a method of dispersal. These viruses do not accumulate within the aphid.

Circulative viruses are actually taken in by the aphid and circulate or replicate in its body before being transmitted to a plant again through the saliva. The method utilized depends not on the aphid, but on the virus (Blackman, 1974). Most work done on the multiplication of phytopathogenic viruses, was done on circulative viruses which replicate in both aphids and plants (Harris, 1980; Gildow, 1982).

1.3.7.6 **Latency of insect viruses**

Latent virus infections have interested researchers ever since the phenomenon was first discovered. Latency is a characteristic of many viruses. It can be defined as the ability of a virus to survive in a host without causing recognizable symptoms, but remain virulent and can be stimulated to pathological activity by certain conditions such as population density, food, and temperature (Podgewaite & Mazzone, 1986). Podgewaite and Mazzone (1986) referred to external influences

capable of activating insect viruses from a latent to an active form as stressors. Epizootic conditions are described as the often unexplained collapse of dense insect populations under field conditions. The possibility that latent viruses are responsible for this situation, prevails.

The mammalian herpes simplex virus is a classical example. However, latent properties also apply to insect viruses. Tubeuf in 1892 (as quoted by Podgewaite and Mazzone, 1986) reported the first case of latency involving insect viruses. He noticed that the polyhedral virus disease of the nun moth *Lymatria monacha* tended to spread slowly in an insect population and did not affect all individuals. Infections of the moth population often changed suddenly to an active virulent form.

Examples of insect viruses in which this phenomenon prevails, is the sigma (σ) virus of *Drosophila* and a polyhedral virus of the silkworm, *B. mori*. Other examples include nuclear-polyhedrosis virus (NPV), granulosis virus (GV), cytoplasmic polyhedrosis virus (CPV), honey bee viruses, CrPV, DCV and *Nodamura* virus (Podgewaite & Mazzone, 1986).

1.3.7.7 Natural epizootics

Mass collapses in natural insect populations of forests have been demonstrated for lepidopterans. The presence of latent viruses were noticed by several researchers in such forest populations. Most insects were shown to develop immunity to viral infections (Podgewaite & Mazzone, 1986). Larvae become increasingly more resistant to virus infections as they grow up. Generally the adults are resistant to infections, although they may transmit the virus to their progeny (Podgewaite & Mazzone, 1986).

Natural dispersal occurs by the movement of healthy carriers and infected hosts, by parasites and predators, and by physical and climatic factors like wind and rain. High humidity was found to be one of the most important environmental factors in the development of virus epizootics in insect populations. Examples of this phenomenon were encountered in the California oakworm *Phnyganidia californica*, the gamma noctuid *Plusia gamma*, the fall webworm *Hyphantria cunea*, the gypsy moth *Lymantria dispar* and the armyworm *Pseudaletia unipuncta* (Podgwaite & Mazzone, 1986).

A virus epizootic tends to develop more easily in insect populations at high density levels than populations at low density levels. However, in some instances

a virus epizootic may develop in insect populations at low densities like in the case of the sawfly, the Great Basin tent caterpillar *Malacosoma fragile* and the armyworm, *P. unipuncta* (Podgwaite & Mazzone, 1986). Various other stressors can influence virus epizootics in insects.

No epizootics due to CrPV or DCV have been reported in natural populations of *Drosophila* and crickets (Scotti *et al.*, 1981).

Insects collected in the field may suddenly succumb to a viral infection under laboratory conditions (Scotti *et al.*, 1981). The close rearing of individuals under laboratory conditions may be responsible for this phenomenon. Fluctuations in the extent of inapparent or non-detectable infections in honey bees in Australia, provide a possible explanation for the occasional KBV epizootic which occurs in Australia and causes a collapse of the bee population (Dall, 1985; Anderson & Gibbs, 1988). If fluctuations are either caused or increased by environmental conditions, an outbreak could be anticipated (Dall, 1985).

Insect viruses provide an unique opportunity to study strain variation during an epizootic occurring in a particular habitat in the absence of a host immune system which could modify the host response and exercise a selective pressure (Scotti *et al.*, 1981).

1.3.8 **Viruses for biological control**

Only a limited number of insects out of a vast number of insect species have been investigated for the presence of viruses. The potential pool of natural pathogenic viruses in this diversity of species is enormous. These naturally occurring viruses may be present as chronic infections which could change suddenly into active and acute forms (Podgwaite & Mazzone, 1986).

Severe effects of small RNA viruses on insect populations have been reported. CrPV infecting a laboratory colony of Australian field crickets, *T. oceanicus*, resulted in 95% death (Reinganum *et al.*, 1970) and frequent epizootics of bee viruses which caused population declines of honey bees have been recorded (Dall, 1985). Detailed data on the effects of picornaviruses on natural insect populations are lacking. Only CrPV has been studied under natural conditions and showed potential as a field control agent of the olive fruit fly *Dacus oleae* (Manousis & Moore, 1987). *Gonometa* virus was isolated from *Gonometa podocarpi*, which caused severe defoliation of *Pinus patula* in Uganda. This virus was successfully applied to control *G. podocarpi* (Harrap *et al.*, 1966; Longworth *et al.*, 1973).

Caution regarding the potential use of picornaviruses as insect control agents was advised when several reports on interactions between insect picornaviruses and antibodies in mammalian sera became known (Tinsley *et al.*, 1984). For this reason and due to their resemblance to vertebrate infecting viruses, picornavirus pesticides have not been considered for commercial purposes (Tinsley *et al.*, 1984; Moore *et al.*, 1985; Flexner *et al.*, 1986). Since evidence is lacking to indicate that CrPV could replicate in vertebrate hosts or mammalian cell lines, the fears of using insect picornaviruses as microbial pesticides were probably unfounded (Scotti & Longworth, 1980). No record of the replication of an insect pathogenic virus in a mammalian host could be found.

The Baculoviruses are the best studied group of viruses of invertebrates and some have been exploited as microbial pesticides (Maramorosch & Sherman, 1985). They are the largest group of insect viruses registered by the United States Environmental Protection Agency (EPA). Before commercial application of these viruses as biological pesticides are considered, all are tested to determine their selectivity, safety and economic viability (Flexner *et al.*, 1986). Applicational problems such as insect stage specificity, host specificity, viral dose, uv irradiation, temperature and pH of the plant surface and activity of the viral pesticide (Falcon, 1985) have limited their utilization. Genetic engineering could possibly solve some of these problems in future. Induced changes could however also affect other properties such as increase their host range, virulence and virion stability (Faulkner & Boucias, 1985), thus inducing new problems. Antigenic responses were detected for granulosis virus of the codling moth *Cydia pomonella* in wood mice, *Apodemus sylvaticus* (Bailey & Fujita, 1987). However, these responses were due to passive exposure and not to insect virus replication in mammalian hosts.

1.3.9 **Aphid species utilized in study**

Rhopalosiphum padi (oat aphid) populations occur in large numbers in the small grain producing area of the Winter Rainfall Region and to a lesser extent in the western and eastern Orange Free State. It is a small aphid with a rounded body, 1.5 to 2 mm in length. Their colour is dark-green and sometimes nearly black. Part of the abdomen between the siphunculi is reddish in colour. *R. padi* feeds on both sides of the leaves and sometimes on the stem near the ground (Annecke & Moran, 1982). It is an early colonizer of grain fields in the Winter Rainfall Region with colonization starting from June through to August depending on climatic conditions.

Diuraphis noxia, also known as the Russian wheat aphid, is well known in the Soviet Union, Turkey and other Mediterranean countries (Erdelen, 1981; Von Wechmar & Rybicki, 1981). This aphid arrived in South Africa during 1978. It is now also found in several of the major small grain producing areas of the United States of America. The *D. noxia* aphid is a relatively small aphid, 1.5 to 1.8 mm in length. The oblong body is small and tapered posteriorly. When viewed sideways under a light microscope, the characteristic "double tail" can be seen. The colour of the aphid is pale gray-green. It feeds on the upper surface of the leaves which roll inwards from the edges and show typical white, yellow and purple streaks parallel to the midvein. The aphids show a preference for young leaves (Annecke & Moran, 1982). In the Winter Rainfall Region, *D. noxia* infest plants early in the season (June/July) and is normally the first colonizer of small grain fields (Laubscher, unpublished data). It has a marked tolerance for cold temperatures as experienced in the eastern Orange Free State. The aphid causes serious phytotoxemia in small grains (Aalbersberg, 1987).

Sitobion avenae (brown ear aphid or English grain aphid) is a relatively large aphid, 2 to 3 mm in length. The colour of the aphid is dark brown, sometimes green to yellow green. *S. avenae* is not indigenous in South Africa. Its occurrence was noted in 1971 (Dürr, unpublished results) and was observed for the first time in the western Cape in 1974 (Laubscher, 1985). Presently the aphid occurs almost everywhere in South Africa where small grain is grown. This aphid feeds mainly on the leaves, but after ear-emergence, *S. avenae* is found in increasing numbers on the ears (Annecke & Moran, 1982; Laubscher, 1985). *S. avenae* is a late colonizer, and normally appears in the grain fields at the end of August or in the beginning of September, and stays more or less until the middle of October.

Myzus persicae (green peach aphid) has a body length of 1.5 to 2.5 mm. The colour in apterae are yellow-green or rarely reddish and the alatae are pale green with a dark patch on the dorsal side of the abdomen (Annecke & Moran, 1982). *M. persicae* feeds on many economically important crops and is an efficient vector of plant viruses.

Brevicoryne brassicae (cabbage aphid) is a soft bodied, slow moving insect about 2 to 2.5 mm long. The colour of the aphid is a pale gray-green or a grayish blue-green, covered with light gray, powdery wax. *B. brassicae* can cause extensive damage to vegetables (Annecke & Moran, 1982).

1.3.10 Techniques utilized in the study

Non-destructive detection of virus in aphid body tissue was done with immunofluorescent technique and immunogold labelling. The use of these techniques will be reviewed briefly.

1.3.10.1 Immunofluorescent technique

Fluorochromes are dyes which will absorb radiation, e.g. ultraviolet light, and thereby become excited. The excited molecules then emit radiation which ceases almost immediately after withdrawal of the exiting radiation. Fluorochromes can be converted to derivatives containing chemically active groups such as sulphonyl chloride or isothiocyanate, and in these forms they can be conjugated with protein. Coons *et al.* (1941) first introduced fluorescent labelled antibodies. Such conjugated antibodies retain their specific immunological reactivity. Fluorochrome labelling of antibodies allows both direct observation and precise localization of the sites of reacting antibody (Holborow & Johnson, 1967).

There are two methods of applying the immunofluorescent technique, the indirect and direct method. In the indirect method, tissue must first be fixed, embedded and sectioned. These sections are incubated in a solution containing excess protein (bovine serum albumin, BSA) to block sites for a specific protein attachment. Then the section is incubated in virus-specific antibody e.g. rabbit antibody, washed, and further incubated on fluorescent labelled anti-immunoglobulin e.g. goat anti-rabbit (GAR) antibody. Finally the sections are washed and viewed in a microscope fitted with a uv lightsource.

In the direct method the labelled antibody is applied to the preparation and will combine with a specific antigen. The excess of labelled antibody is washed away. With a heterologous reaction, no combination occurs, and washing removes the labelled antibody (Walker *et al.*, 1971).

1.3.10.2 Immunogold labelling

The first use of colloidal gold for immunoelectron microscopy (IEM) was reported by Faulk and Taylor (1971), and has proven to be a very useful marker in electron microscopic and light microscopic immunocytochemistry (Horisberger, 1981; Horisberger, 1983; De May *et al.*, 1986; Bendayan, 1985).

Colloidal gold as a marker in immunocytochemistry has several advantages over established markers like ferritin and radioactive isotopes. The extreme electron density of gold particles makes them clearly visible as black dots on electron micrographs. The different sizes ranging from 5 to 80 nm (Slot & Geuze, 1981; Slot & Geuze, 1985) make them suitable for labelling two or even three antigens in the same specimen. Some molecules (immunoglogulins, enzymes) that are primary reagents in specific recognition, cannot always be visualized microscopically, yet their indirect visualization was one of the main topics in IEM (Carrascosa, 1988).

Three methods exist for the preparation of gold particles, all of which involve the reduction of chloroauric acid with a suitable agent such as trisodium citrate, tannic acid and white phosphorus (Frens, 1973; Slot & Geuze, 1981). The gold particle sizes depend on the procedure chosen. Several biologically active proteins (protein-A, enzymes) can be absorbed to the surface of the gold particles without loss of their bio-activities (Roth, 1983). The same gold marker used in electron microscopy may be used in light microscopy, with enhancement by silver staining (De May *et al.*, 1986).

A requirement for the detection of antigens in thin sections is the retention of antigenic properties of polypeptides to be detected, and good ultrastructural preservation. The conventional fixation, dehydration and embedding schedules including osmium tetroxide and uranyl acetate fixation and a polar embedding media, metacrylate, Spurr or Epon, tend to partially or completely destroy antigenic properties of proteins. The success of the immunogold marker system is due to the development and improvement of alternative methods for fixation, dehydration and embedding of tissue. Recently developed polar embedding resins like Lowicryl K4M and London Resin white or gold (LR white; LR gold) have improved preservation of cellular structures and retention of antigenicity (Carlemalm *et al.*, 1982; Slot & Geuze, 1983; Van Lent, 1988).

These new developments in the preparation of tissue for electron microscopy have led to an extensive utilization of the immunogold marker system in animal and plant virology (Patterson & Verduin, 1987; Appiano *et al.*, 1987). For the first time a reasonably easy and reliable technique is available for the *in situ* detection of animal and plant viral antigens. A couple of successes have been documented utilizing this system i.e. Garnier *et al.* (1986) with turnip yellow mosaic virus (TYMV), Saito *et al.* (1987) and Tomenius *et al.* (1987) with tobacco mosaic virus (TMV) and Stussi-Garaud *et al.* (1987) with alfalfa mosaic virus (AlMV).

Immunogold labelling as a method for *in situ* localization of structural and nonstructural viral proteins was the first (immuno)cytochemical technique with potential for routine application in the light and electron microscopical study of plant and animal virus-infected cells (Russel & Rohrmann, 1990; Van Lent, 1988).

CHAPTER 2

DETECTION OF APHID LETHAL PARALYSIS VIRUS BY IMMUNOFLUORESCENT TECHNIQUE

DETECTION OF APHID LETHAL PARALYSIS VIRUS BY IMMUNOFLUORESCENT TECHNIQUE

SUMMARY

The indirect immunofluorescent technique was used to detect ALPV in dissected aphids. Aphids used in this study were taken from known infected *R. padi* and *D. noxia* colonies and aphids collected from naturally infested small grains. ALPV - specific antibody was used to bind to the virus in the tissues, which in turn was detected with goat anti-rabbit fluorescein iso-thiocyanate-conjugated antibody. Detection of virus by immunufluorescence was found to be a more sensitive method when compared to DAS-ELISA. The greater sensitivity was of importance when testing for low levels of virus in infected aphid body tissues where infection could cause detection problems. The immunofluorescent technique was a relatively inexpensive method for routine tests.

2.1 INTRODUCTION

Although ALPV was first identified in South Africa in 1987 (Williamson *et al.*, 1988), retrospective analysis of collected specimens and antisera prepared earlier, showed that the virus had already been present in natural populations of *D. noxia* collected in the Orange Free State in 1978 and 1979. ALPV usually occurred together with RhPV, another aphid virus, as a mixed infection (Williamson *et al.*, 1988; 1989; Von Wechmar unpublished results).

To determine the pathogenesis of ALPV and RhPV and the effect of the viruses on the general biology and survival of the aphids, large numbers of single aphids had to be screened for the presence of the viruses. Two methods were evaluated. This chapter presents information on the effectivity and sensitivity of DAS-ELISA (Appendix B.3.4) and the indirect immunofluorescent method for screening large numbers of aphids for virus. The methods and preliminary results were reported previously (Laubscher and Von Wechmar, 1991). Since RhPV is not as pathogenic to aphids as ALPV, it was considered less important and was not studied in the same detail (Williamson *et al.*, 1989).

2.2 MATERIALS AND METHODS

2.2.1 Origin of infected aphids

a) A laboratory reared virus-infected *R. padi* aphid clone (Appendix D.1) was maintained at a controlled temperature regime of 24°C / 20°C day/ night and 12L:12D hours day length (Appendix D.2.1).

b) Field collected aphids sampled at weekly intervals during the small grain growing season (Chapter 5) included *R. padi*, *D. noxia*, *M. dirhodum* and *S. avenae*.

2.2.2 Aphid maintenance and propagation

The propagation and maintenance of the *R. padi* aphid clone was mainly as described by Von Wechmar (1990c). Details of aphid maintenance and propagation are described in Appendix D.2.1. Aphids were maintained mainly on barley seedlings (*Hordeum vulgare*), cv. Clipper. To maintain active colonies, transfer to new seedlings were made on a six-day cycle.

2.2.3 Virus detection with DAS- ELISA

Detection was done with DAS-ELISA (Appendix B.3.4) using antisera specific for either ALPV or RhPV available from previous studies by Williamson (1988) (Appendix B.3.1). For testing, single aphids (nymphs or adults) were crushed in 400 µl of phosphate buffered saline pH 7.0 (PBS) (Appendix A.3.4) and 200 µl of this used for testing the presence of ALPV and RhPV respectively in a parallel test. For calibration of DAS-ELISA, dilutions of purified virus of known concentration were used (Appendix B.1.1 and 1.3).

R. padi aphid clones showing no apparent or detectable virus infection were used as negative controls. The same clones were used previously by C. Williamson (Williamson *et al.*, 1989). These aphids were kept in self-contained incubators at a temperature regime of 10 / 7°C day/night and 12L:12D hours of day length (Appendix D.2.1).

Aphids of non-infected clones of *Brevicoryne brassicae* and *Myzus persicae* were included to check whether aphid tissues showed non-specific fluorescence.

2.2.4 Virus detection with the indirect immunofluorescent technique

The original immunofluorescent technique of Walker *et al.* (1971), was adapted for use with aphids. The technique was chosen because it is known to be sensitive to detect antigens in tissue preparations or viable cells.

Goat anti-rabbit gamma globulins (Appendix B.3.2 and 3.3) prepared by the method of Clark and Bar-Joseph (1984) were labelled with fluorescein isothiocyanate (Merck) (GAR IgG-FITC) by the dialysis method of Otsuki and Takebe (1969). Preference was given to the indirect immunofluorescent technique to exclude nonspecific reactions with aphid body proteins.

Single aphids were dissected in normal saline, containing 0.05% sodium azide. The ovariols with developing embryos were separated from the abdominal tissue. The dissected aphid was washed twice with pH 7.0 PBS. Ten microliters of ovalbumin (0.1%) (to break surface tension of the droplets) were then added followed by addition of five microliters of antibody as a 1/16 dilution and incubated for 30 min to one hour at 37°C. The titre of the antiserum used was 1/256 when tested by the Ouchterlony double diffusion test.

The specimen was washed twice with PBS to remove unbound antibodies. GAR IgG-FITC labelled antibodies were added at 1/50 dilution and the specimen incubated for 30 min at room temperature in the dark. The specimen was washed twice with PBS and left to dry at room temperature. One drop of PBS was added to the dried specimen prior to covering with a glass slip. Specimens were viewed after two hours (or longer) with a ZEISS- inverted uv microscope at magnifications of 288x and 720x (Plan and Nuofluar lenses respectively) and photographed with a Contax RTS camera on 400 ASA colour slide film.

Controls consisted of the following : aphids from a clone apparently free of virus infection treated as above; infected aphids treated with normal rabbit serum and GAR IgG-FITC; infected aphids treated with GAR IgG-FITC-labelled antibodies only and infected aphids without antibody treatment to check for autofluorescence.

2.2.6 Utilization of the indirect immunofluorescent technique to determine pathogenesis in natural aphid populations

Aphids were collected weekly from small grain fields in the Swartland region in the western Cape Province. The survey was done at five different localities and stretched over a five month period, from June to October, and was repeated in two consecutive years, 1988 and 1989. This method was also utilized to determine the role of ALPV as a growth limiting factor in aphid populations under natural conditions together with other natural enemies of aphids (Chapter 5 section 5.3.6)

Collection was done as follows : leaves with live aphids, randomly picked, were placed in Petri dishes, and kept in a cool place overnight. The next day single aphids were transfered into preprepared specimen vials (35 mm x 55 mm) (Appendix D.2.4) to maintain them in a live condition awaiting dissection. The vials contained sterilized vermiculite moistened with Knopp's inorganic nutrient solution (Riker & Riker, 1936) (Appendix A.11) and Clipper barley seedlings grown from surface sterilized seed (Appendix D.2.2). Vials were kept at 4^{o}C to slow down the reproduction rate. Single aphids from these groups were tested for ALPV and RhPV with indirect immunofluorescent technique and DAS-ELISA (Appendix B.3.4).

Fungal-infected-aphids were among the weekly collections for routine laboratory assays by DAS-ELISA and immunofluorescent antibody technique. For the latter test, single aphids were dissected (section 2.2.4) to retain as much as possible of

the internal organs intact.

2.3 RESULTS

2.3.1 Detection with indirect immunofluorescent technique

The dissection of 40 - 80 aphids could usually be done in one day and the specimens stored in a moist chamber at 4°C overnight. The washing, staining and viewing of dissected specimens was done the following day.

Unborn embryos inside infected viviparous aphids, showed a brilliant yellow-green fluorescence (Figs 2.1, 2.2, 2.3, 2.4 and 2.6). Embryos, at the same stage of development taken from the same mother, always showed the same level of brilliance. However, in the same ovariole it could be observed that embryos at different stages of development, appeared to contain different levels of virus infection (Fig. 2.1) with older embryos fluorescing more strongly than the younger ones.

The intestine was never observed to show fluorescence in over 500 dissected aphids examined (Fig. 2.2). The epidermis and underlying mesodermal tissue of the adult aphid also showed a brilliant yellow-green fluorescence (Fig. 2.3). Fluorescence in different body tissues of the embryos from dissected adults are shown (Fig. 2.4).

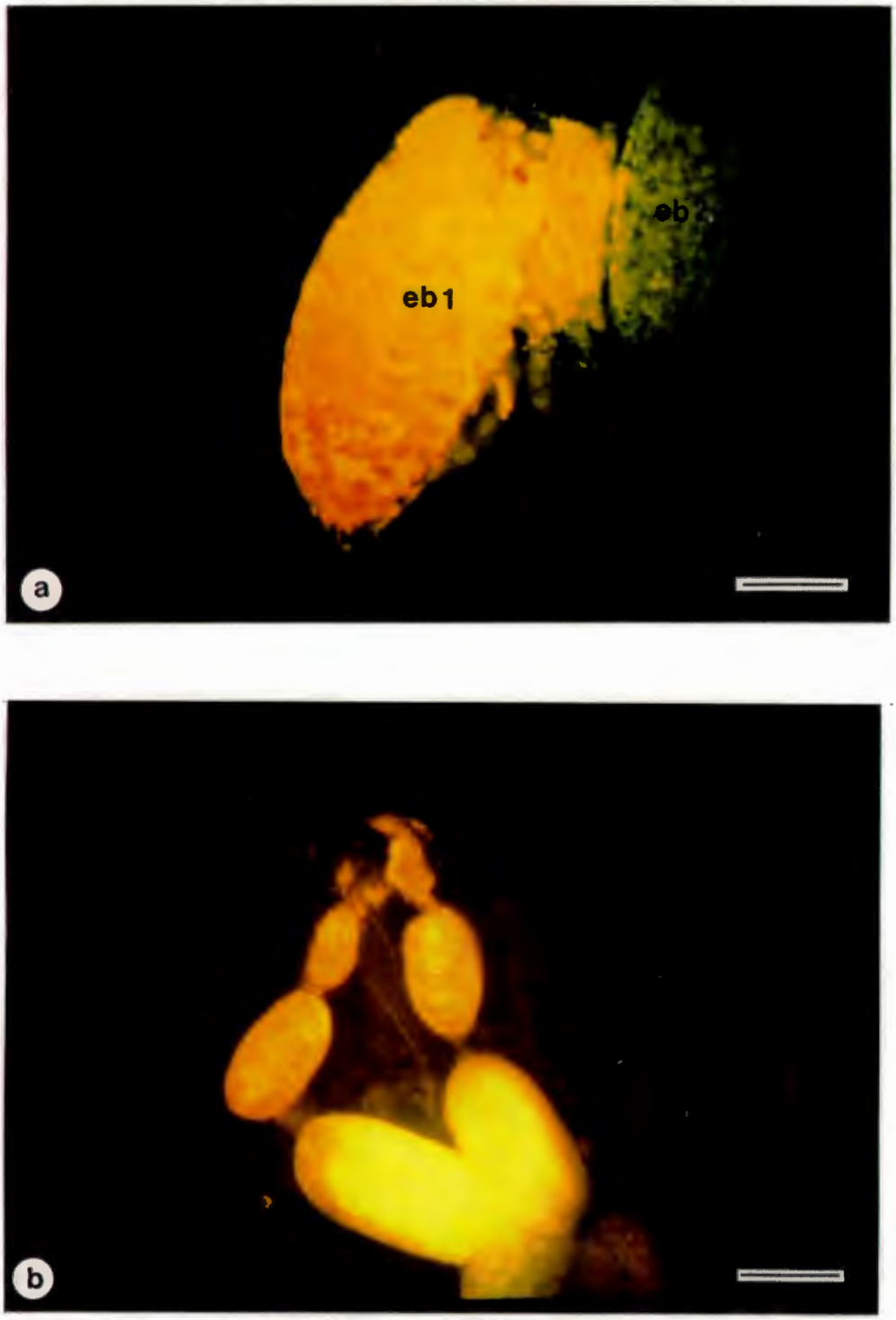


Fig.2.1. Dissected aphids treated with anti-ALPV serum followed by GAR IgG-FITC labelled antibody to detect ALPV *in situ*. Colour photographs reproduced from original colour slides. a) Embryos in the same ovariol at different stages of development (eb1 and eb2) appeared to contain different levels of virus infection. Bar marker represents 26 μm. b) A group of embryos in the same ovariole showing different levels of infection. Bar marker represents 66 μm.

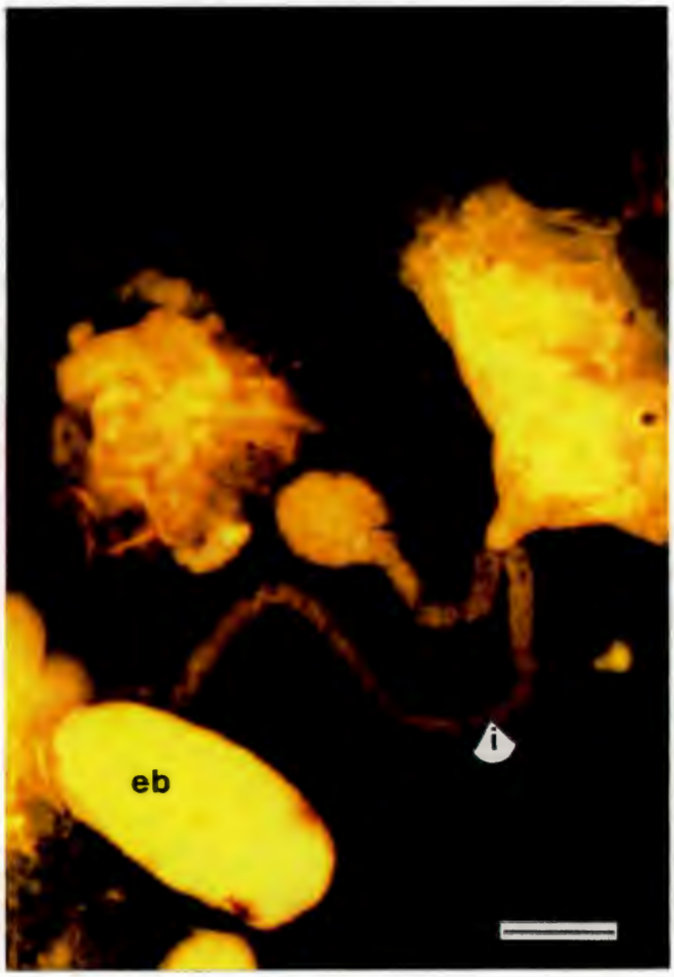


Fig. 2.2. Non-fluorescing intestine (i) of an ALPV-infected aphid adult, compared to the positive fluorescence of an embryo (eb). Bar marker represents 66 μm.

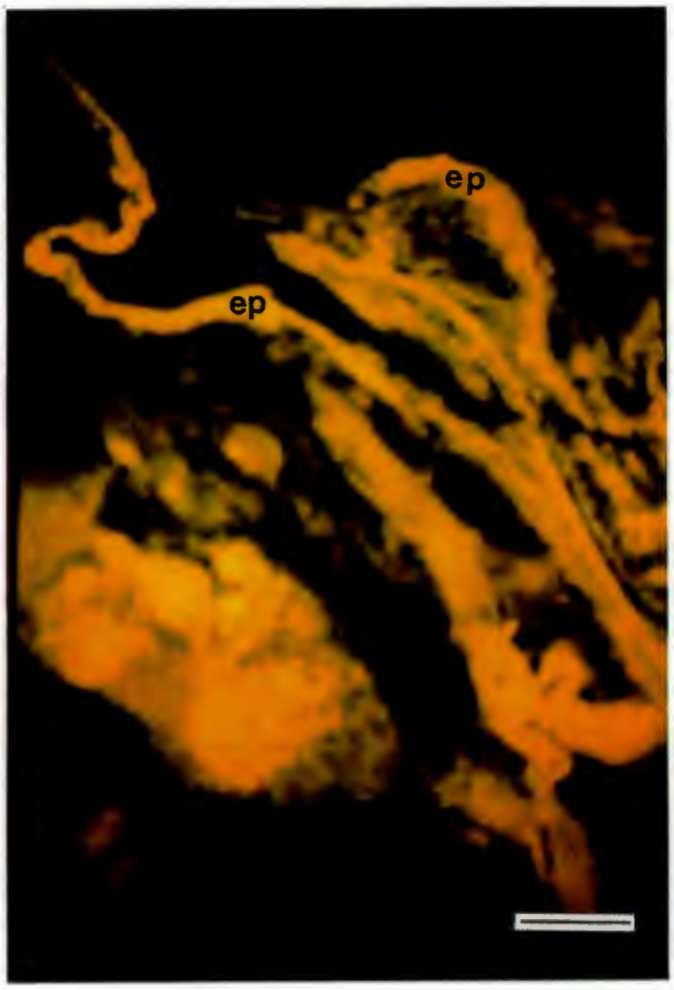


Fig. 2.3. Epidermis (ep) of a dissected aphid adult showing fluorescence. Bar marker represents 26 μm..

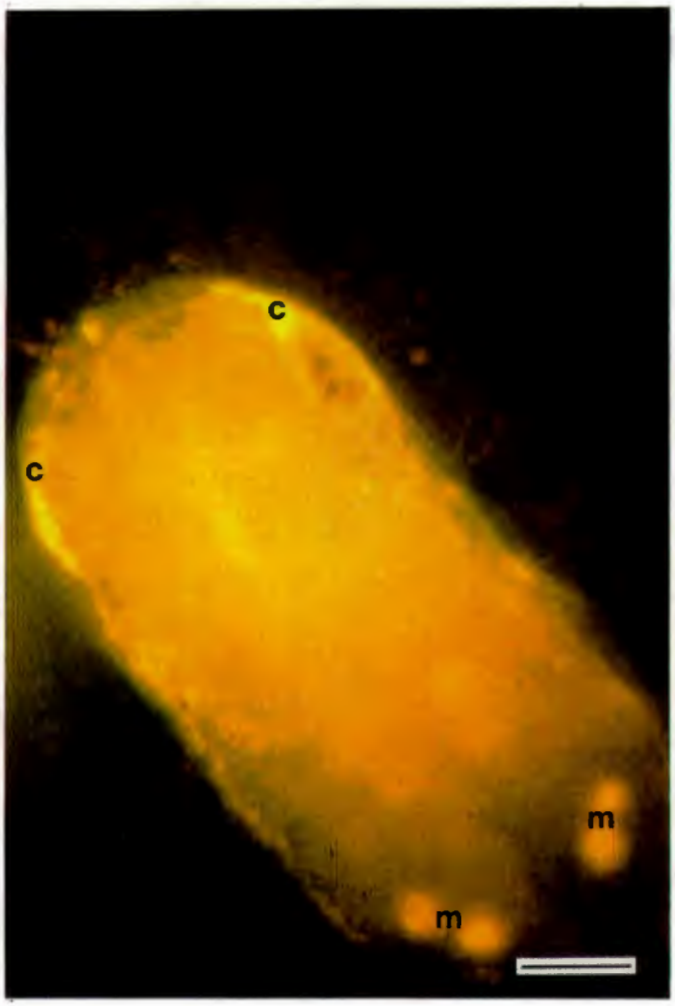


Fig. 2.4. Strong fluorescence of chitin (c) and mycetome (m) in an aphid embryo. Bar marker represents 66 μm.

Brilliant yellow-green fluorescence was associated with positive virus infected aphids (Figs 2.1, 2.2, 2.3, 2.4 and 2.6). None of the dissected aphids taken from apparently uninfected clones showed any such fluorescence (Fig. 2.5). Photos taken of these specimens are dark. Specimens prepared with normal rabbit serum and GAR IgG-FITC labelled antibodies did not fluoresce (Fig. 2.5).

To check for the presence of autofluorescence in aphid tissues generally, specimens of non-infected clones of *B. brassicae* and *M. persicae* were examined as discussed above. The aphids showed no fluorescence. Infected aphids prepared with normal rabbit serum or any non-specific antibody treatment also did not fluoresce.

2.3.2 Field survey

The indirect immunofluorescent technique was utilized in parallel with DAS-ELISA which was used to investigate the difference in levels of the two aphid viruses occurring in bodies of field collected aphids (*R. padi*) from Langgewens (Table 2.1).

Fig. 2.5. Non-fluorescing control showing embryos (eb) and part of parent body in background (p). Bar marker represents 66 μm.

Table 2.1. Presence of ALPV and RhPV in a natural aphid population (*R. padi*) at Langgewens screened with indirect immunofluorescent technique and DAS-ELISA

DATE*	INDIRECT IMMUNOFLUORESCENCE	DAS-ELISA	
	% aphids positive	% aphids positive	
	ALPV	ALPV	RhPV
31/7/89	90	30	6
3/8/89	97	44	8
7/8/89	95	6	12
14/8/89	75	34	50
17/8/89	77	26	36

* Date of field collection

Fungal infected aphids were also tested with immunofluorescent technique for the presence of ALPV. The content of the fungal hyphae fluoresced strongly positive with anti-ALPV serum followed by GAR-IgG-FITC (Fig. 2.6). An increase of fungal infection coincided with a sudden drop of aphid numbers. This drop also coincided with the highest percentage virus-infected aphids in the natural population (Chapter 5). Tests with anti-RhPV serum were done for fungus infected aphids and were found to be positive (result not shown).

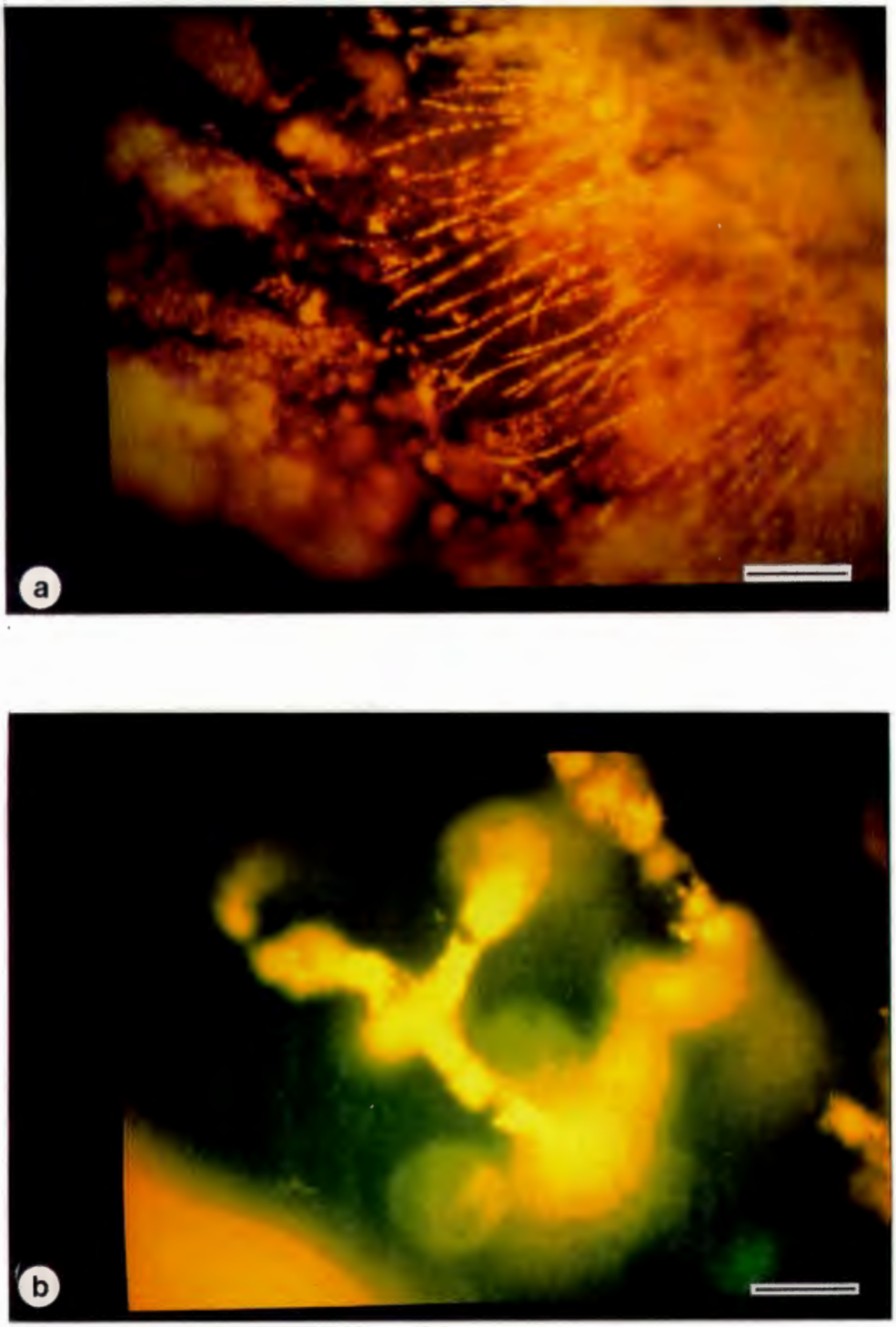


Fig. 2.6. Fluorescence of dissected fungal-infected-aphids. a) Fungal hyphae on the body of the aphid. Bar marker represents 66 μm. b) Hyphae around the leg of an aphid. Bar marker represents 26 μm.

2.4 DISCUSSION

No autofluorescence was noticed. The absence of fluorescence in the aphid gut tissue may be caused by a sheath, called the tunica propria which covers these organs (Fig. 1.4) (Ponsen, 1972). This membrane is comprised of different layers and is a rather diffused osmiophilic layer (Wigglesworth, 1956; Forbes, 1964) which could prevent penetration of the antibody and GAR IgG-FITC-label.

The comparison of results obtained by immunofluoresce and DAS-ELISA for aphids collected in 1989 at Langgewens, indicates the difference in sensitivity of the two methods used (Table 2.1). Due to the greater sensitivity and reliability of the indirect immunofluorescent technique, this method was prefered for sensitive detection of ALPV specifically. The purpose for comparing the two techniques was basically to check the reliability of fluorescent labeled antibody for virus detection in aphid tissues. The minimum virus detectable with DAS-ELISA on crushed aphid tissues, was 23 ng/200 µl. With immunofluorescence the sensitivity was much greater, although accurate quantitative comparisons could not be made (Table 2.1).

Aphids were dissected carefully to maintain organs intact in an effort to determine whether one or the other fluoresced stronger due to the presence of virus. The same applied to the embryos and the germarium. The low levels of infection noted (Fig. 2.1)(Table 2.1), could not be detected by DAS-ELISA and would have been overlooked as positive infections. These infections had previously been considered to be inapparent infections and could be detected by cDNA hybridization, but not by DAS-ELISA (Williamson, 1988). The relative high cost of cDNA hybridization precluded its use in this study.

Detection of ALPV infection by immunofluorescent technique had the advantage that the distribution of virus infection in the different aphid body tissues could be observed and the degree of infection could be assessed. Early infections were always first visible in the mycetome region (Fig. 2.4). Infected mycetocytes were usually more clearly discernable in *S. avenae* embryos compared to other species. Apart from small differences, the pathogenesis of different aphid species when examined by immunofluorescent technique was very similar. Further advantages of immunofluorescent technique were the large number of aphids that could be processed on a regular, weekly basis.

CHAPTER 3

DETECTION BY IMMUNOGOLD CYTOCHEMICAL LABELLING OF APHID LETHAL PARALYSIS VIRUS IN THE APHID *Rhopalosiphum padi* (HEMIPTERA : APHIDIDAE)

DETECTION BY IMMUNOGOLD CYTOCHEMICAL LABELLING OF APHID LETHAL PARALYSIS VIRUS IN THE APHID *Rhopalosiphum padi* (HEMIPTERA : APHIDIDAE)

SUMMARY

ALPV was visualized in ultra thin sections of aphid body tissues by an immunogold cytochemical technique. ALPV antigen was detected in the ovariole tissue, tracheocytes, symbionts of the mycetocytes, fat body cells, brain tissue, nerve tissue and stomach epithelial tissue. Virions were detected predominantly in the cytoplasm but were also found in the nucleus. ALPV antigen was not detected in muscle fibres or mitochondria.

3.1 INTRODUCTION

Little is known about the relationship between aphid infecting viruses and their hosts. Aphids differ fundamentally from other insects in their biology, feeding behaviour and digestive physiology (Minks & Harrewijn, 1987). They therefore pose unique questions concerning virus-host associations, in particular with regard to vertical transmission of viruses during parthenogenetic reproduction.

Localization studies of picorna-like viruses in insect tissues have been performed by direct observation by electron microscopy. The following insect viruses have been studied: cricket paralysis virus (CrPV) (Scotti *et al.*, 1981), sacbrood virus (SBV) (Mussen & Furgala, 1977), Kashmir bee virus (KBV) (Dall, 1987) and *Drosophila* C virus (DCV) (Jousset *et al.*, 1977).

However, most of the detailed studies on insect/virus relationships have been performed on nuclear polyhedrosis virus (NPV) infecting lepidopteran adults and larvae (Smith-Johannsen *et al.*, 1986; Vail & Hall, 1969; Mathad *et al.*, 1968). NPV is easily identified due to the large virion size and the presence of inclusion bodies (Faulkner, 1981; Tanada & Hess, 1984). As picornavirus particles are similar in size to cellular organelles such as ribosomes, using similar techniques is difficult. This situation is further confused as insects may be co-infected with more than one small RNA virus (Anderson & Gibbs, 1988; Dall, 1985; Williamson *et al.*, 1988).

In this study, immunogold label is used to specifically identify ALPV in aphid tissue. The immunogold technique also enhances the detection of virions.

A comparison of the tissues infected by ALPV with other picorna-like virus infections will be made and the possible mode of transmission during parthenogenetic reproduction will be discussed.

3.2 MATERIALS AND METHODS

3.2.1 Antisera

The preparation and specificity of the antisera were described previously by Williamson *et al.* (1988) (Appendix B.3.1).

3.2.2 Aphid maintenance and propagation

Before selecting the aphids for embedding the clone was monitored for several weeks under constant conditions. *R. padi* aphids infected with ALPV and RhPV were reared at 20 / 22°C and 12L:12D as described by Von Wechmar (1990c) (Appendix D.2.1). Aphids were transferred weekly to fresh barley seedlings. During this period aphids were screened by DAS-ELISA at regular intervals to determine maximum incidence of infection for collection of aphids for embedding (Table 2.1 Chapter 2). Similarly aphids were monitored by immunofluorescent technique to check the degree of infection in single aphids of that particular clone. The time for collecting aphids for embedding was chosen to be such that virus could be detected by fluorescence in different organs but before the entire body was infiltrated with a large quantity of virus. For purposes of embedding it was considered important to rather have a lower level of infection to be sure that cross-contamination of tissues with excess virus did not occur. To achieve this purpose the work reported in chapter two was considered essential.

For embedding, adult aphids were randomly selected from the group of aphids. Control aphids were taken from a colony that did not react with antiserum to ALPV or RhPV when tested with DAS-ELISA (Appendix B.3.4) and the immunofluorescent technique (Chapter 2).

3.2.3 Embedding

When examining virus infection of various tissues, it is ideal to dissect out certain organs (Forbes, 1964). Due to the small size of the aphid, dissection of whole organs was difficult.To overcome this problem, whole aphids were embedded. This had the added advantage that haemolymph containing virus did not leak out to contaminate other tissues.

Details of the embedding technique used are given in Appendix C.1.2. To facilitate penetration of the fixative and resin, the antennae and legs were cut off and the body cut into two pieces at the juncture of the abdomen and the thorax. Aphids were fixed in 2% gluteraldehyde, postfixed in 1% osmium tetroxide and dehydrated in an alcohol series followed by acetone. Embedding was done in Spurr's resin (Spurr, 1969) and ultrathin sections (60-70 nm) were cut on an ultramicrotome (Reichert-Jung) with a glass knife. Sections were collected on 300 mesh carbon coated copper grids.

3.2.4 Immunogold labelling of antigen in ultrathin sections

The labelling methods of Stussi-Garaud *et al.* (1987) and Van Lent (1988) were adapted, compared, and utilized for immunogold labelling (IGL) (Laubscher *et al.*, 1991). Five nanometer goat-anti-rabbit-gold (GAR-IgG-gold) labelled antibodies were used for IGL (Janssen Life Sciences Products).

Sections were incubated for 30 min at room temperature on 20 µl drops of 1% BSA in PBS (pH 7.2) (Appendix A.3). They were then transferred to drops of 0.01 mg/ml anti-ALPV-IgG in PBS-BSA and incubated on GAR-gold which was diluted in PBS-BSA to give a suspension with A_{520} nm = 0.15. The ultrathin sections were not incubated on a saturated aqueous solution of sodium metaperiodate as reported by Van Lent (1988).

In the Stussi-Garaud *et al.* (1987) method the grids were incubated on drops of specific anti-ALPV serum or immunoglobulins (IgG) diluted with phosphate buffer, (pH 7.2) (Appendix A.1.2) and 0.05% Tween 20 (PT20)(Appendix A.6) to 1/10 000 for four hours. The grids were then washed with PT20 four times for 10 min and incubated in GAR-IgG-gold (A_{520}nm = 0.15) for one hour. The grids were then washed twice for 10 min with PT20, followed by two washes with double distilled water. The grids were negatively stained with 2% uranyl acetate for 3 min and rinsed immediately with double distilled water.

Controls consisted of infected aphids incubated in normal rabbit serum and antiserum to tobacco mosaic virus (TMV). Additional controls consisted of uninfected aphid sections incubated with ALPV antiserum and treated identically to the other samples. Prepared specimens were examined in a Jeol CX100 or Zeiss EM109 electron microscope at 120 and 80KV respectively. Sections were photographed at 10 000, 20 000, 30 000 or 50 000X magnification.

3.3 RESULTS

Results presented in this section are derived from sections obtained from ten individual embedded and sectioned aphids. Single aphids for embedding were from groups of aphids that reacted positively with ALPV antiserum in DAS-ELISA and by immunofluorescent technique (Laubscher & Von Wechmar, 1991)(Chapter 2).

3.3.1 **Embedding**

The ultrastructure of the aphids was well preserved in Spurr's resin. Membranes and organelles could be seen in thin sections, but the detection of virus particles among other cellular organelles without IGL was impossible. Good definition and contrast of tissues was obtained with the embedding method used.

3.3.2 **Immunogold labelling of antigen in ultrathin sections**

The concentration of binding that was achieved with the Stussi-Garaud *et al.* (1987) method was more superior to Van Lent's (1988) adapted method. There was however not a complete decrease in the antigenicity of antigens by utilizing this method. The presence of virions was identified by the typical clustered decoration with gold label. An average binding of two to six or more gold particles to antigen-antibody complexes was achieved in ultrathin sections (Laubscher *et al.*, 1991). The preparation of immunogold labelled antibodies and their binding to ALPV was optimized in the same laboratory by A. Hackland (unpublished results)(Laubscher *et al.*, 1991). A comparison of 10 and 5 nm gold particles showed that 5 nm gold particles were more suitable. This finding and the small size of the virions was decisive in chosing 5 nm gold label.

The detection of ALPV in different aphid body tissues is described below.
Cuticle: Dense concentrations of IGL bound to virions in the cuticle were repeatedly noted in sections cut from different aphids. Labelled virions were present in the epicuticular and inner endocuticular layers (Fig. 3.1). IGL was uniformly distributed and no specific foci were identified.
Fat body cells: One of the most abundant cell types in the aphid body are the fat body cells. IGL identified virions scattered throughout this tissue (Fig. 3.2). A clear difference in concentration was noted between the cuticle and the fat body cells, the latter being less densely labelled (not shown).
Muscle tissue: IGL was absent in muscle tissue examined in the mesodermal tissue indicating that it was free of virions (Fig. 3.3). The absence of gold-labelled virions in muscle tissue appeared to be consistent for the various muscle tissues examined in the mesoderm and the digestive tract (not shown).
Brain tissue: A moderate density of IGL was observed in brain tissue (Fig. 3.4). The IGL appeared to be associated with membranes and clearly showed a clustered arrangement of gold-labelling.
Nerve tissue: Sparse IGL was observed on membranes in axons of nerve tissue, indicating a low virus concentration in this tissue (Fig. 3.5).

Stomach: Dense labelling was observed in the stomach epithelial tissue (striated border) with the foci of virions adjacent to the stomach lumen (Fig. 3.6) (Fig. 1.4). Less dense labelling was observed in the stomach tissue adjacent to the tunica propria (Fig. 1.4), with an absence of labelling in the vacuolated areas of these cells (Fig. 3.6).

Ovariole tissue: Figure 3.7 illustrates ovariole tissue at a lower magnification to show the arrangement of nuclei in germarial tissue and the distribution of gold-labelled virions throughout. ALPV virions with IGL were visible in the nuclei of the germarial tissue of the ovarioles (Fig. 3.7). There were no specific foci of virions present in the nucleus and binding was fairly evenly distributed (Fig. 3.8). Labelled virions were also present at the cell wall surface of the germarial cells. More gold particles appeared to be concentrated in the cytoplasm of the germarial cells than in the nuclei.

Symbionts: Figure 3.9 illustrates symbionts in the mycetocytes at low magnification. Virions detected by IGL were observed inside the symbionts (Fig. 3.10). A low concentration of bound gold particles appeared in the fine, mid-dense matrix of the cytoplasm of the symbionts. No specific binding of gold particles was noticed in the nucleoplasm consisting mainly of chromatin (Fig. 3.10). Labelling was also noticed in the cytoplasm of the mycetocytes (Fig. 3.10).

Tracheocyte: A high concentration of virions detected by IGL was noted in the tracheocytes of the trachea (Fig. 3.11) and dense labelling was noted on tracheocyte membranes. These trachea were present in the mesodermal tissue of the aphid. Adjoining fat body cells showed dense scattering of gold-labelled virions indicating a high concentration of virus in the fat body cells.

Haemocytes: Few virions detected by gold particles were identified in the haemocytes associated with the mesodermal tissue (Fig. 3.12). Similarly, few gold-labelled virions were noticed in the haemolymph (results not shown).

Mitochondria: Gold-labelled virions were not detected in the mitochondria of the stomach epithelial tissue (striated border) (Figs 1.4 and 3.6) or fat body cells (Fig. 3.2). In degenerating mitochondria of the above tissues gold-labelled virions were observed (not shown).

Control: The typical clustered arrangement (two or more particles) of gold label was absent in all the control samples examined (Fig. 3.13).

3.4 DISCUSSION

Five nanometer gold particles gave good results for labelling antigen-antibody complexes in ultrathin sections. Complete loss of antigenicity as stated by Roth (1982) and Bendayan and Zollinger (1983) was not experienced for Spurr.

The detection of ALPV in the cuticle and epidermal tissue (Fig. 3.1) confirms previous results obtained by immunofluorescent studies in which strong fluorescence was regularly noted in the cuticle and epidermis of infected aphids (Laubscher & Von Wechmar, 1991). Similar observations were made with cricket paralysis virus (CrPV) and Kashmir bee virus (KBV) infected insects (Mussen & Furgala, 1977; Dall, 1987). The presence of IGL attached to virions in the intercuticular space may indicate that free virus may occur on the surface of new developing cuticular layers. Shed skin could therefore serve as sources of contagion and may have dramatic implications in the overall epidemiology of ALPV in aphid populations. Similarly, virions trapped in the shed skin of an aphid, pose a source of contagion in dense populations.

ALPV was not detected in any of the muscle tissues examined (Fig. 3.3). Similarly CrPV and sacbrood virus (SBV) particles and RhPV were not observed in muscle tissue (Mussen & Furgala, 1977; Scotti *et al.*, 1981; Gildow & D'Arcy, 1990) but KBV was noticed in the alimentary tract musculature (Dall, 1987).

Labelled ALPV was observed in stomach epithelial cells. The same phenomenon was noted for CrPV, RhPV, SBV and KBV (Mussen & Furgala, 1977; Scotti *et al.*, 1981; Dall, 1987; Gildow & D'Arcy, 1990). Brain tissue was moderately dotted by gold particles (Fig. 3.4), indicating an infection of this tissue. The presence of virions in the stomach epithelial cells and in brain tissue confirm earlier findings of Hatfill *et al.* (1990) in which an apparent high concentration of virus was detected with nucleic acid *in situ* hybridization. Mussen and Furgala (1977) did not find SBV in brain tissue, but Dall (1987) and Scotti *et al.* (1981) showed that KBV and CrPV respectively were present in the nerve system.

IGL of ALPV was also noted in nerve tissue (Fig. 3.5). The detection of virions in the brain and nerve tissue, and not in the muscle tissue, indicates that the observed paralytic effect on aphids is probably the result of infection of the nervous system and not of the musculature. Gildow and D'Arcy (1990) did not observe RhPV particles in nerve tissue.

IGL occurred more frequently in the cytoplasm of the germarial cells of the ovarioles than in their nuclei (Fig. 3.7 and 3.8). Infection of ovariole tissue by CrPV, SBV, KBV and *Drosophila* C virus (DCV) was not reported (Jousset *et al.*, 1977; Scotti *et al.*, 1981; Dall, 1987). *Drosophila* virus P (DPV) was detected in the nuclei of germ cells of female *Drosophila melanogaster* (Teninges & Plus, 1972). The accumulation of RhPV in the cytoplasm was also stated by Gildow and D'Arcy (1990).

Ponsen (1977) showed that during the degeneration of the fat body cells and mycetocytes in *Myzus persicae* aphids, disintegrating cytoplasm of the fat body cells and symbionts were released into the haemolymph. It could be argued that a similar process occurred in *R. padi* aphids and that disintegration of virus-infected tissues (fat body cells and symbionts) (Figs 4.2 and 4.9) would release ALPV into the haemolymph. Similar to KBV and DCV (Dall, 1987; Jousset *et al.*, 1977), the extracellular ALPV circulates in the haemolymph. The release of RhPV into the heamolymph was shown by Gildow and D'Arcy (1990). The presence of circulating ALPV and RhPV in haemolymph could in turn be the source of infection to organs and tissues not yet infected. With immunofluorescent studies of numerous aphids it was repeatedly observed that the transition of early infection of mycetocytic infection (Fig. 2.4) to overall infection of aphid tissues appeared to be a rapid one. It would appear thus that haemolymph played an important role in the distribution of ALPV.

IGL was consistently observed within the tracheocytes (Fig. 3.11), which are present in the hemocoel of the aphid. This phenomenon was also observed in immunofluorescent studies (Laubscher & Von Wechmar, 1991). CrPV and SBV infection in tracheocytes were absent (Reinganum *et al.*, 1970; Mussen & Furgala, 1977).

Haemocytes were found closely associated with fat body cells, mycetocytes, symbionts and the ovarioles. IGL was shown in these haemocytes (Fig. 3.12). ALPV conforms with CrPV and SBV (Scotti *et al.*, 1981; Mussen & Furgala, 1977) in that it also infects haemocytes.

Immunofluorescent studies have shown that ALPV infections were present in several generations of embryos in different stages of development (Laubscher & Von Wechmar, 1991). The IGL of virions in the cytoplasm and nuclei of germarial cells indicate that transmission of virions from germarial tissue to the developing embryos may be cytoplasmic and/or nucleoplasmic.

Symbionts inside the mycetocytes were well labelled (Fig. 3.9). This was not noted with CrPV, SBV, KBV and DCV (Dall, 1987; Jousset *et al.*, 1977; Scotti *et al.*, 1981). Labelled virions were also observed in the cytoplasm of symbionts (Fig. 3.9 and 3.10). Strong fluorescence also indicated the presence of virions in the mycetocyte tissue which harbour the symbionts (Laubscher & Von Wechmar, 1991).

The presence of ALPV particles in symbionts of mycetocytes and germarial tissue

indicates that viral transmission to the progeny could follow two routes. First, symbionts may play an important role in the transmission of virions to the progeny. Successive generations of embryos are present inside the ovarioles in the abdomen of the parthenogenetic aphid (Dixon, 1985; Blackman, 1987). During embryogenesis, symbionts are introduced into the developing embryo (Hinde, 1971; Couchman & King, 1980). As ALPV was found inside these symbionts it is probable that the virus is introduced with the symbionts. The other possible route of infection starts directly in the germarium of an infected aphid mother. Since ALPV virions were detected in the cytoplasm and nuclei of the germarial cells of the aphid mother, it can be argued that these cells are directly responsible for the transmission of ALPV to the progeny.

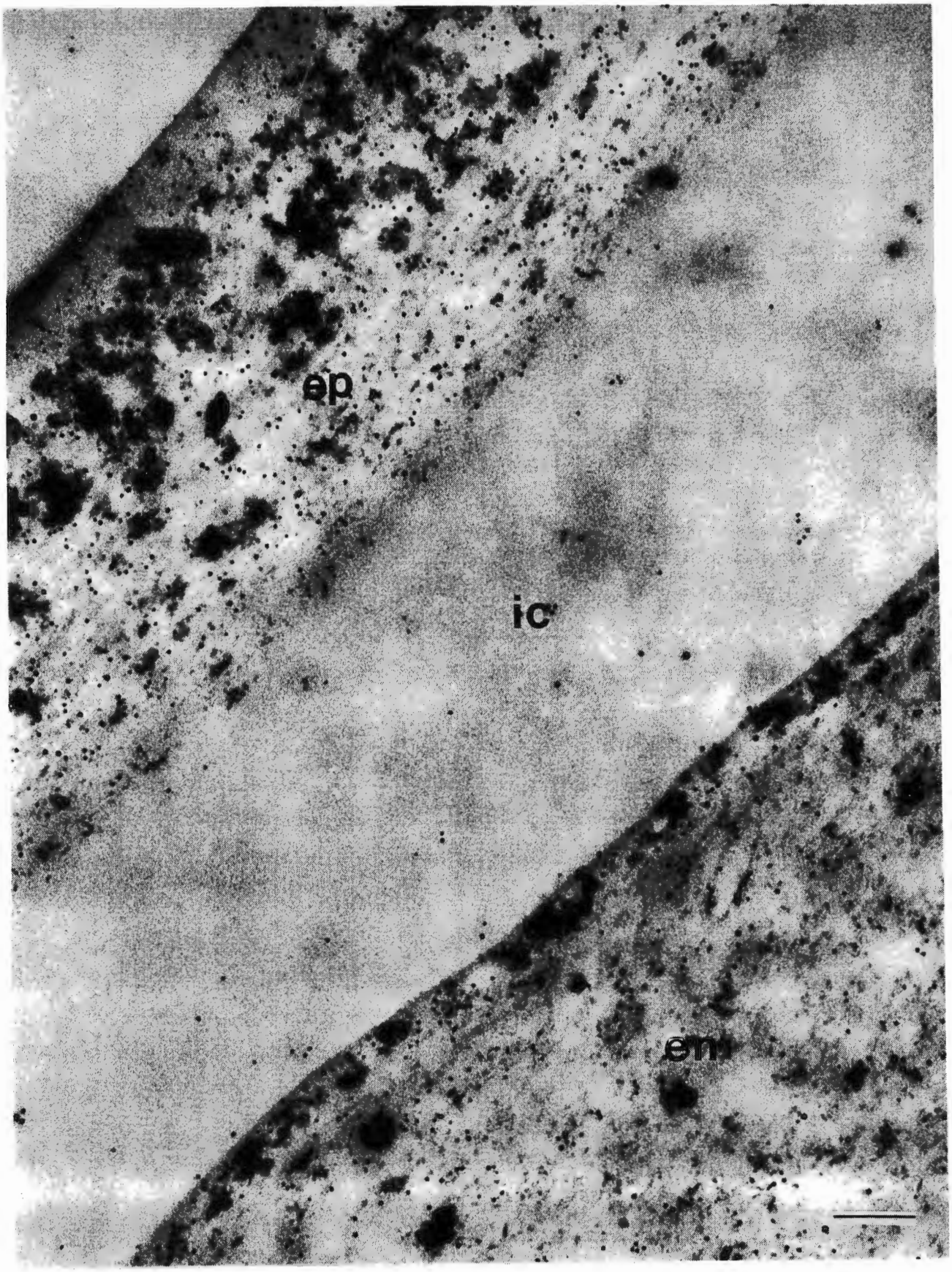


Fig. 3.1. Electron micrograph of cuticle of ALPV-infected aphid with gold label in the epicuticula (ep), endocuticula (en) and intercuticular space (ic). Bar marker represents 160 nm.

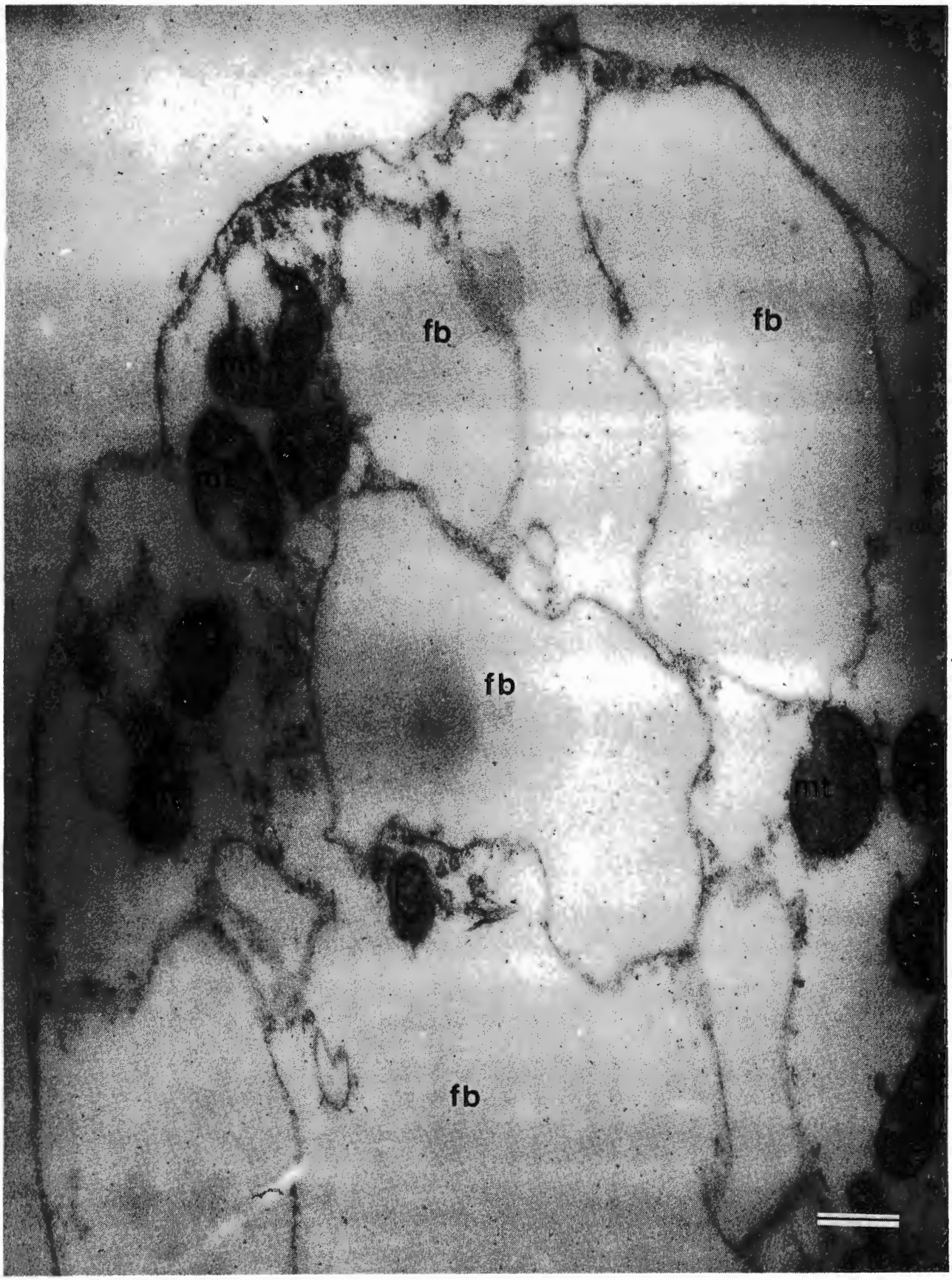


Fig. 3.2. Electron micrograph of ALPV-infected aphid fat body cells (fb) with gold label. Mitochondria (mt) do not show gold label. Bar marker represents 160 nm.

Fig. 3.3. Electron micrograph of muscle tissue (mu) situated in the mesodermal tissue (m) of an ALPV-infected aphid. Gold label was not detected in this tissue. Bar markers represent 160 nm.

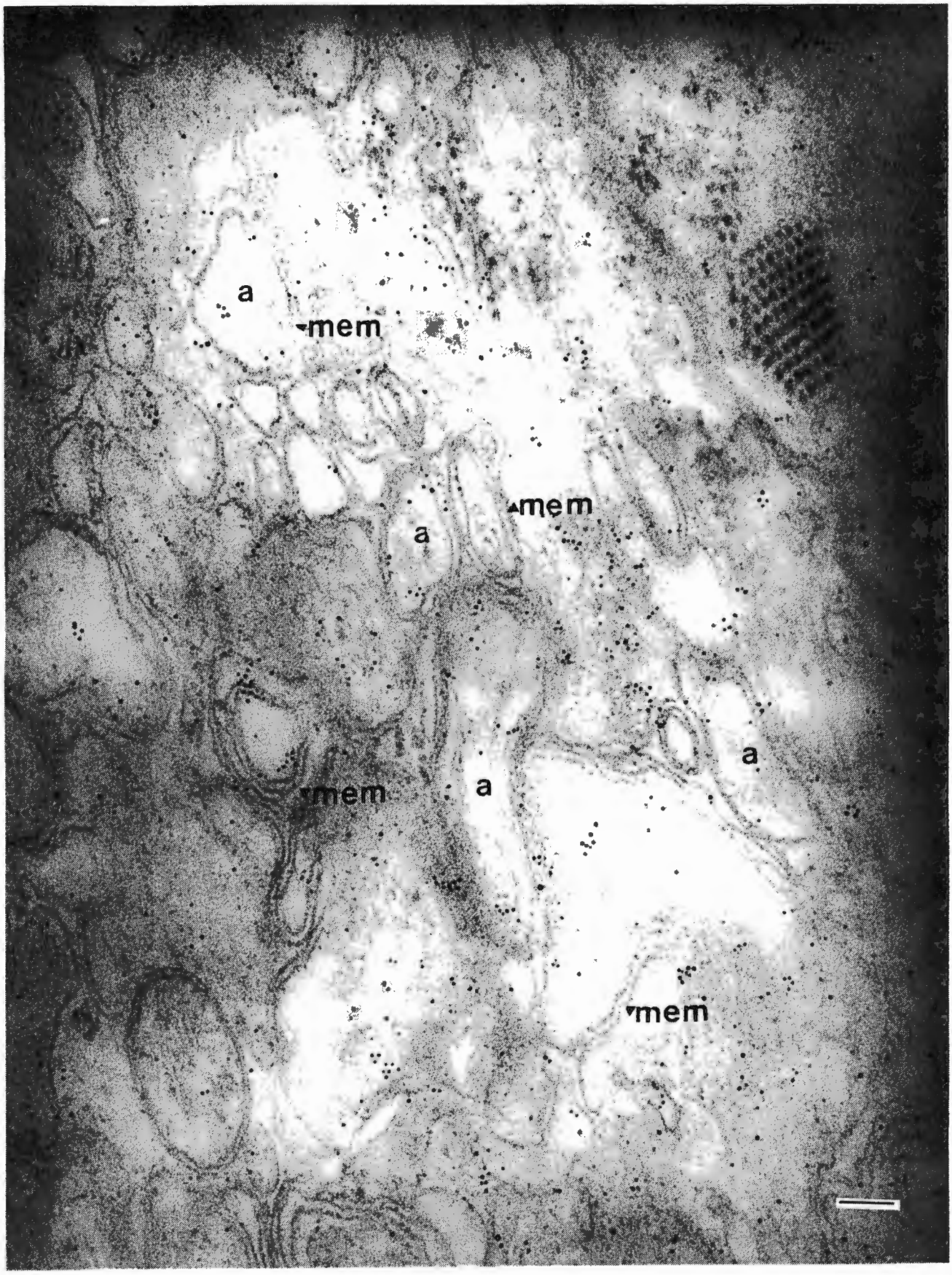


Fig. 3.4. Electron micrograph of brain tissue (membrane (mem) (arrows) and axon (a)) with bound immunogold label clearly visible in the ALPV-infected tissue. Bar marker represents 80 nm.

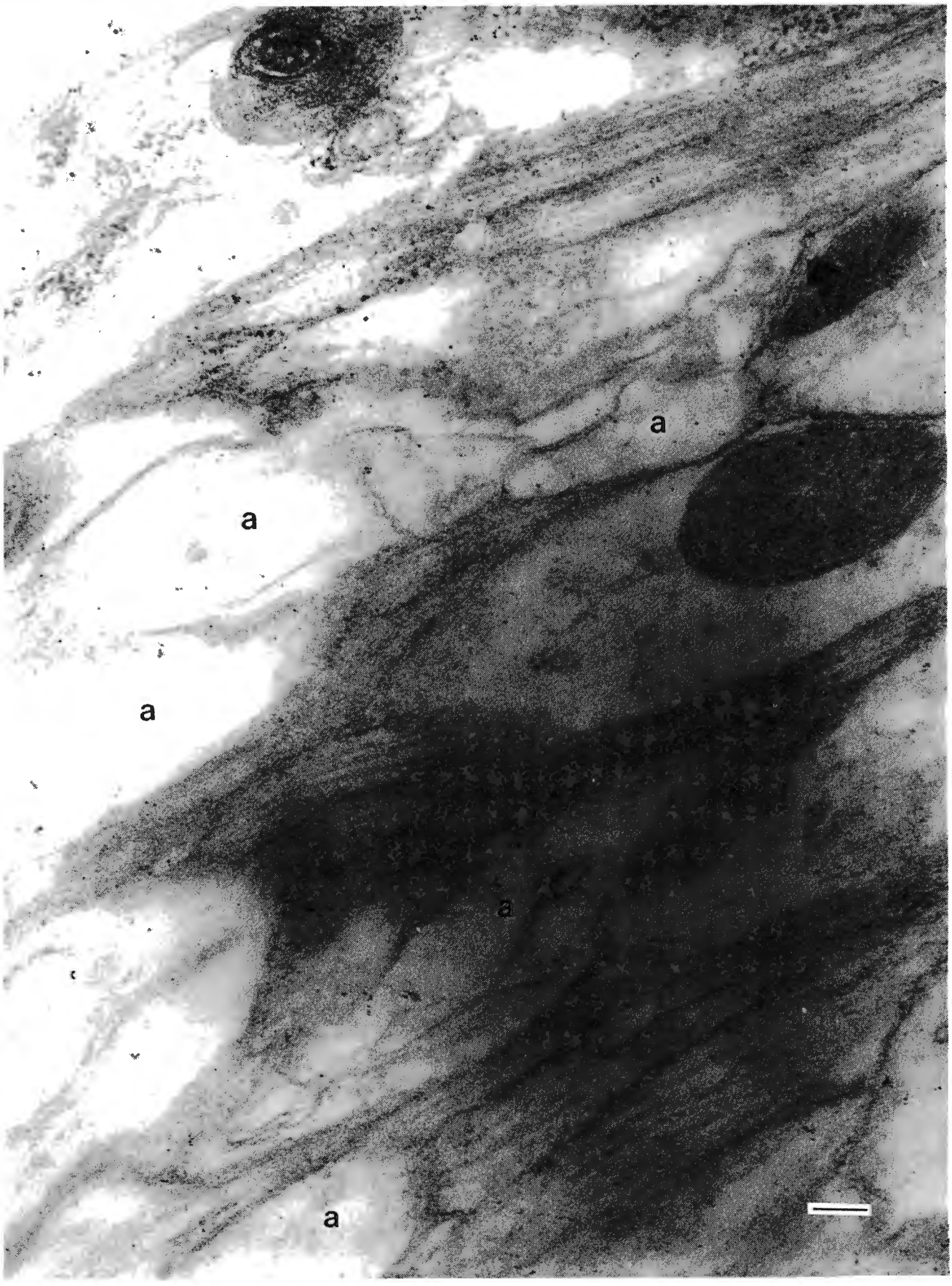


Fig. 3.5. Electron micrograph of nerve tissue showing gold label on the ALPV-infected axons (a). Bar marker represents 200 nm.

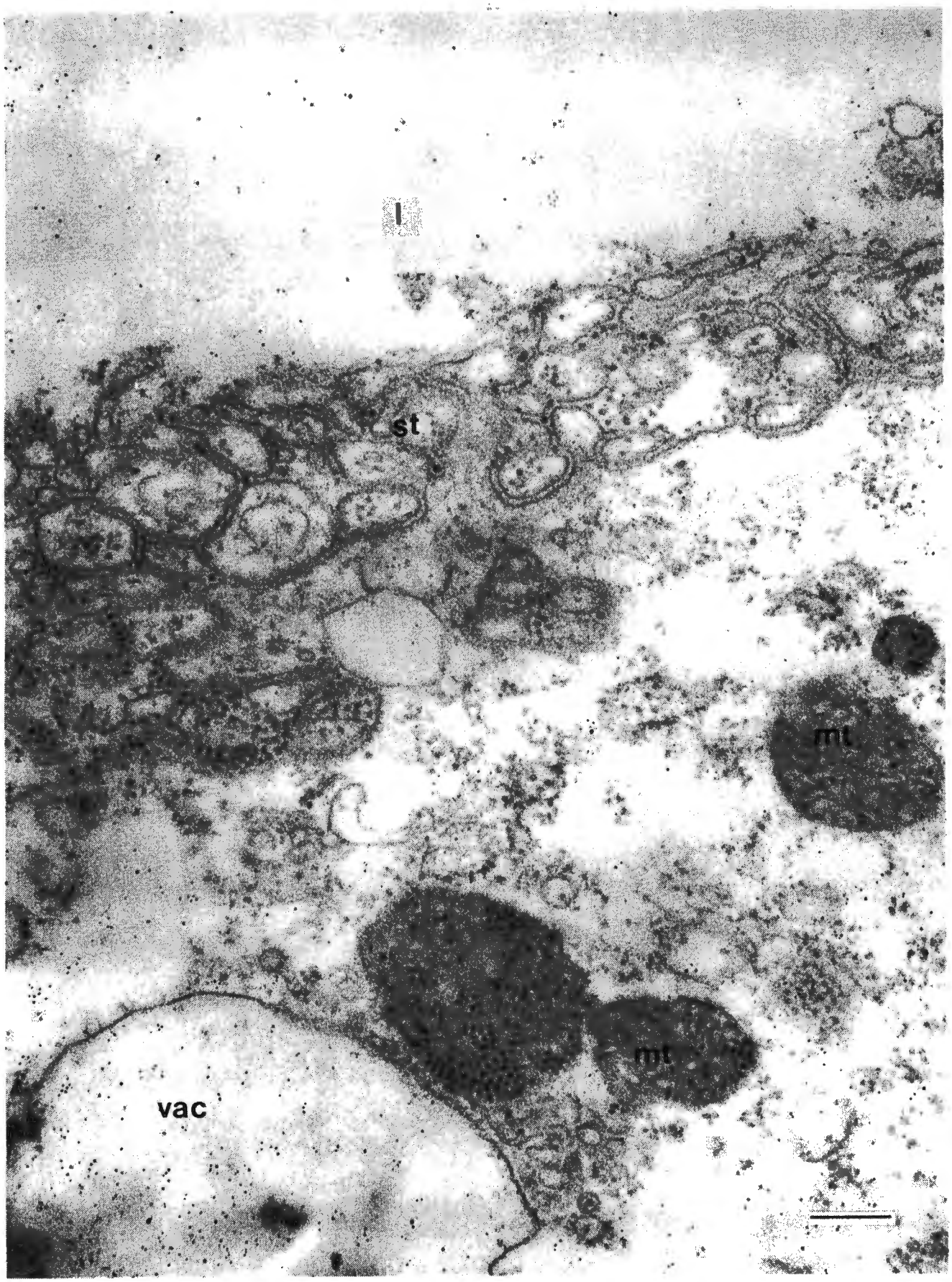


Fig. 3.6. Electron micrograph of stomach epithelium of ALPV-infected aphid showing immunogold label in the lumen (l), striated border (st), stomach tissue and vacuole (vac). No label was detected in the mitochondria (mt). Bar marker represents 160 nm.

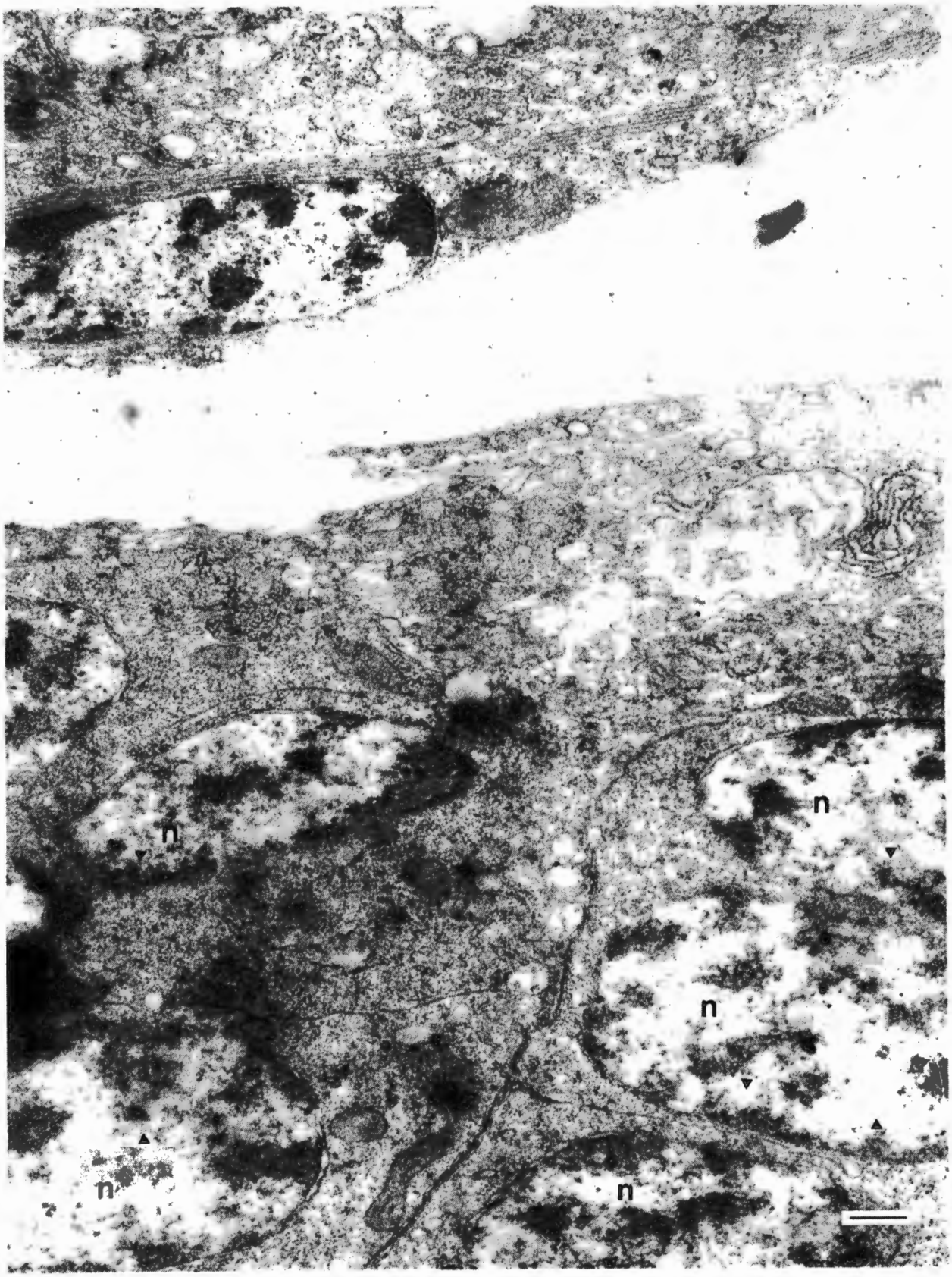


Fig.3.7. Electron micrograph of ALPV-infected germarial cells and mycetocytes. Germarial cells show labelling (arrows) in the nuclei (n). Bar marker represents 700 nm.

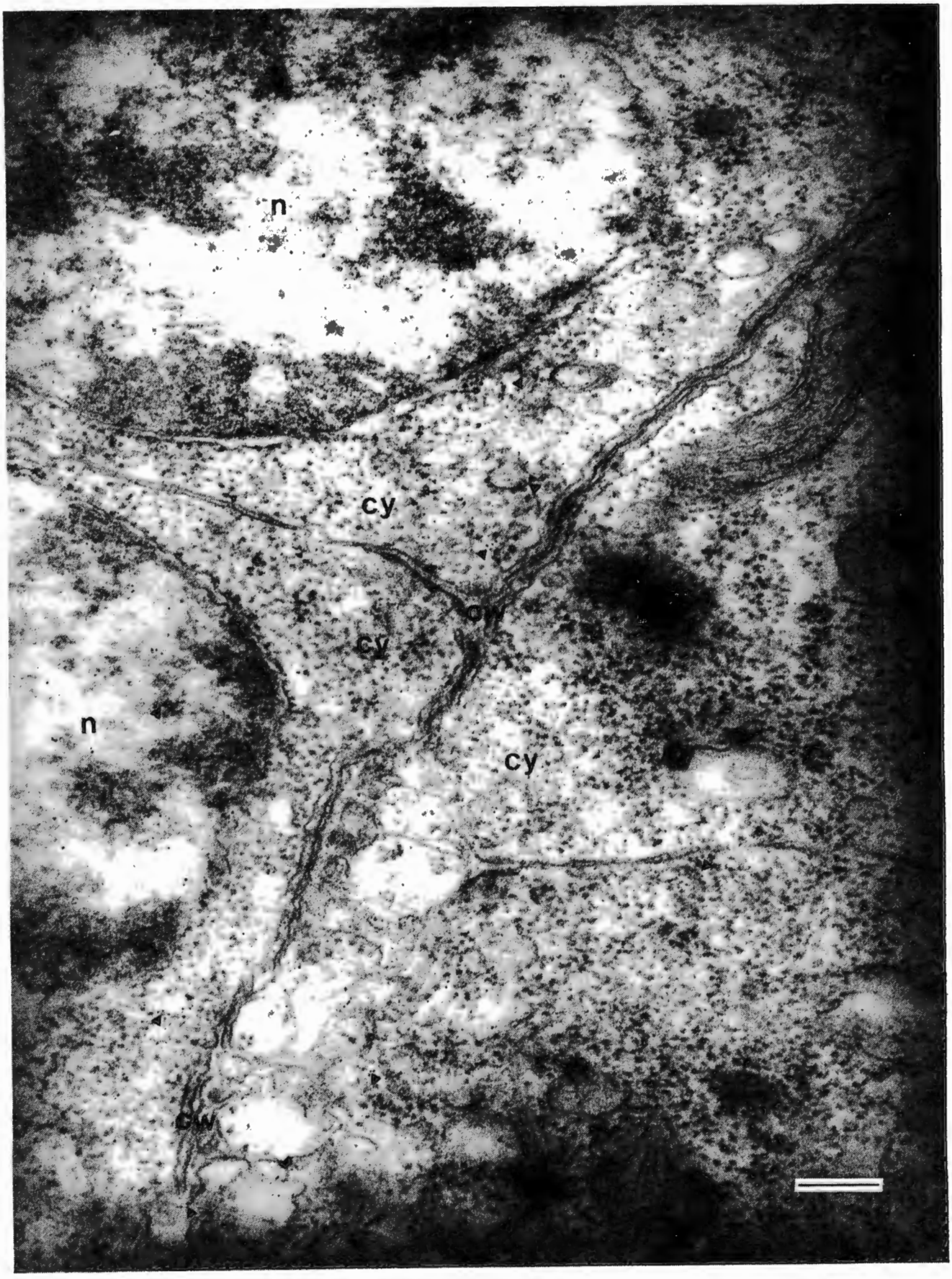


Fig. 3.8. Electron micrograph of ALPV-infected germarial cells showing immunogold label (arrows) in the cytoplasm (cy), nuclei (n) and cell wall (cw). Bar marker represents 160 nm.

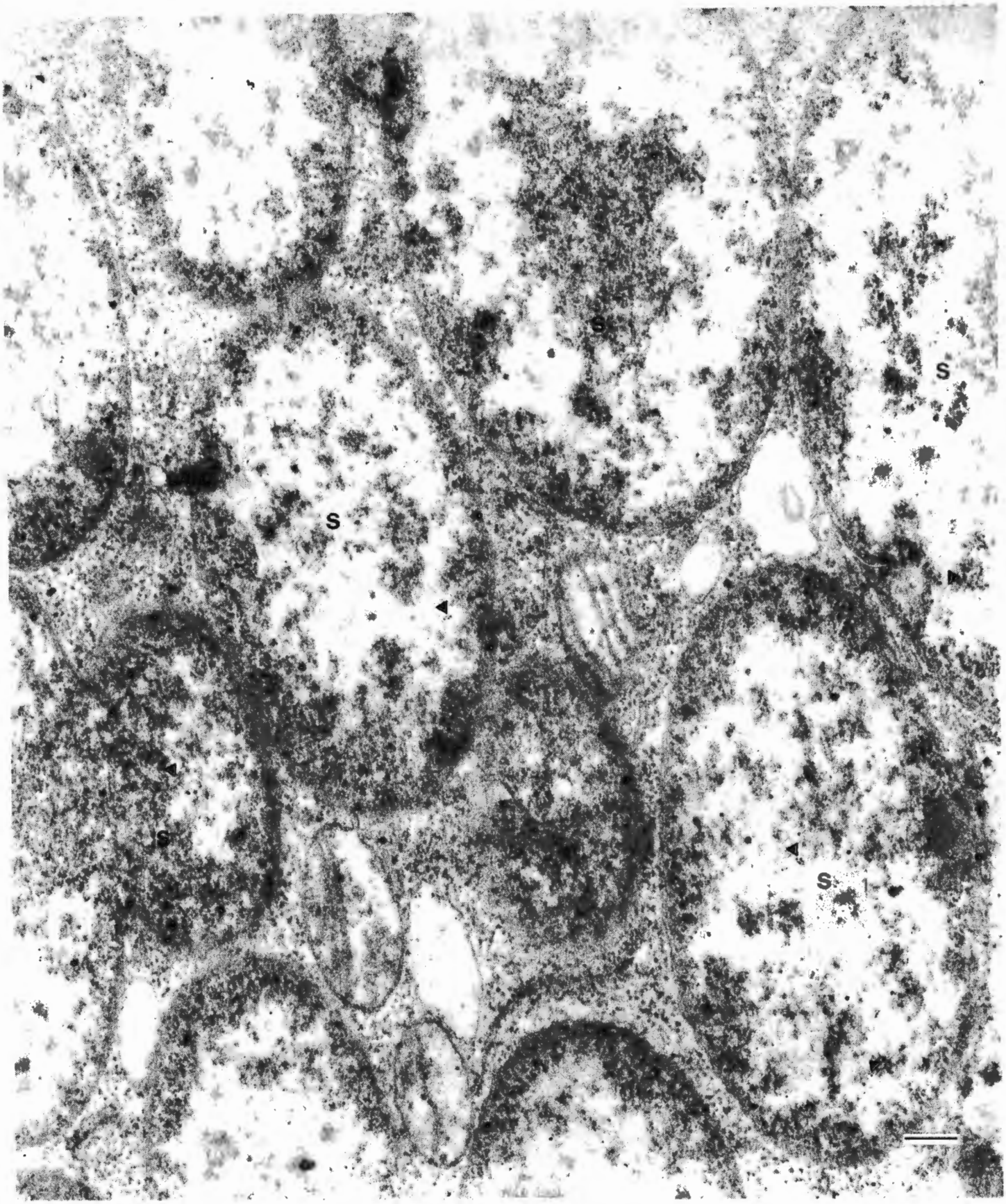


Fig. 3.9. Electron micrograph of a mycetocyte of an ALPV-infected aphid with immunogold label (arrows) in the symbionts (s). Bar marker represents 700 nm.

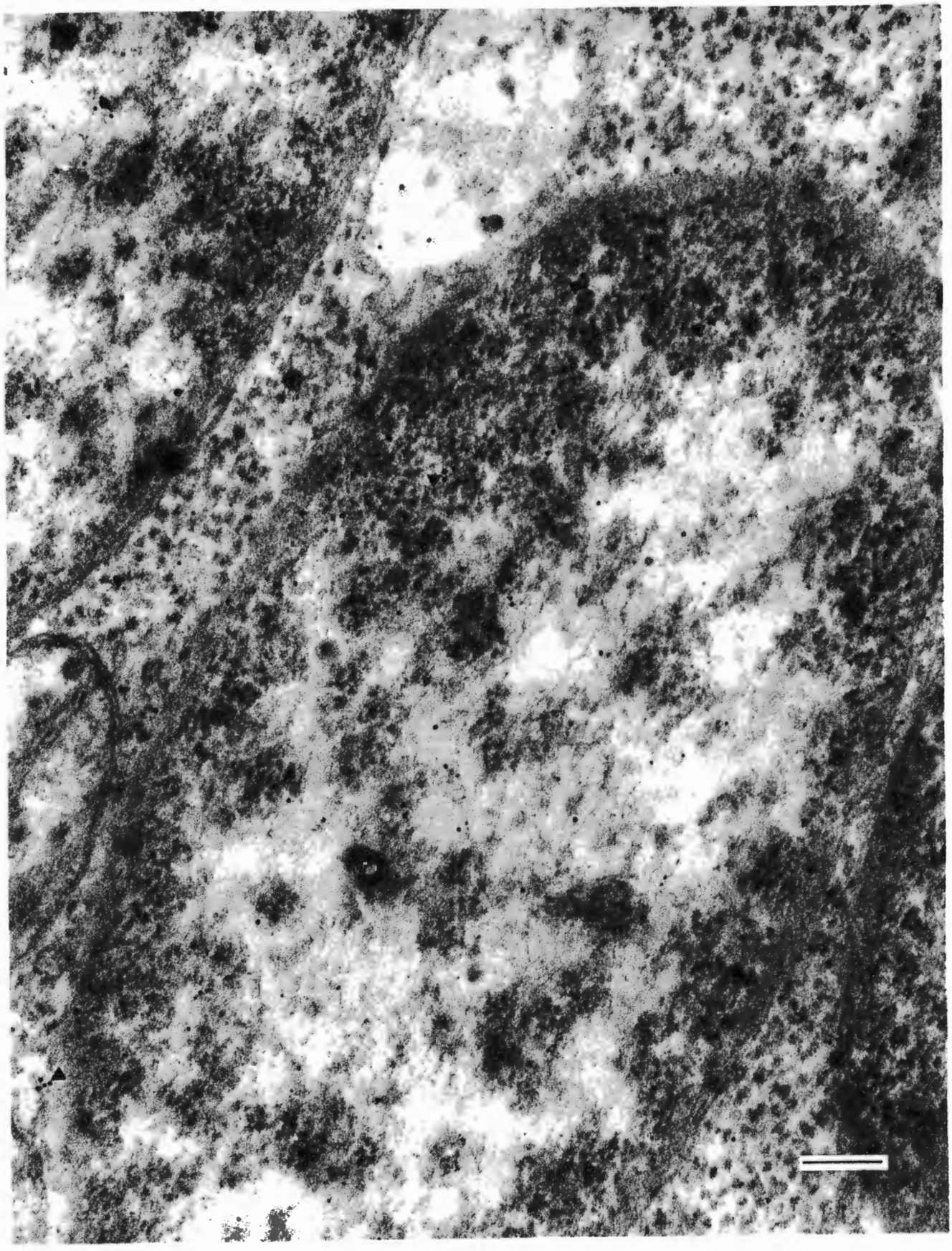


Fig. 3.10. Electron micrograph of ALPV-infected symbiont (same as Fig. 3.9, but higher magnification) with immunogold label (arrows) in the nucleoplasm. Bar marker represents 160 nm.

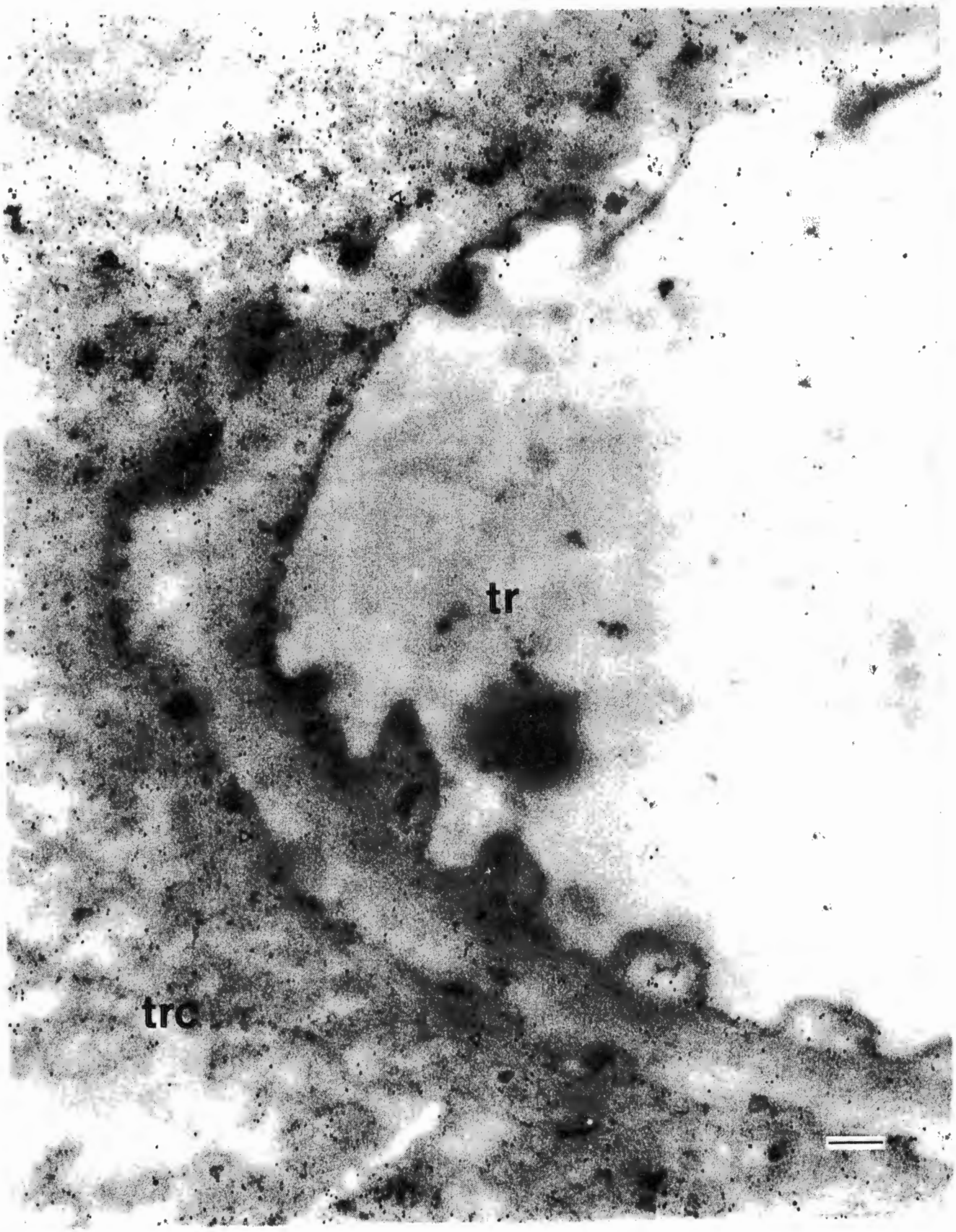


Fig. 3.11. Electron micrograph of a tracheol (tr) in a tracheocyte (trc) in the fat body cells of an ALPV-infected aphid (arrows indicate immunogold label). Bar marker represents 200 nm.

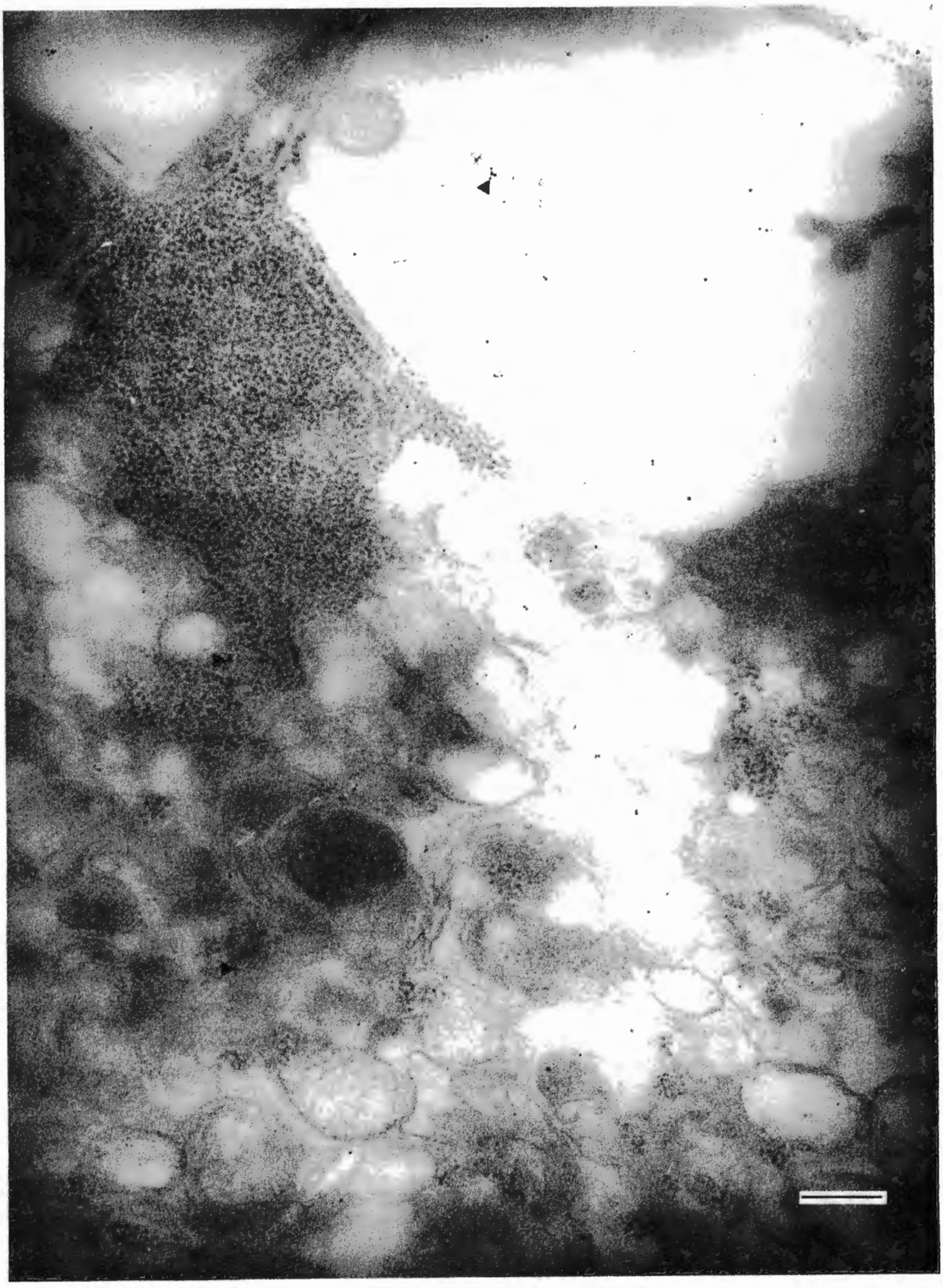


Fig. 3.12. Electron micrograph of a haemocyte in the mesodermal tissue of an ALPV-infected aphid. Arrows indicate immunogold label. Bar marker represents 160 nm.

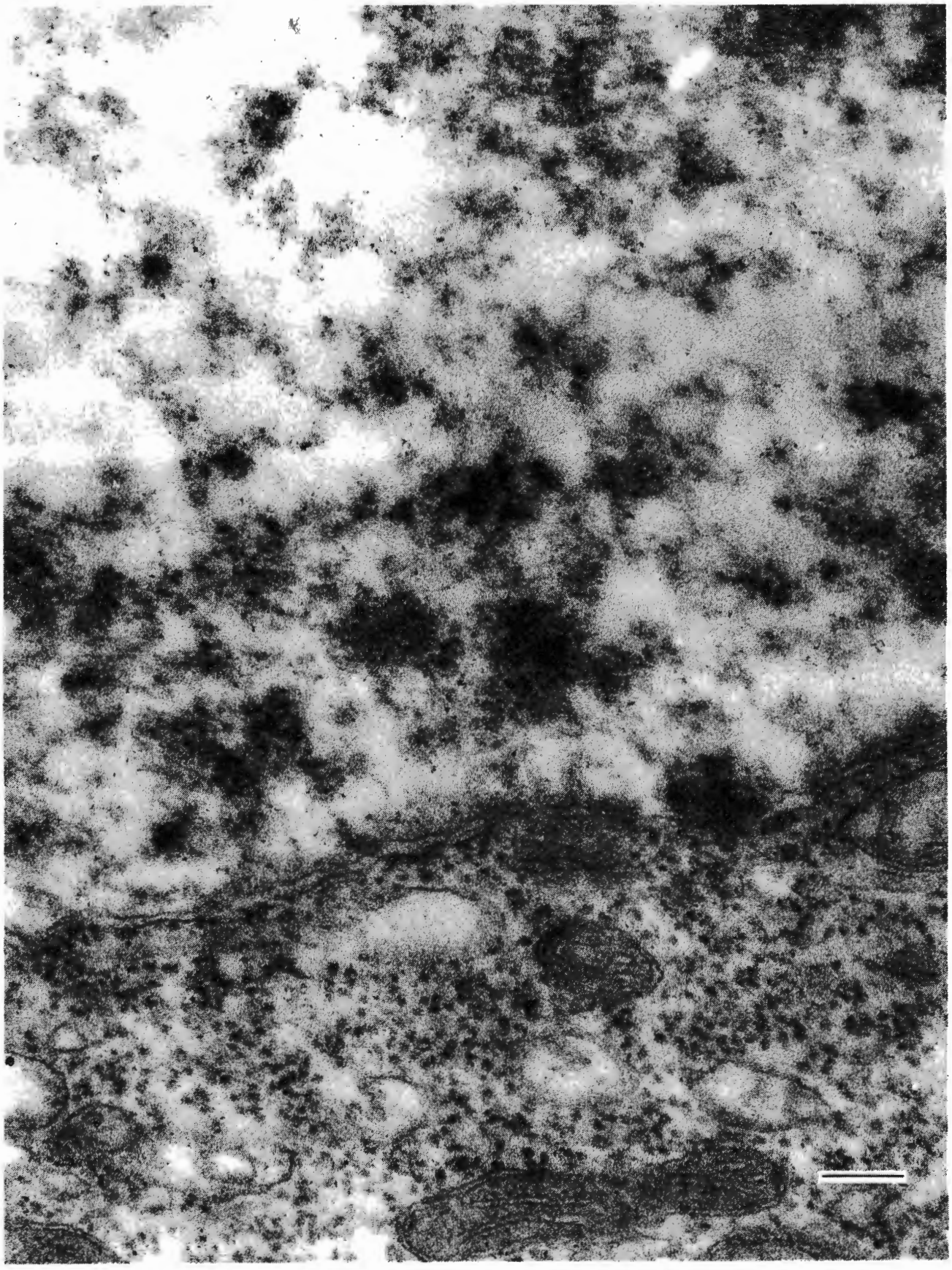


Fig. 3.13. Electron micrograph of germarial tissue of an ALPV-infected aphid treated with non-specific anti-tobacco mosaic virus (TMV) serum. No gold labelling was detected. Bar marker represents 160 nm.

CHAPTER 4

INFLUENCE OF APHID LETHAL PARALYSIS VIRUS AND *Rhopalosiphum padi* VIRUS ON APHID BIOLOGY AT DIFFERENT TEMPERATURES

INFLUENCE OF APHID LETHAL PARALYSIS VIRUS AND *Rhopalosiphum padi* VIRUS ON APHID BIOLOGY AT DIFFERENT TEMPERATURES

SUMMARY

ALPV and RhPV are transmitted transovarially. Different incidences of transmission of ALPV were obtained for *R. padi* (30%) and *S. avenae* (17%) progeny and ALPV infections significantly reduced the longevity and fecundity of these aphids. Infected apterous *R. padi* aphids were more fecund than alate aphids of the same clone. The percentage of viral infection in different aphid species (*R. padi*, *S. avenae* and *D. noxia*) was positively correlated with temperature; higher temperatures significantly increased the incidence of ALPV and RhPV and *vice versa*.

4.1 INTRODUCTION

Little is known about the biology of aphids infected with ALPV and RhPV although both viruses have been fully characterized (Williamson *et al.*, 1988; 1989). The viruses are isometric ssRNA viruses which are very similar to insect and mammalian picornaviruses. ALPV and RhPV infect several aphid species including *R. padi, S. graminum, M. dirhodum, R. maidis, D.noxia* and *S. avenae* (Williamson *et al.*, 1988; 1989). Aphids infected with ALPV show signs of uncoordinated movement and paralysis which is followed by death (Williamson *et al.*, 1988). ALPV and RhPV are transovarially transmitted and transmission can also take place through contact between infected individuals (Williamson, 1988).

D'Arcy *et al.* (1981a) reported on preliminary studies conducted with *R. padi* aphids infected with RhPV. They found that RhPV was vertically transmitted to 15 to 28 percent of the next generation and infected aphids had a reduced number of offspring and longevity. During routine propagation of aphids over ten years in the laboratory it appeared that temperature had a major effect on virus yield (Williamson & Von Wechmar, unpublished results), and on the survival of naturally infected aphid clones under changing weather conditions (Von Wechmar, unpublished results).

This chapter reports on the fecundity of apterous aphids in virus-infected clones, the transovarial passage of ALPV in the progeny of apterous and alate parent aphids and the effect of temperature on the incidence of ALPV and RhPV under crowded conditions.

4.2 MATERIALS AND METHODS

4.2.1 Aphid maintenance and propagation

The propagation and maintenance of aphids was described by Von Wechmar (1990c) (Appendix D.2.1). For long term maintenance, colonies were kept at 4 / 6^{o}C. A 12 hr light and 12 hr dark (12L:12D) cycle was chosen to eliminate the influence of daylength on the aphid biology (Blackman, 1987). When large numbers of aphids were needed, they were transfered to fresh plants and maintained at laboratory temperature (20 / 22^{o}C). If not otherwise specified, this temperature was constant for experimental purposes.

Several aphid clones were utilized (Appendix D.1). One clone originated from

Bethlehem (*R. padi*-Beth.) in the Orange Free State and the other one from Leipoldtville (*R. padi*-Leip) in the Cape Province. Both clones were collected during 1988. *S. avenae* (Clones A and B) were collected at Riviersonderend (*S. avenae*-Rse) in the Cape Province during 1982. Two *D. noxia* clones, one from Brits and the other from Ventersdorp in the Transvaal (*D. noxia*-Brits and -Vent), were collected during 1988.

4.2.2 Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA)

To determine the presence of virus in single aphids, DAS-ELISA was done as described by Clark and Bar-Joseph (1984) (Appendix B.3.4).

Freshly prepared antigen, purified from laboratory-reared *R.padi*-Beth aphids was utilized for positive controls (Appendix B.1.3). Anti-ALPV serum, anti-RhPV serum and conjugates were as described in Appendix B.3.1, 3.2 and 3.3.

4.2.3 Fecundity and transovarial transmission

4.2.3.1 Comparison of two aphid species

The fecundity of virus-infected aphid clones was compared to that of an apparently non-infected aphid clone. Aphid clones testing negative by DAS-ELISA were assumed to be virus-free. This aspect will be discussed later. Aphids utilized were *R. padi*-Beth and *S. avenae*-Rse (Clone A). These aphids were maintained at 8 / 10°C night/day (N/D) at 12L:12D. Experiments were performed at 20 / 22°C N/D at 12L:12D. An uninfected clone of *S. avenae*-Rse (Clone B) maintained at a similar temperature range was used as a negative control.

To obtain aphids of equal age, six apterous adults from each clone were placed on a single barley (*Hordeum vulgare* cv. Clipper) leaf in separate Petri dishes containing wet filter paper and left for 24 hr at 20 / 22°C.

Nymphs born during this period were collected, maintained at ambient temperature, and at maturity ten single apterous aphids were put separately into Petri dishes with moistened filter paper. Adults that died during the first 24 hr period were replaced with new adults from the same age group.

Nymphs born during the first 12 hr were removed at 2 hr intervals and transferred

to new vials containing seedlings. For most of the experiments aphids were kept in specimen vials covered with plastic lids fitted with a piece of gauze (Appendix D.2.4). Sterilized vermiculite was put into the vial and moistened with sterile inorganic nutrient solution (Knopp's) (Appendix A.11). Five surface sterilized seeds were placed into each vial for germination at ambient temperature.

Aphids born during the night were kept separately. Each single aphid was allowed to reproduce for 5 days. The progeny reproduced during each 24 hr period was left until they reached the fourth instar or adult stage at which time they were counted, put into Eppendorf tubes and frozen at -20°C. At the end of each experiment the parents were also frozen at -20°C. Progeny and parents were tested by DAS-ELISA (Appendix B.3.4) for the presence of ALPV.

4.2.3.2 Comparison of different morphs in one aphid species

The effect of ALPV infection on the fecundity of different aphid morphs was examined. For this purpose six alate and six apterous adult females from the *R. padi*-Beth maintenance colony were allowed to reproduce for 24 hr and the progeny maintained. Of the mature progeny ten new alatae and apterae were selected. Monitoring of these adult alatae and apterae and sampling of the progeny was conducted as described in the previous experiment (section 4.2.3.1). The experiment was terminated after all the adults had stopped reproducing. The progeny was counted and the presence of ALPV in the progeny and parents was determined by DAS-ELISA.

4.2.4 The effect of temperature on virus incidence

4.2.4.1 Fluctuating temperature regimes

To examine the effect of different temperature regimes on the incidence of ALPV in progeny aphids the following two experiments were conducted:
In the first experiment, three different aphid clones (*R. padi*-Leip, *D. noxia*-Brits and -Vent) were used. Prior to setting up experiments, aliquotes of 40 aphids of each maintenance clone (10 / 15°C) were checked by DAS-ELISA for the presence of ALPV.

Temperature regimes were chosen to closely resemble natural conditions which occur in the small grain producing area of the Swartland in the Western Cape. Five different temperature regimes were chosen i.e. 8 / 16°C (N/D), 4 / 16°C (N/D); 8 / 24°C (N/D); 4 / 20°C (N/D) and 4 / 4°C (N/D)(control). Adjustable

selfcontained incubators were used for this experiment (Appendix D.2.4).

The experiment was conducted in 8 X 16 cm canning bottles containing germinated seedlings (Appendix D.2.4). Twenty surface sterilised barley seeds (cv. Clipper) were germinated in each bottle. Approximately 50 adult aphids of each clone were put into each bottle and allowed to settle on the barley seedlings overnight. Each temperature regime consisted of four bottles arranged in two groups.

Aphids were allowed to reproduce for 14 days at each temperature regime. Aphids (approximately 50) were sampled after two and four weeks. Sampled aphids were tested singulary by DAS-ELISA for the presence of ALPV and RhPV.

The second experiment compared another set of temperature regimes at 12L:12D i.e. 10 / 20°C (N/D), 15 / 24°C (N/D) and 10 / 15°C (N/D). Due to the faster rate of reproduction at higher temperature regimes only twenty adult aphids were put into each bottle and each temperature regime consisted of four replications. Aphids (about 50) were sampled after 2 weeks and screened individually by DAS-ELISA.

4.2.4.2 Constant temperature

The influence of constant temperatures and crowding on virus incidences in three aphid species (*R. padi*-Beth, *S. avenae*-Rse and *D. noxia*-Beth) was examined.

Three constant temperatures, 10, 15 and 20°C, were used. The initial maintenance colonies were kept throughout at a fluctuating regime of 10 / 15°C N/D. They were tested for ALPV and RhPV by DAS-ELISA. Aphids kept at 20°C were monitored for 77 days and aphids kept at 15°C and 10°C were monitored for 70 days. Three separate self contained incubators, each at a constant daylength cycle of 12L:12D were utilized for each aphid species. One plastic tray, containing 12 pots was put into each cabinet. Each pot contained about 40 Clipper seedlings densely populated with aphids taken from the maintenance colony. Aphids were allowed to settle and reproduce until they reached a level of about 20 aphids per seedling. Plants were harvested weekly and the aphids were shaken off and about 500 aphids per pot (all ages) were used to re-infest a new set of seedlings. The remainder of the aphids were stored at -20°C and aliquotes subsequently tested by DAS-ELISA.

With each harvest a sample of 20 leaves with aphids was taken randomly from the

S. avenae-Rse colony. These aphids were carefully removed from the leaves with a small brush and put into 95% alcohol. Alatae and apterae were separated and counted.

Results obtained were analyzed by a statistical package, BMDP (1983). The viral incidence was correlated with the number of alate and apterous aphids in the original source population. The relationships between points obtained with the different temperature regimes were calculated and utilized to describe the relationships obtained.

4.3 RESULTS

4.3.1 Fecundity and transovarial transmission

4.3.1.1 Comparison of two aphid species

Seventeen percent (5/30) and 30% (13/44) of *S. avenae* and *R. padi* respectively reacted positive for ALPV. Of these 9% (4/44) *R. padi* nymphs and 6% (2/30) *S. avenae* nymphs had A_{405} nm readings in DAS-ELISA close to 1.0 for single aphids (A_{405} 1.0 is equivalent to 1.5 mg/ml of purified ALPV used for the control in DAS-ELISA). Generally *R. padi* nymphs contained more ALPV than *S. avenae*.

During the experiment it was observed that approximately 7% (3/44) *R. padi* progeny were aborted embryos. These aborted embryos tested negative for ALPV by DAS-ELISA.

ALPV appeared to greatly reduce the number of offspring produced by *S. avenae* as shown in Table 4.1.

Table 4.1. Fecundity of virus infected *S. avenae* adults compared to uninfected *S. avenae* adults and the presence of ALPV in the progeny

CLONE	NUMBER OF NYMPHS[3]/ADULT	NYMPHS[3]/DAY	%POSITIVE (ALPV)
C[1]	5.7 ± 1.8	1.4 ± 0.38	16.6
C[2]	22.2 ± 2.1	4.5 ± 0.4	0.0

1. *S. avenae* - Rse - ALPV-positive
2. *S. avenae* - Rse - uninfected clone as tested by DAS-ELISA. Clone was kept at 8 / 10°C (N/D)
3. mean number of nymphs ± standard error

Sixty eight percent (13/19) of the dead aphids (*R. padi* and *S. avenae*) collected during the experiment were ALPV-positive. A strong correlation between virus infection and aphid death was observed as indicated in Table 4.2.

Table 4.2. Detection of ALPV by DAS-ELISA in *S. avenae* and *R. padi* aphids that died during the analysis

CLONE	NO. DEAD		NO. ALPV POSITIVE	
	Adult	Progeny	Adult	Progeny
Sa-Rse[1]	8	3	5	1
Rp-Beth[2]	0	8	0	7
Total	19		13	

1. Sa-Rse *S. avenae* - Riviersonderend.
2. Rp-Beth *R. padi* - Bethlehem.

4.3.1.2 **Comparison of different R. padi morphs**

A small reduction was observed in the number of nymphs produced by apterous adult *R. padi* aphids when compared to that of alate aphids (Table 4.3).

Tabel 4.3. Fecundity and mean life span of infected alate and apterous *R. padi* aphid clones compared to other *R. padi* groups and a *R. padi* control

CLONE	NUMBER OF NYMPHS[6]/ADULT	MEAN[7] LIFE SPAN	% NYMPHS POSITIVE BY ELISA ASSAY ALPV RhPV
Rp-Beth(Al)[1]	45.4 ± 3.5	9.7 ± 1.1	21.1 0.6
Rp-Beth(Ap)[2]	49.5 ± 6.2	6.9 ± 0.98	12.1 0.0
Rp-W[3]	42 ± 3.5	19 ± 1.4	48 35
Rp-Eur[4]	60 ± 2.4	19 ± 1.2	ND ND
Rp-Cntrl[5]	63 ± 2.2	25 ± 0.7	0 0

1. Rp-Beth(Al) *R. padi* - Bethlehem (alate)
2. Rp-Beth(Ap) *R. padi* - Bethlehem (apterous)
3. Rp-W *R. padi* - results obtained from Williamson (1988).
4. Rp-Eur *R. padi* - results obtained from Dean (1974).
5. Rp-Cntrl *R. padi* - results obtained from Williamson (1988).
6. mean number of nymphs ± standard error
7. mean days ± standard error

A higher percentage of nymphs from alate aphids were virus-infected when compared to those (of nymphs) from apterous aphids (Fig. 4.1).

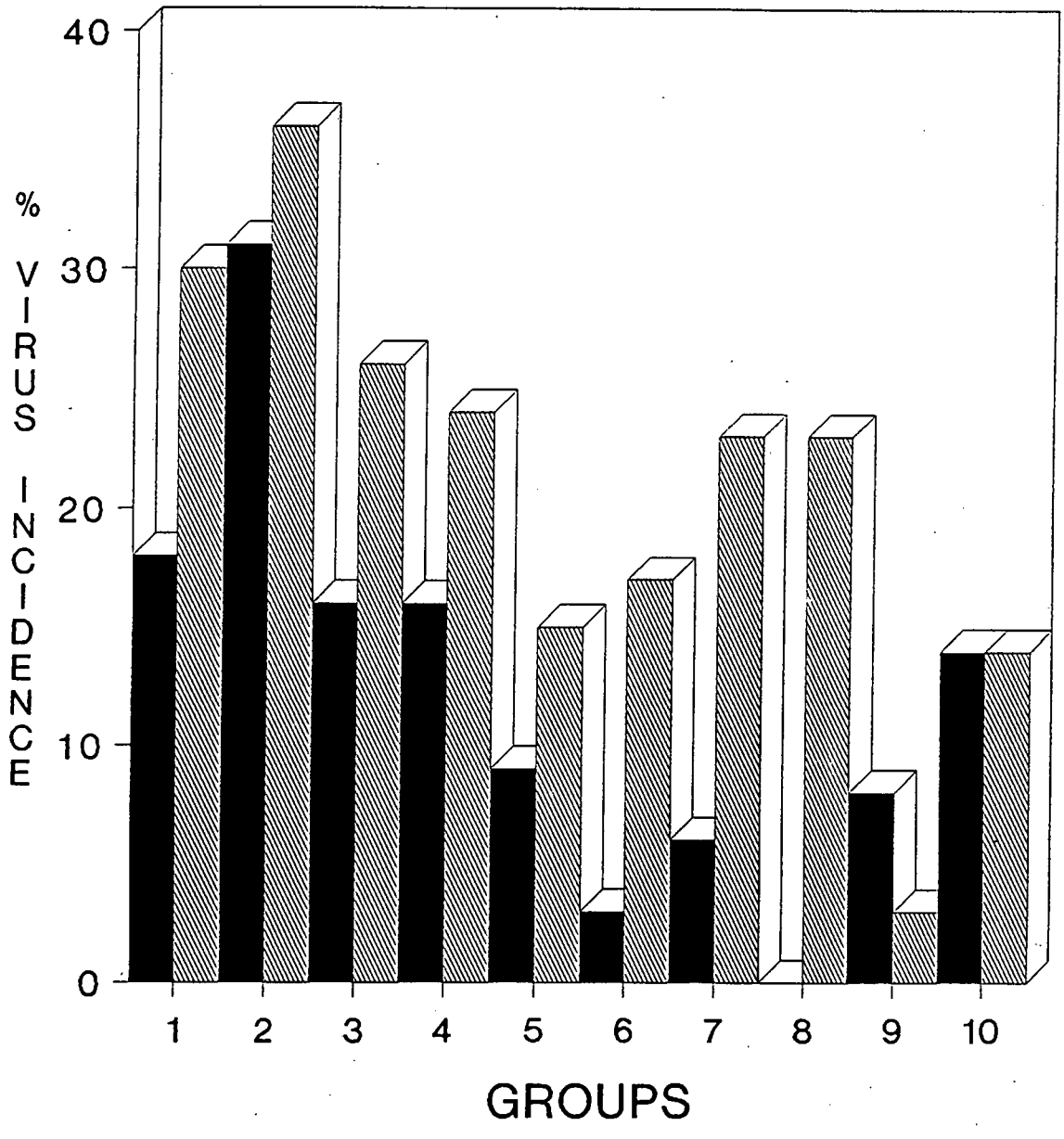


Fig. 4.1. Incidence of infection in the progeny of alate (▨) and apterous (■) *R. padi* aphids.

No ALPV or RhPV was detected in the apterous adults by DAS-ELISA. However, ALPV was detected in 2/10 of alate adult aphids (*R. padi*), but all were negative for RhPV.

4.3.2 Effect of temperature on incidence of virus

4.3.2.1 Fluctuating temperature

All aphids taken from the maintenance clones prior to setting up the experiments, tested negative for ALPV and RhPV by DAS-ELISA. No virus was detected in *D. noxia*-Vent at all temperature regimes and RhPV was detected in *D. noxia*-Brits at only one temperature regime (4 / 20°C) (Table 4.4). ALPV was detected in *R. padi*-Leip only at 4 / 16°C.

Table 4.4. ALPV and RhPV incidences (%) for different aphid clones kept at fluctuating temperature regimes tested by DAS-ELISA.

TEMP.	Dn-B[1]		Dn-V[2]		Rp-L[3]	
	AL[4]	Rh[5]	AL	Rh	AL	Rh
4 : 16°C	0	0	0	0	0	0
4 : 20°C	0	3	0	0	0	46
8 : 16°C	0	0	0	0	0	48
8 : 24°C	0	0	0	0	0	34
10 : 15°C	2	0	2	0	5	49
15 : 20°C	29	2	11	1	5	95
15 : 24°C	13	0	13	3	24	93

1. Dn-B *D. noxia* - Brits
2. Dn-V *D. noxia* - Ventersdorp
3. Rp-L *R. padi* - Leipoldtville
4. AL ALPV(%)
5. Rh RhPV(%)

The original maintenance clones at 10 / 15°C and an additional control maintained at 4 / 4°C were virus free when tested at the end of the experimental cycle.

A high incidence of RhPV was detected in *R. padi*-Leip at all temperatures. A

difference in the incidence of ALPV in *D. noxia*-Brits and *D. noxia*-Vent was observed. The percentage of ALPV-infected aphids increased steadily with its highest incidence at 15 / 24°C (Table 4.4). In both *D. noxia*-Brits and *D. noxia*-Vent ALPV was detected at much higher incidences than RhPV.

4.3.2.2 **Constant temperature**

In this experiment the incidences of virus infection in alate and apterous aphids kept at a constant temperature was investigated. The results obtained indicate that there was a positive correlation between the number of alatae aphids and ALPV infection ($r=0.81$; $P<0.05$). For apterae a negative correlation between aphid numbers and incidence of ALPV ($r=-0.82$; $P<0.05$) was found. For RhPV no correlation was obtained between incidence and numbers of alate and apterous aphids. The incidence of RhPV in *S.avenae* was too low to be analyzed statistically.

The second factor examined in this experiment was the incidence of virus infection in a dense population kept at a constant temperature. Figure 4.2 (p. 94) shows the results obtained with temperatures of 20, 15 and 10°C respectively using a statistical package (BMDP, 1983) to calculate the relationship between the points obtained for each clone. Distinct differences were obtained for the three aphid species used.

Figure 4.2a illustrates an increase in the incidence of ALPV infection with time for *R. padi*, *D. noxia* and *S. avenae* kept at 20°C over a 77 day period. *R. padi* reached the highest incidence of infection with a slope b = 0.76. The rate of increase of ALPV infection was lower for *D. noxia* compared to *S. avenae* reflected by a slope of b = 0.12 compared to a slope b = 0.49 respectively.

The percentage of RhPV-infected *R. padi* increased linearly over time (77 days) (Fig. 4.2b). This increase is represented by a slope b = 0.78. However, in the case of *D. noxia* and for *S. avenae* there was virtually no increase in the incidence of RhPV. This is reflected in a slope of b = 0.02 and 0.04 respectively.

Results obtained at 15°C are in sharp contrast to those obtained at 20°C (Fig. 4.2c). A negative relationship for the incidence of ALPV over time was observed for all the aphid species studied. The slopes obtained were b = -0.51 for *R. padi*, b = -0.48 for *D. noxia* and b = -0.49 for *S. avenae*.

A similar pattern was found for the percentage infection with RhPV (Fig. 4.2d) for *R. padi* with a slope of b = -0.40. At this temperature *D. noxia* and *S. avenae* showed

a very low incidence of infection for RhPV reflected in a slope of b = 0.03 and b = 0.002 respectively.

A different trend was observed at 10°C (Fig. 4.2e), when compared to the results obtained at higher temperatures. The incidence of ALPV infection of *R. padi* did not differ dramatically from *S. avenae* although the percentage of ALPV infection declined faster in *R. padi* compared to *S. avenae* (slope b = -0.27 and b = -0.12 respectively). ALPV was not detectable in *D. noxia* maintained at 10°C.

The general trend of RhPV infection in *R. padi* (at 10°C) did not differ markedly from results obtained with aphids kept at 15°C (slopes of -0.55 and -0.40) (Fig. 4.2f). RhPV was not detectable in *S. avenae* and *D. noxia* maintained at lower temperature and the incidence of infection in *R. padi* decreased more rapidly than at higher temperature.

5.4 DISCUSSION

In this chapter evidence is provided of transovarial transmission of ALPV with 30% of the progeny of *R. padi* and 17% of the progeny of *S. avenae* being infected. This correlates with previous evidence (Chapter 2) in which unborn nymphs were found to contain ALPV using indirect immunofluorescent technique (Laubscher and Von Wechmar, 1991). No RhPV was detected in the nymphs tested by DAS-ELISA which confirms results obtained by Williamson (1988) for *R. padi*. The difference in level of transovarial transmission of ALPV between *R. padi* and *S. avenae* may be due to a species difference. From DAS-ELISA results it appeared that *R. padi* nymphs contained more virus than did *S. avenae*, indicating that the transovarial transmission of ALPV was more successful in *R. padi*. Other small RNA-containing viruses like CrPV and DCV were found not to be transmitted transovarially (Scotti *et al.*, 1981; Podgwaite & Mazzone, 1986; Moore *et al.*, 1980). They speculated that transmission took place through cannibalism of infected cadavers (Reinganum, 1975). Other picorna-like viruses like sacbrood virus (SBV) and Kashmir bee virus (KBV) are transmitted transovarially (Dall, 1985).

As nymphs may become infected from the mother during birth, testing transovarial transmission by examining propagated nymphs may be misleading (Von Wechmar *et al.*, 1990). In addition it has previously been shown that DAS-ELISA is not sensitive enough to detect low levels of virus (Chapter 2) (Laubscher & Von Wechmar, 1991). Therefore, using this method to estimate percentage transovarial transmission may not be accurate and the best method would be to screen for virus in embryos using a more sensitive technique such as indirect

immunofluorescent technique (Laubscher & Von Wechmar, 1991).

In this study aborted *R. padi* nymphs were negative for ALPV in DAS-ELISA tests. However, using the more sensitive indirect immunofluorescent technique (Chapter 2), these nymphs were found to be positive (Laubscher & Von Wechmar, 1991). As viral replication would be halted in a dead host, this may explain why the concentration of virus in these nymphs was below the level of detection by DAS-ELISA.

A dramatic difference in reproduction rates was noted between alate and apterous infected *R. padi* aphids. This may have important implications for the epidemiology of ALPV, as although the alate aphids showed a lower reproduction rate (Table 4.3), their life span is longer, thus ensuring a higher likelihood of horizontal spread. The lower rate of reproduction for alatae was previously noted for *S. avenae* under similar circumstances (Laubscher, unpublished results).

The phenomenon of mixed infection was also encountered. This was shown where alate *R. padi* aphids were found to be ALPV-positive and RhPV-positive, while the apterous adults tested were ALPV-positive and RhPV-negative. These aphids were from the same clone, which indicates that differences can exist among individuals of the same clone confirming earlier findings of Williamson *et al.* (1988).

The incidence of ALPV and RhPV in aphids was found to be temperature dependent. At low temperature regimes (constant and fluctuating) the viruses could seldom be detected by DAS-ELISA. It is probable that the viruses were present in very low concentrations. Williamson (1988) showed that *D. noxia* clones (*D. noxia*-Beth and *D. noxia*-Brits maintained at 7 - 10^{o}C), although testing negative with DAS-ELISA, were found to be positive when tested with a more sensitive technique i.e. cDNA hybridization. Earlier work in this laboratory made extensive use of low temperature regimes for easy aphid maintenance. Some aphid clones maintained at low temperature were considered virus free. On transfer to higher temperatures to increase population development, these clones often changed to virus positive when tested by sensitive techniques (Williamson, 1988). Throughout this chapter we emphasize that the virus status was determined by DAS-ELISA and does not imply that all the aphid clones used were entirely free of virus infection (Williamson, 1988 and Von Wechmar, unpublished results).

It also appears that aphid species differences can influence the presence and incidence of ALPV and RhPV. *D. noxia* and *S. avenae* are poor hosts for RhPV,

compared to ALPV. *R. padi*, on the other hand, appears to be a good host, having high infection rates for both viruses.

Temperature along with crowded conditions dramatically affected the incidence of ALPV and RhPV in various aphid species. There was a drop in the incidence of ALPV and RhPV as the temperature decreased. No attempt was made to analyze these results statistically and the results obtained must be seen as relationships between the incidence of infection obtained for temperature tested. Our work showed that temperature together with population density has a considerable influence on ALPV and RhPV incidences in specific (field collected) laboratory populations and may be causal and/or contributory factors for the decline in numbers. The influence of increased population densities (crowding) under laboratory conditions were stated by Podgwaite and Mazzone (1986) and increased death rates were experienced by Williamson, (1988). Temperature and crowding are considered to be stressors or stimulators (Podgwaite & Mazzone, 1986).

From these results it can be seen that temperature has a dramatic effect on the presence of virus in aphid clones. Considering natural temperatures, one will note that certain seasons may be too cold for RhPV and ALPV to increase to significant thresholds, but sudden seasonal changes may lead to the increase of either or both viruses in natural aphid populations. Experiments to examine such incidences and their effect on aphid populations will be reported elsewhere (Chapter 5).

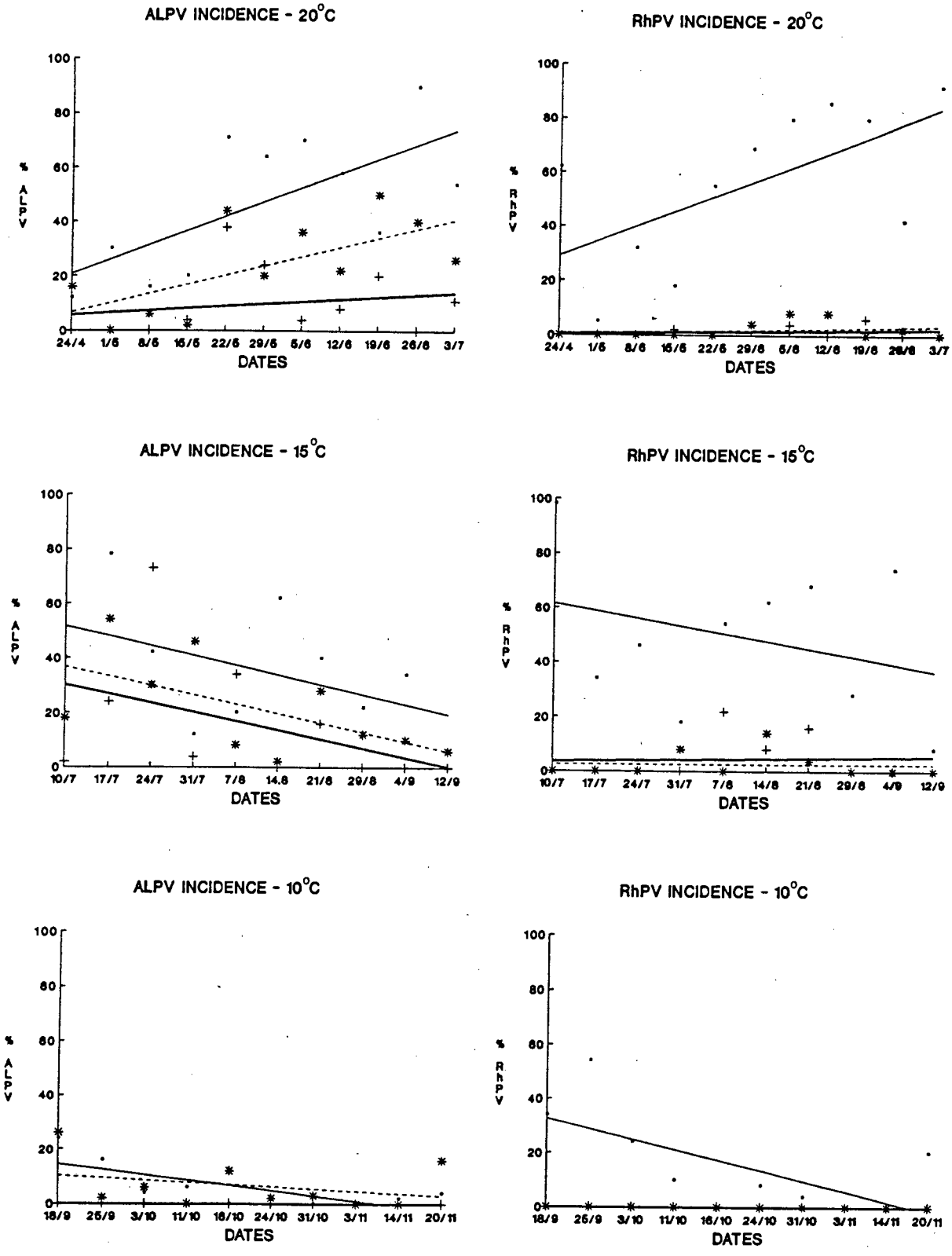


Fig.4.2. Lines fitted to show the incidence (%) of ALPV and RhPV at different constant temperatures (10, 15 and 20°C) in three aphid species, *R. padi* (-•-), *D. noxia* (-+-) and *S. avenae* (-*-): (a) ALPV 20°C *R. padi*: Y = 15.45 + 0.76X; *D. noxia*: Y = 5.05 + 0.12X; *S. avenae*: Y = 3.42 + 0.49X; (b) RhPV 20°C *R. padi*: Y = 23.56 + 0.78X; *D. noxia*: Y = 0.47 + 0.02X; *S. avenae*: Y = 0.25 + 0.04X; (c) ALPV 15°C: *R. padi*: Y = 55.2 - 0.51X; *D. noxia*: Y = 33.6 - 0.48X; *S. avenae*: Y = 40.27 - 0.49X; (d) RhPV 15°C *R. padi*: Y = 64.4 - 0.40X; *D. noxia*: Y = 3.47 + 0.03X; *S. avenae*: Y = 2.53 + 0.002X; (e) ALPV 10°C *R. padi*: Y = 16.27 - 0.27X; *D. noxia*: Y = 0; *S. avenae*: Y = 11.2 - 0.12X and (f) RhPV 10°C *R. padi*: Y = 36.53 - 0.55X; *D. noxia*: Y = 0; *S. avenae*: Y = 0, where Y = percentage virus and X = time.

CHAPTER 5

ASSESSMENT OF APHID LETHAL PARALYSIS VIRUS AS AN APPARENT POPULATION GROWTH LIMITING FACTOR IN GRAIN APHIDS IN THE PRESENCE OF OTHER NATURAL ENEMIES

ASSESSMENT OF APHID LETHAL PARALYSIS VIRUS AS AN APPARENT POPULATION GROWTH LIMITING FACTOR IN GRAIN APHIDS IN THE PRESENCE OF OTHER NATURAL ENEMIES

SUMMARY

The influence of ALPV, RhPV, natural enemies and fungal infection on the population growth of *R. padi* and *S. avenae* aphids in wheat fields of the western Cape Province of South Africa was investigated at two sites. Time-specific life tables were compiled for *R. padi* aphids at one site only. During the logarithmic phase of development of *R. padi* aphids, natural enemies were not present in high numbers and the apparent large scale mortality observed appeared to be due to other causes. During the decline phase of this aphid population, the population size was reduced by 49%. This reduction coincided with a calculated high death factor of 70 aphids per plant. A dramatic decline in *R. padi* aphid numbers and a high incidence of ALPV present in this aphid population was experienced during this period. Virus assays were done by DAS-ELISA and indirect immunofluorescent technique. Entomophthorales type fungal infections of aphids also reached its highest level during the decline phase, but at a later stage than ALPV infection, with a calculated level of 21 aphids per plant. This indicated that the presence of ALPV limited population development in *R. padi* aphids. Similar results were obtained with *S. avenae* aphids. Relative numbers of *D. noxia* were low throughout and declined from the fifth week after infestation.

5.1 INTRODUCTION

ALPV, an aphid pathogenic virus, was first characterized in 1988 (Williamson *et al.*, 1988). It was found to be present in aphids in the major small grain producing regions of South Africa (western and southern Cape, and eastern and western Orange Free State), and other minor regions. In natural aphid populations it is usually present as a mixed infection with another aphid picorna-like virus namely *Rhopalosiphum padi* virus (RhPV) (Williamson *et al*, 1988; Williamson *et al.*, 1989).

In the United States RhPV was shown to infect several aphid species including *R. padi, S. graminum, M. dirhodum, R. rufiabdominalis* (D'Arcy *et al.*, 1981a), and in a separate independent study in South Africa, was shown to infect *R. padi, R. maidis, D. noxia, M. dirhodum* and *S. avenae* (Von Wechmar and Rybicki, 1981, Williamson *et al.*, 1989).

The presence of these viruses in aphids collected at geographically distant sites was reported by Williamson *et al.* (1988). Some of the field-collected aphids tested positive for ALPV and negative for RhPV, suggesting that the two viruses could survive independently. Both viruses were detected in field-collected *R. padi* aphids examined after an inexplicable decline in numbers of *R. padi* in the Riviersonderend district of the south western Cape Province, suggesting that these viruses may limit aphid population development in nature (Williamson *et al.*, 1988).

A pilot study conducted on the occurrence of ALPV and RhPV in aphids in the Swartland region of the western Cape during 1988 showed that these viruses were ubiquitous and this stimulated further investigation into the occurrence and the biology of these viruses under natural conditions. At Riebeek West oat plants densely colonised with *R. padi* aphids, attracted attention. These infested plants showed red-orange discolouration of leaves, severe stunting of growth and premature death. Virus extracted from plants infested with aphids, yielded a high concentration of ALPV (Appendix B.1.2). Aphids collected from these diseased appearing plants had high concentrations of ALPV per individual aphid when tested by DAS-ELISA and with an indirect immunofluorescent technique (Laubscher and Von Wechmar, 1991) (Chapter 2). Due to this finding this region was chosen for a further in-depth study to analyze a naturally occurring situation.

Aphid numbers and species were monitored during the growing season from June to October 1989. Their numbers were compared with the incidence of virus in the predominant aphid species. The presence of other natural pathogens and

predators of aphids at the study site was recorded and their possible effect on the aphid population was examined.

5.2 MATERIALS AND METHODS

5.2.1 Field survey

The field survey to assess the effect of natural virus infection on wheat colonizing aphids (natural aphid infestation) was done at two localities in the western Cape about 50 km apart; at Langgewens (33 17' S ; 18 42' E), an experimental farm of the Department of Agricultural Development for wheat and other alternative dry-land crops in the Swartland, and a wheat farm in the Pools area (32 48' S ; 18 53' E). Sample plots at the two field sites were 30 m x 3 m. Wheat (*Triticum aestivum*), cv. Palmiet was sown at each locality. Fifty plants were sampled from each field plot at weekly intervals at each locality. Plants were collected in a u-pattern at meter intervals moving along the lenght of the field. Aphids on each sample of 50 plants were counted in the field. At each sampling date counts of parasitized aphids (mummified) and fungal infected aphids were recorded *in situ*. Entomophthorales type fungi were not identified. Other aphids present early during the sampling period were *D. noxia* and small counts of *M. dirhodum* and *S. avenae* later in the season at Langgewens. At the beginning of sampling *R. padi* aphids predominated at this locality. A similar pattern was encountered at Pools but *S. avenae* aphids predominated and *D. noxia* was absent. The sampled plants from both localities were taken to the laboratory and kept at 4^{o}C. The following day *R. padi* aphids were separated from the plants and divided into two groups for virus assays. One group was frozen at -20^{o}C for later analysis by DAS-ELISA and aliquotes of the other group were dissected and tested by indirect immunofluorescent technique. The number of *S. avenae* and *D. noxia* aphids at Langgewens were very small and were ignored for further processing. Time recording is given as weeks, with week one being the time when the first aphids of the season were noticed. The survey was conducted from June to October 1989.

5.2.2 Moericke traps

Four Moericke traps (Moericke, 1951) per locality were placed at ground level at a northern, western, southern and eastern position at a distance of 1.5 m away from the sample plots. The traps consisted of four yellow bowls (28 cm in diameter X 13 cm deep), filled with two litres of water containing 20 ml detergent to reduce surface tension and 5 g of benzoic acid to preserve the insects. Moericke trap catches were collected weekly by transferring caught insects to 70% ethanol and

the aphids, predators and parasitoids identified and counted in the laboratory.

5.2.3 Virus detection with DAS-ELISA

Detection was done by DAS-ELISA (Appendix B.3.4) using two antisera specific for ALPV and RhPV respectively (Appendix B.3.1). From the weekly samples collected at Langgewens fifty aphids were used per test. Single aphids of *R. padi*, the dominantly occurring aphid species, were tested for ALPV and RhPV. Negative controls for DAS-ELISA were *R. padi* aphids taken from a laboratory clone of *R. padi*-Leip (Appendix D.1) maintained at a low temperature regime and showing no detectable level of virus infection (Laubscher and Von Wechmar, 1991). DAS-ELISA was not done on samples collected at Pools.

5.2.4 Virus detection with immunofluorescent technique

The indirect immunofluorescent technique (Chapter 2)(Laubscher and Von Wechmar, 1991) was used for the detection of viral infection in aphids. Groups of 40 aphids per sample were dissected and examined individually utilizing anti-ALPV serum and fluorescein isothiocyanate (FITC) labelled goat-anti-rabbit (GAR) serum. *R. padi* aphids from Langgewens were tested for ALPV only. Forty *S. avenae* aphids from Pools were tested for APLV and six for RhPV. Aphids which do not host the viruses (*Brevicoryne brassicae* and *Myzus persicae* (Williamson et al, 1988)) were included as negative controls to check whether aphid tissues showed non-specific fluorescence or autofluorescence.

5.2.5 Time-specific life tables

5.2.5.1 Sampling procedure for twin samples

The purpose was to assess the effect of predators, parasites and aphid-infecting viruses as well as other possible factors on the population dynamics of *R. padi* at Langgewens. Based on weekly counts three key points were selected on the population growth curve, i.e., during the exponential growth of the population (week six), at the beginning of its decline (week seven) and during the decline phase (week eight).

For each twin sample two estimates of population density were made one instar-period apart (i.e. 56 to 60h apart) (Dean, 1974; Hughes, 1962; 1963). This was done by randomly collecting 50 plants infested with aphids. The whole plant was carefully cut into pieces and placed into a bottle containing 96% ethyl alcohol to

facilitate handling of aphids and transport. All aphids were removed from the plant pieces in the laboratory and counted. Four hundred *R. padi* aphids were then taken at random from each twin sample. These were mounted and the instar age identified. Twenty additional plants were randomly collected during the first sampling date of each twin sample to assess ALPV infection by indirect immunofluorescent technique, insect parasitism and fungal infection.

The method used for calculating the time-specific life table was adapted from Hughes (1962; 1963) who previously studied the population dynamics of the cabbage aphid, *B. brassicae*. Hughes' method is generally considered to be accurate for this purpose (Hughes, 1972).

5.2.6 Meteorological data

To be able to interpret biological data obtained from the time-specific life tables the impact of meteorological factors were examined. Data on mean daily temperature, rainfall and wind speed for Langgewens was obtained from the agrometeorological section at the Elsenburg Agricultural Development Institute of the Department of Agricultural Development (Table 6.1). Except for a lower rainfall, the climatic conditions at Pools resemble those at Langgewens (50 km apart) and were therefore not analyzed seperately.

5.3 RESULTS

5.3.1 Field survey

The following grain aphid species were found in the Swartland region: *R. padi*, *D. noxia*, *R. maidis*, *M. dirhodum* and *S. avenae*. No *Schizaphis graminum* was found.

Population development of the four aphid species, sampled at Langgewens is shown in Fig. 5.1a (p. 110). The species *R. padi* and *S. avenae* were the most abundant, followed by *M. dirhodum*. *D. noxia* numbers were low and started to decline from the fifth week after infestation. The level of fungal infections and parasitoid activity was also compared (Fig. 5.1b). The number of parasitised aphids was much lower than those infected by fungus.

Fig. 5.1c illustrates the seasonal appearance of three aphid species, *R. padi*, *M. dirhodum* and *S. avenae* at Pools. *S. avenae* aphids were present in higher numbers than *R. padi*. Fungal infections and parasitoid activity are shown in Fig. 5.1d. Similar to aphids screened at Langgewens, the number of parasitised aphids was

much lower than those infected by fungus.

5.3.2 **Moericke traps**

The natural enemies identified at each site included *Hippodamia variegata*, *Cheilomenes lunata*, *Scatophaga* spp., *Eupeodes corollae* and *Aphidius colemani*. Natural enemies caught during 1989 at Langgewens and Pools by Moericke traps are listed in Tables 5.1 and 5.2 respectively. At Langgewens dung flies (*Scatophaga* spp.) and parasitoids (*A. colemani*) were the most abundant natural enemies present (Table 5.1).

Table 5.1. Moericke trap catches of natural enemies of aphids at Langgewens-1989

DATE	*H. variegata*[1]	*C. lunata*[2]	*S.* spp.[3]	*E. corollae*[4]	*A. colemani*[5]
24/7	3	0	6	0	27
31/7	8	0	14	0	69
7/8	4	0	8	1	6
14/8	5	0	21	0	187
21/8	12	0	119	1	134
28/8	1	0	135	0	257
4/9	9	0	18	0	155
11/9[6]	-	-	-	-	-
18/9	16	0	55	4	516
25/9	16	0	48	1	353
2/10	8	0	41	1	355
9/10	7	0	39	0	371
16/10	45	1	18	0	128

1. *Hippodamia variegata*
2. *Cheilomenes lunata*
3. *Scatophaga* spp. - dungfly
4. *Eupeodes corollae* - syrphid fly
5. *Aphidius colemani* - parasitoids
6. Data of week 11/9 lost

An analysis of the Moericke trap catches showed that parasitoid numbers reached their maximum level during week 14 and *R. padi* during week seven (Fig. 5.2c). A

comparison of Moericke trap catches and *in situ* counts of parasitized aphids showed that the level of activity peaked at week 14 and nine respectively (Fig. 5.2d). Natural enemy numbers retained this pattern at Pools, but the numbers were much lower (Table 5.2).

Table 5.2. Moericke trap catches of natural enemies of aphids at Pools-1989

DATUM	*H. variegata*[1]	*C. lunata*[2]	*S.* spp.[3]	*E. corollae*[4]	*A. colemani*[5]
14/7	4	1	16	1	6
31/7	11	0	7	0	1
7/8	10	1	6	1	0
14/8	1	0	40	0	68
21/8	1	0	30	0	58
28/8	2	0	11	0	54
11/9	4	0	9	0	11
18/9	0	0	6	2	3
25/9	2	0	7	3	15
2/10	3	0	67	1	31
9/10	4	0	63	1	23
16/10	6	0	42	0	6

1. *Hippodamia variegata*
2. *Cheilomenes lunata*
3. *Scatophaga* spp. - dungfly
4. *E. corollae* - syrphid fly
5. *A. colemani* - parasitoids

5.3.3 **Virus detection with DAS-ELISA**

The incidences of ALPV and RhPV infection were compared with the number of *R. padi* aphids recorded (Fig. 5.2a). Population decline of *R. padi* aphids corresponded with maximum ALPV levels detected in this population. The incidence (%) of ALPV was lower than that of RhPV (Fig. 5.2a). In Fig. 5.2b fungal infections were compared with the incidence of ALPV and RhPV. The maximum level of fungal infection overlapped with viral fluctuations recorded for aphids infected with ALPV and RhPV.

5.3.4 Virus detection with indirect immunofluorescent technique

Indirect immunofluorescent technique was also used for detecting ALPV in *R. padi* aphids collected at Langgewens (Fig. 5.3a). For *S. avenae* aphids collected at Pools, tests were done on dissected aphids for ALPV and RhPV (not shown) (Fig. 5.3b). The incidence of ALPV increased linearly with increase in aphid numbers at Pools. RhPV was also present in this population. Not enough aphids could be collected at the late stage of infestation of *S. avenae*, thus further analysis was not possible.

5.3.5 Meteorological data

Prevailing weather conditions during the period when twin samples were collected were normal (Figs 5.4a - d). No excessive temperature fluctuations, wind speed or rainfall (Table 5.3) was measured.

Table 5.3. Climatic data for Langgewens and Pools 1988 and 1989.

	Year	Jan.	Feb.	Mrch.	Apr.	May	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.
Langgewens													
Max. daily Temp. (^{o}C)	1988	29.4	33.4	28.8	23.9	22.7	17.8	16.5	18.8	20.3	23.0	28.2	30.5
	1989	31.8	31.1	28.1	26.2	20.8	17.9	16.9	18.1	19.6	22.9	27.8	28.7
Min. daily Temp. (^{o}C)	1988	15.3	18.9	16.6	13.7	13.0	9.2	7.9	9.5	8.4	9.9	13.8	16.3
	1989	17.5	18.4	15.6	14.9	11.5	9.3	7.8	8.7	9.3	9.6	14.4	15.3
Rain (mm)	1988	0.5	0.0	13.8	43.2	29.5	73.6	51.9	69.7	38.8	8.7	6.9	15.9
	1989	2.9	17.5	59.2	58.3	38.8	46.8	68.2	82.5	48.2	29.7	34.7	2.4
Pools													
Max. daily Temp. (^{o}C)	1988	31.9	35.9	30.0	24.6	23.6	18.5	16.4	19.6	21.6	25.0	30.9	31.7
	1989	34.2	33.1	29.9	27.8	22.0	18.3	17.4	18.6	20.6	25.0	30.0	31.3
Min. daily Temp. (^{o}C)	1988	15.6	18.5	16.4	12.5	11.3	7.2	5.4	6.8	7.3	8.9	13.4	14.7
	1989	17.3	17.4	14.8	13.4	9.7	7.2	5.8	7.1	7.8	8.2	13.1	14.9
Rain (mm)	1988	0.8	0.3	25.8	52.8	15.3	52.6	26.6	47.7	20.7	9.0	4.3	0.5
	1989	0.8	6.9	28.6	31.9	14.2	61.7	51.9	52.5	36.0	14.9	4.8	0.0

5.3.6 Time-specific life tables

This study was conducted at Langgewens. Different factors that could play a role in limiting the development of a natural aphid population (*R. padi*) such as emigration, parasitism, fungal pathogens and predation were analyzed.

Time-specific life tables were constructed from *R. padi* aphids sampled during the three stages of population build-up. Figs 5.5a and b present results obtained at Langgewens illustrating the logarithmic and decline phase of aphid population development.
The impact of predators (residual mortality), emigration, fungal infections, and parasitism was 15, 2, 6 and 0.4 aphids per plant respectively during the logarithmic phase. The calculated potential increase rate during this period was 0.86 (Fig. 5.5a). Calculated losses due to deaths were 5.6 aphids per plant.

During the decline phase the impact of predators (residual mortality), emigration, fungal infections and parasitism was 69, 38, 21 and 0.2 aphids per plant respectively. The potential increase rate was 0.34. Estimated losses due to deaths were 70 aphids per plant (Fig. 5.5b).

6.4 DISCUSSION

The predominant aphid species at Langgewens was *R. padi* which reached maximum numbers during late July and beginning of August (Fig 5.1a). *R. padi* started to increase three weeks after the first sampling date and took seven weeks to reach its highest infestation level. A decline in the population was observed immediately after ALPV had reached a high incidence i.e. when 30% of the individuals tested from a *R. padi* sample gave positive results (Fig 5.2a). In Australia a similar unexpected decline in numbers of bees was associated with a viral epizootic caused by sacbrood virus (SBV) and Kashmir bee virus (KBV) (Dall, 1985). The incidence of ALPV showed a similar pattern of fluctuation of viral incidence as was described by Dall (1985). RhPV peaked at week nine and 11. It reached a level of 60% when compared to ALPV which fluctuated between 30 and 35% (Fig. 5.2a). However, the incidence of RhPV was not associated with population decline. A similar finding was obtained in the laboratory by Williamson (1988) where it showed that RhPV only effects fecundity and longevity. It can be argued that the lower level of ALPV could be attributed to the fact that virus diseased aphids had already dropped off from the plants as a consequence of paralysis and the lethal effect of the virus (Williamson *et al.*, 1988; Von Wechmar *et al.*, 1990) thus influencing the assay. It was impossible to sample

aphids on the soil surface. Nocturnal predators can be excluded (Laubscher, unpublished results).

Since the numbers of the other aphid species were too low for analysis, and the fact that they maintained this profile during the growth season, they were only counted on plants in the field. *D. noxia* numbers grew steadily and six weeks later started to decline. This took place one week before *R. padi* numbers started to decline. The numbers of *D. noxia* were much lower than *R. padi* and the highest number recorded was 36 aphids.

Fungal infestation at Langgewens started to appear from the fifth week and reached its peak during the eighth week of sampling, before it started to decline. This maximum level of fungal infestation occurred only after the incidence of ALPV had reached its initial peak and the aphid population was already in the decline phase (Fig. 5.2b). These results suggest that the viral incidences may influence fungal pathogenesis. It is possible that the viremic condition in sick or paralysed aphids predisposes them to fungal parasitism. Alternately the fungus could play a role in the dissemination of ALPV. Laubscher *et al.* (1990) showed that ALPV virions were present in fungal hyphae cultured from parasitized aphids thus indicating that the fungi may act as vectors.

The activity of natural enemies at Langgewens was very low. Parasitoids, mainly *A. colemani*, reached their highest level of 44 "mummies" after eight weeks (Fig. 5.1b). This gave a maximum average number of parasitoids per plant of 0.9 compared to the maximum average number of aphids per plant of 320. Therefore the level of parasitoid activity could clearly not influence the natural aphid population build-up.

The meteorological data obtained did not indicate adverse conditions during the population build-up of *R. padi* at Langgewens. There appeared to be no relationship between weather conditions and aphid numbers (Fig. 5.4a - d)(Table 5.3).

At Pools the situation was different. *R. padi* numbers were much lower than at Langgewens. The highest number of *R. padi* recorded was approximately 600 after the seventh week (Fig. 5.1c) whereas the highest number of *R. padi* at Langgewens was approximately 16 000 (Fig. 5.1a) during the same time. *S. avenae* reached much higher numbers than at Langgewens indicating its relevance as a problem species in this area (Fig. 5.1c). For this reason analysis of viral incidence was done only in this aphid species. *S. avenae* reached its highest level of infestation after 17 weeks.

At the same time immunofluorescent analysis showed a linear increase in the incidence of ALPV (Fig. 5.3b). However, by the eighteenth week all aphids had disappeared and further analysis was not possible (Fig. 5.3b). Results obtained from Moericke traps did not indicate a massive emigration from this locality. *A. colemani* does not parasitize *S. avenae* (Laubscher, unpublished results) and can be excluded as a population limiting factor. Dung flies (*Scatophaga* spp.) were the only predators present at Pools in high numbers. The highest count was 67 (Table 5.2), and that was reached during the 16th week. At this level of infection it is unlikely for this predator to cause such a dramatic decline in the aphid population (Laubscher, unpublished data). Fungal levels (Fig. 5.1d) were also too low during this period to have any impact on the population development of *S. avenae*. Thus the sudden decline was unexpected. The only other factor examined that could influence a population decline was ALPV.

Since there were many limiting factors involved which could influence population development of aphids, an attempt was made to analyze some of these natural factors. Various methods exist by which such an analysis could be done. It was decided to utilize Moericke traps and time-specific life tables (Hughes, 1972). Although only a general flight pattern of aphids and their species distribution was obtained from field data using water traps (Coon & Rinick, 1962), they gave an indication of the presence of natural enemies. The Moericke traps showed that parasitoid activity was late in the season, reaching its peak during September (Fig. 5.2c). This could have been the reason for the low numbers of *M. dirhodum* at these localities. However, parasitoid and predator activity did not coincide with the dramatic decline of *R. padi* (Figs 5.2c and d; Table 5.1).

The aphid population of *R. padi* sampled at Langgewens, showed a stable age distribution during the logarithmic phase (Fig. 5.5a). Diseased aphids that had already developed paralytic symptoms and dead aphids on the soil surface, were not considered in the survey. Predators were present in very low numbers (Table 5.1) and the estimated impact (residual mortality) was that 15 individuals of *R. padi* per plant was removed during one instar period. However, the measured impact of parasitoids, fungal infections and emigration were less than one, six and two aphids per plant per instar period (Fig. 5.5a). As all these factors were active at a very low level, the impact on aphid number is likely to be minimal. The calculated potential population maximum which could have been attained during one instar period by the aphid population was lowered by 19%. As this situation cannot be fully ascribed to residual mortality factors (predation) as described above, it appears that ALPV had a major effect on aphid numbers as indicated by the increase in incidence of viral infection (Fig. 5.3a).

During the decline phase there was a dramatic increase in the relative number of fourth-instar aphid nymphs with wingbuds. There was also an increase in emigration (38 aphids per plant calculated). Fungal infections increased to 21 aphids per plant (calculated) and the average loss due to parasitism was also very low i.e. less than one aphid per plant during one instar period. The residual mortality rate (predation) calculated for one instar period increased to 69 aphids per plant. It is unlikely that this was due to predation, as predator numbers were very low (Table 5.1). The potential maximum which could have been reached by the population during one instar period was lowered by 49%. The cumulative effect on the population growth is illustrated in Fig. 5.1a. In this study fungal activity (Fig. 5.1b) is responsible for a large portion as a growth limiting factor (6 and 21 aphids per plant calculated).

Hughes (1972) included the reduction of reproduction by deaths of potentially reproducing females in his time-specific life table, but he mentioned no viral or bacterial diseases which could have influenced aphid reproduction and development. This death factor (calculated) was included in the calculations (Fig. 5.5b), and during the logarithmic phase the impact was six aphids per plant. During the decline phase the death factor increased to 70 aphids per plant.

Overall, there was a lack of population increase which was clearly illustrated by the low potential increase rates obtained (0.86 and 0.34). Similarly, Aalbersberg *et al.* (1988) who investigated the influence of natural enemies on *D. noxia* aphids in the eastern Orange Free State, could not find a relationship between mortality and natural enemy activity. Epidemiological studies conducted over several years (Von Wechmar, unpublished results) have shown that ALPV is endemic in the eastern Orange Free State, and it is likely that the presence of this virus could explain this low potential increase rate.

ALPV and RhPV are two fully characterized aphid pathogenic viruses (Williamson *et al*, 1988; 1989). RhPV appears to be a persistent viral infection causing reduction in longevity and reproduction (D'Arcy *et al.*, 1981b; Williamson, 1988) whereas pathogenesis with ALPV is more severe, causing aphid paralysis and rapid death (Williamson *et al.*, 1988). This finding may explain the measured low population growth of *D. noxia* and the earlier decline of the *D. noxia* population. A comparison of the graphical data in Figs 5.1a, 5.2a and 5.3a illustrates that the level of ALPV was already reasonably high at a time when the *D. noxia* population reached its peak. *D. noxia* is more tolerant to the low temperature season compared to other grain aphids (Von Wechmar, unpublished

results; Aalbersberg, 1987; Von Wechmar *et al.*, 1990). Under natural conditions *R. padi* and *D. noxia* aphids often occur as mixed populations on the same leaf blade. Considering the fact that the *D. noxia* population declined at a time when the virus incidence was already high, raises the question whether the virus infection of *D. noxia* aphids played a role in the initial contagion that caused the *R. padi* population to decline seven weeks later. The information presented here on the field occurrence of the two viruses appears to indicate that they are important factors in the limitation of aphid population growth under natural conditions in the Swartland.

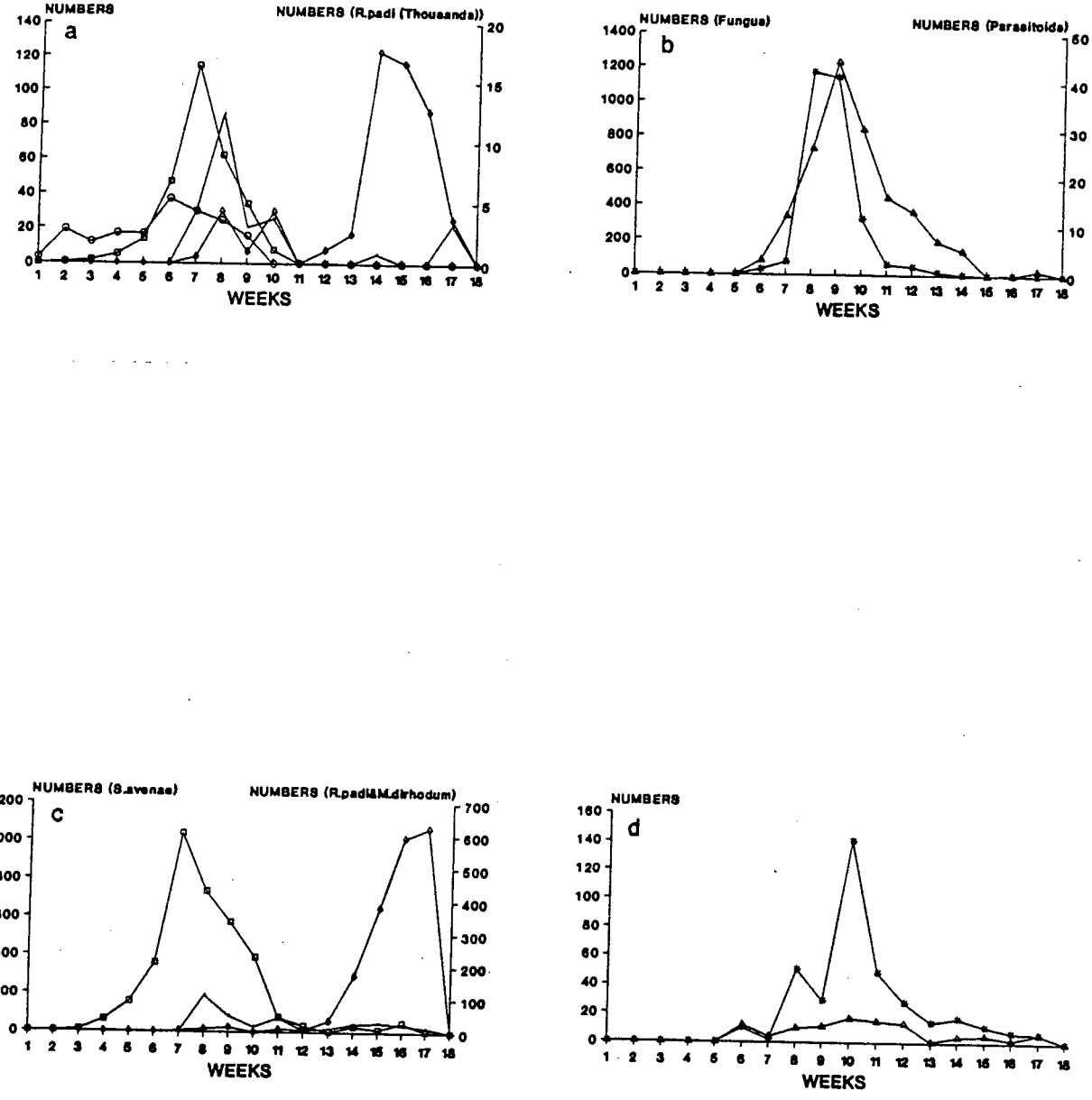


Fig. 5.1a - b. The weekly numbers of aphids and natural enemies of aphids on the 50 sampled wheat plants at Langgewens and Pools.

a.) Numbers of *M. dirhodum* (—), *D. noxia* (-o-), *R. padi* (-□-) and *S. avenae* (-◇-) present at Langgewens.

b.) Parasitoid (-△-) and fungal (-*-) activity at Langgewens.

c.) Numbers of *M. dirhodum* (—), *R. padi* (-□-) and *S. avenae* (-◇-) aphids present at Pools.

d.) Parasitoid (-△-) and fungal (-*-) activity at Pools.

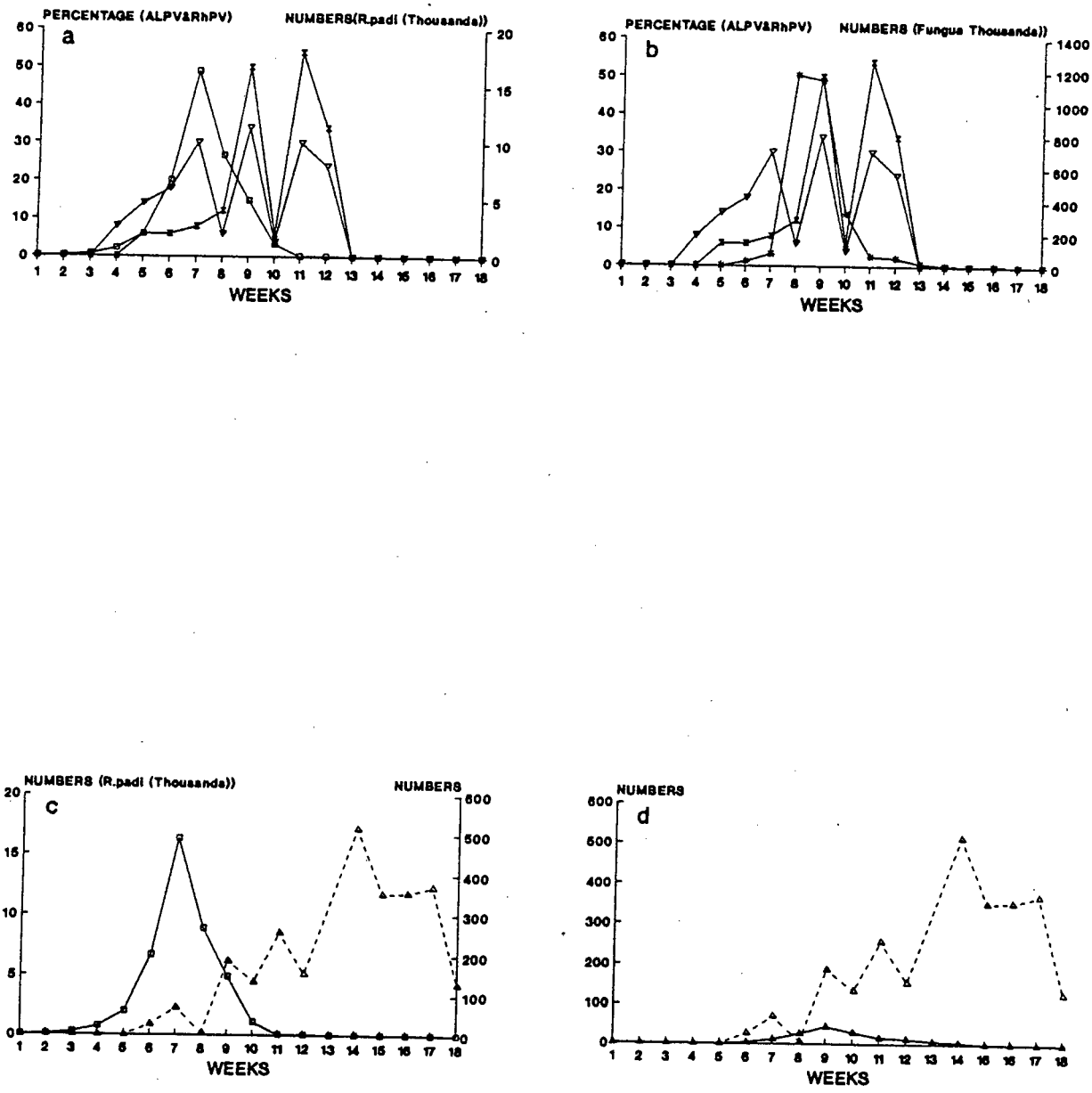


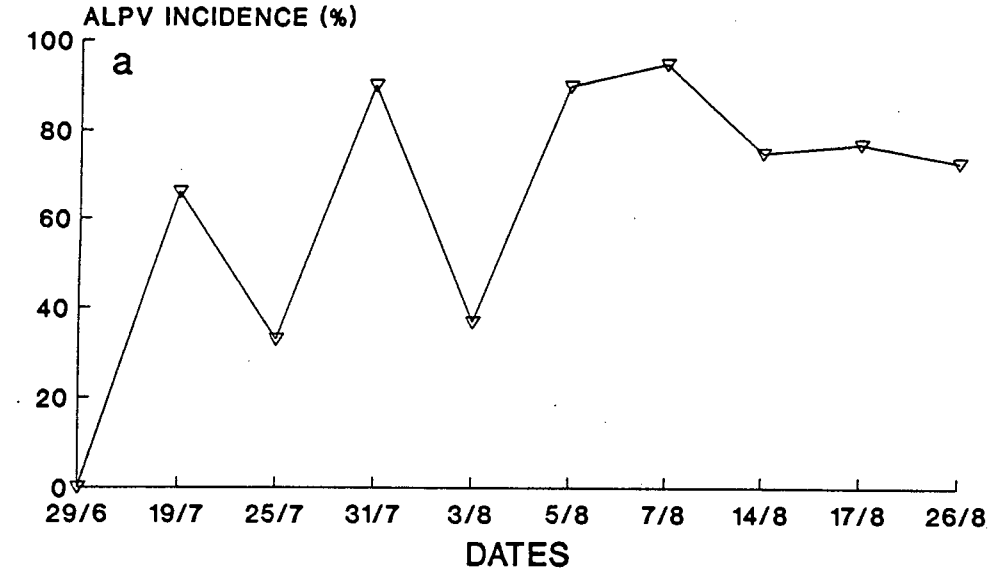
Fig. 5.2a - b. Comparison of viral incidence, parasitoid numbers and fungal parasitism at Langgewens.

a.) ALPV (▽) and RhPV (▼) incidences compared to *R. padi* (□) numbers.

b.) Comparison of fungal parasitism (✱) with ALPV (▽) and RhPV (▼) incidences.

c.) Moericke trap (△) catches of parasitoids compared to *R. padi* (□) aphid numbers (*in situ* counts) at Langgewens.

d.) Moericke trap (△) catches of parasitoids compared to numbers of parasitized aphids (▲) counted at Langgewens.



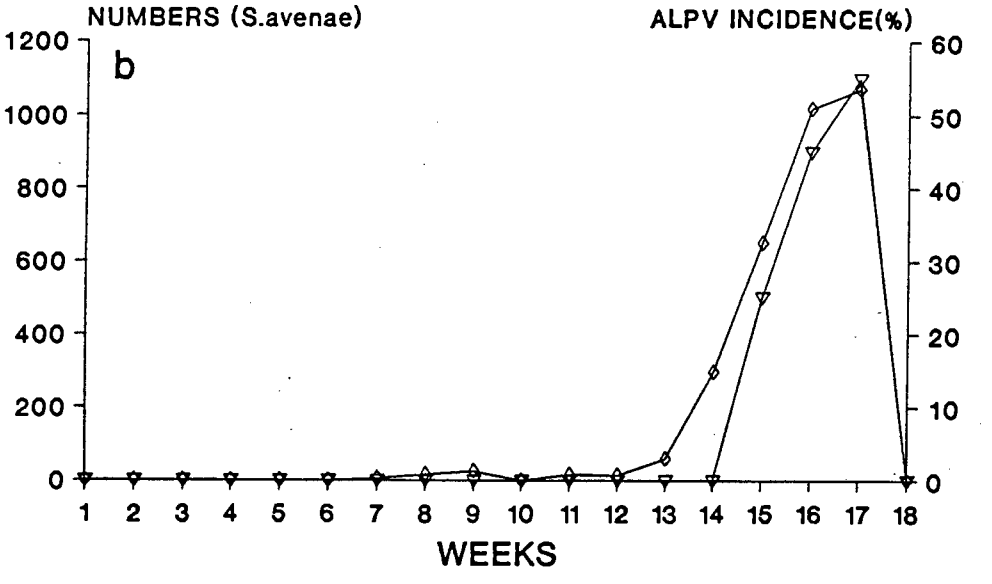
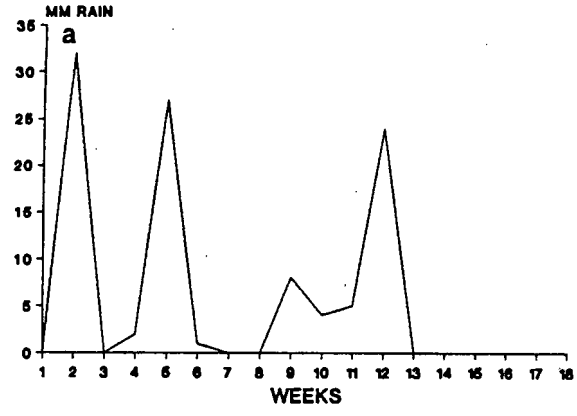
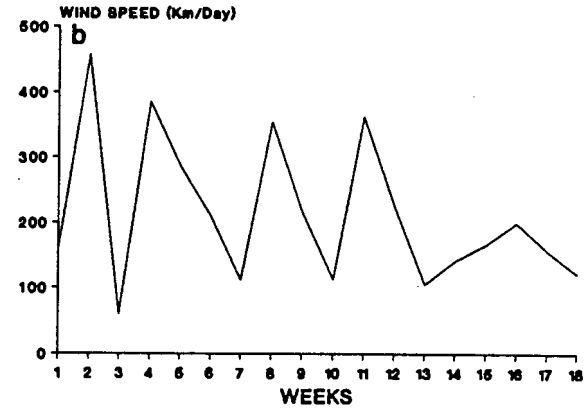
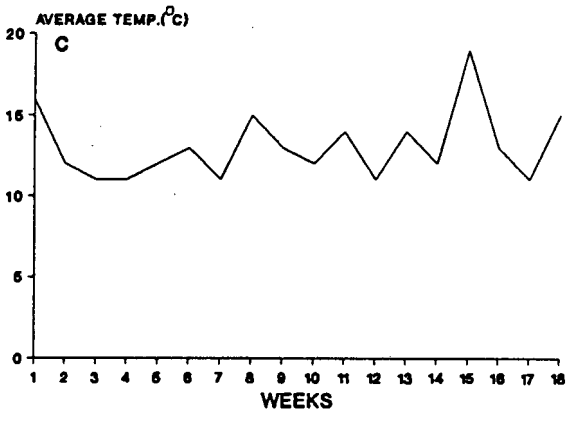


Fig. 5.3a and b. Incidence of ALPV at Langgewens and Pools as analyzed by immunofluorescent technique. Time scale based on actual dates and not weeks.
a.) Incidence of ALPV (▽) in *R. padi* aphids sampled at Langgewens. Dates on which sampled aphids were tested, include the results utilized for calculating the time-specific life tables.
b.) Incidence of ALPV (▽) in *S. avenae* aphids (◇) sampled at Pools.







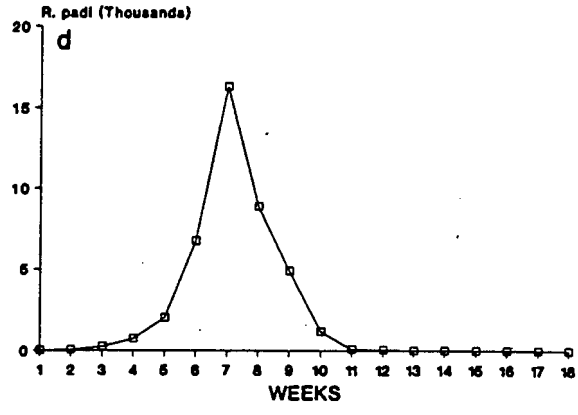


Fig. 5.4a - d. Climatic conditions at Langgewens during 1989.

a.) Rainfall in mm.

b.) Windspeed km / day.

c.) Average temperature in ^{o}C.

d.) *R. padi* (⊟) aphid numbers.

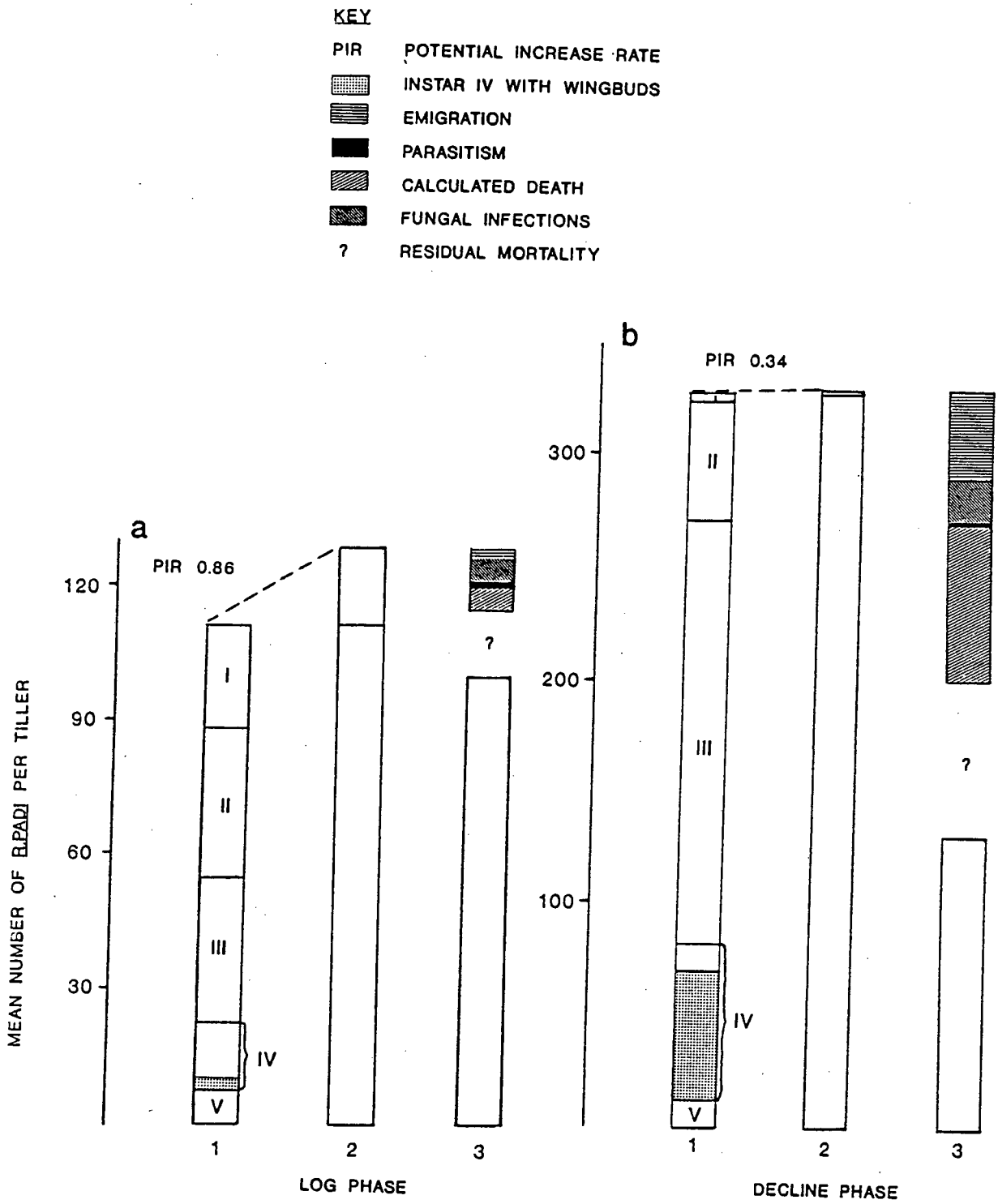


Fig. 5.5a and b. Time-specific life tables of *R. padi* aphids sampled during various phases of aphid development in a Palmiet wheat field during 1989 at Langgewens.
a.) Logarithmic phase of population development;
1) population composition of the first twin sample,
2) potential population one instar period later and
3) actual population one instar period later.
b.) Decline phase of population development; 1) population composition of the first twin sample, 2) potential population one instar period later and 3) actual population one instar period later.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

To be able to study the pathogenicity of ALPV in aphids under natural conditions it was essential to develop a sensitive method for virus detection in single aphids. The indirect immunofluorescent technique was found suitable for this purpose. This method had the advantage that it was tissue non-destructive so that individually infected organs and tissues could be identified in the adult aphid as well as the degree of infection of embryos at different stages of development in the ovarioles. It was also possible to distinctly detect the initial location of virus in mycetocyte tissues. Reproductive mothers were usually found to contain embryos with different levels of infection. A further advantage was the greater sensitivity of the indirect immunofluorescent technique when compared to the tissue destructive DAS-ELISA method.

Virus detection by indirect immunofluorescent technique also assisted with the selection of diseased aphids for immunogold cytochemistry studies. It was important to select aphids at an early stage of infection before the virus level increased to a point where all tissues were engulfed by virus. To observe the onset and/or presence of early infection in a population the indirect immunofluorescent technique proved to be particularly useful. Because the degree of virus infection can change very rapidly, the speed of the indirect immunofluorescent technique was a distinct advantage. The sudden build-up of virus in a population could be assessed within 48 hr. To be able to monitor the presence/absence of ALPV in cereal aphid populations, for purposes of biocontrol, this assay could easily be utilized by the applied sector.

The presence/absence of the virus could be used to assess whether it is necessary to apply expensive pesticides or not. Where a high incidence of virus is present in the population it may run itself out within a couple of days at average temperatures above 16^{o}C as was found in the biological study presented in Chapter 5. The stability of the virus level in individual aphids even after the decline of the population, ensures that later infestations are exposed to the contagion of the virus and will contribute to keep numbers low. The significance of

this is that in a normal pest management program interference with applications of chemical pesticides would be superfluous to limit secondary late colonizers.

Considering the potential economic uses of ALPV in a pest management program of aphids, it would be of interest to know whether young wheat colonized by virus-infected aphids could compensate later for nutrient loss experienced during the logarithmic phase of aphid population development in the absence of chemical pesticides. Once the decline phase sets in, further continued biological control with ALPV would limit aphid population development of late colonizers, and could allow the plant to recover. It is not known whether the rate of plant recovery in the western Cape after aphid population collapse is sufficient to compensate for the earlier external stress of large aphid numbers. This will have to be determined in a separate study. To be successful the practical utilization of such a pest management system would have to be monitored very closely, and would also have to be examined under different climatic conditions.

The temperature studies showed that between 15 and 20 ^{o}C the replication of ALPV was apparently activated. With DAS-ELISA assays it was usually not possible to detect ALPV in diseased aphid clones maintained below this temperature.

Aphids maintained at low temperature for an extended time, testing virus-negative at low temperature, rapidly switched to testing virus-positive when transfered to day/night temperatures of 15 / 10^{o}C with increased virus levels at higher day/night regimes i.e. 20 / 15^{o}C and 24 / 15^{o}C. These temperature regimes represent averages of natural winter and early spring conditions in the western Cape and explained the rapid increase in virus-positive aphid numbers recorded in early spring when average day/night temperatures increased, compared to cool conditions at the start of the season when few virus-positive aphids were found and virus incidences were generally low.

Temperature itself plays an important role in aphid physiology and development. The additional effect of temperature on virus replication appears to influence the level of infection of unborn embryos in ovarioles with the result that embryos developing in one aphid mother could be infected with different levels of virus.

Virus transmission from parent to embryo follows two routes. One route is by contagion of virus positive germarial tissue, the other by passage of virus-positive symbionts from parent to embryo. Evidence for the presence of virus in germaria and in symbionts was obtained by indirect immunofluorescent technique and

immunogold cytochemistry. It is possible that fluctuating temperature regimes may be responsible for the phenomenon where diseased and apparently non-diseased embryos could be visualized in one virus infected aphid mother. Other factors that may also influence virus replication and the passage of virus to the embryos were not examined.

In the western Cape several cycles of hot berg wind (a hot, dry wind from the north) conditions can be experienced during August to September, lasting 1-2 days resulting in a sudden decline of aphid populations where significant aphid numbers diminish to non-recordable numbers. Climatic conditions in the Swartland region vary considerably from year to year, thus having a significant effect on the level of virus infected aphids present in populations. Extended periods of low temperature will result in a gradual build-up of apparently non-infected aphid numbers (latent infection). DAS-ELISA did not detect ALPV in such aphids whereas the greater sensitivity of indirect immunofluorescent technique revealed the presence of low thresholds of virus. Previously such populations were considered to be virus-negative (Williamson, 1988; Williamson *et al.*, 1988; Von Wechmar, unpublished results). Sudden changes in temperature could turn an apparently negative colony into a positive colony causing a fast decline in aphid numbers. This was observed when a detailed analysis was made of the factors influencing the biology of virus infected populations of *D. noxia, R. padi* and *S. avenae.*

ALPV was visualized in specific aphid body tissues by indirect immunogold labelling (IGL) on ultrathin sections of virus diseased aphids. Viral infections were detected in ovariole tissue, tracheocytes, cytoplasm, symbionts of mycetocytes, fat body, brain, nerve, and stomach epithelial tissue. No ALPV was detected in the muscle fibres and mitochondria of cells. Very similar labelling patterns as were shown by Booth *et al.* (1989) for Thogoto virus, a tick-born virus, were obtained with aphid brain tissue. The detection of ALPV in thin sections of embryos of infected mothers was supported by evidence previously obtained by cDNA hybridization on thin sections (Hatfill *et al.*, 1990) and by immunofluorescent technique mentioned above. These results compared well with reports of other insect picorna-like viruses. For instance Dall (1987) detected KBV in the foregut, epidermis, haemocytes and tracheal epithelium of bees. Mussen and Furgala (1977) found SBV in the tracheal epithelial cells, in the nerve tissue and haemocytes. Similarly Reinganum *et al.* (1970) showed the presence of CrPV in nerve tissue. Scotti *et al.* (1981) detected DCV in tracheal cells and cells surrounding the ganglia in *D. melanogaster*, and in the cytoplasm of cells surrounding the gut of *C. capitata.* DPV was detected in the ovaries of *D. melanogaster* (Teninges & Plus, 1972) and

Chao *et al*. (1986) found $CrPV_{ark}$ in epidermal cells, haemocytes, fat body cells and midgut epithelial cells. RhPV was shown by Gildow and D'Arcy (1990) in the stomach epithelium and cytoplasm of cells, but not in nerve tissue.

The presence of ALPV in nerve and brain tissue and not in the muscle fibres indicates that the paralytic effect on aphids is the result of virus diseased nerve tissue. It is possible that the infected cuticle and epidermal tissue of infected aphids may assist the spread of viral infections through a natural aphid population as a contagion. During the feeding process aphids die *in situ* as a result of high ALPV incidences. These cadavers as well as the body content are a good source of contagion for other aphids. This was confirmed by Von Wechmar *et al*. (1990).

Species differences between different aphid species i.e. *R. padi* and *S. avenae* can influence the epidemiology of ALPV in aphid populations as illustrated in Chapter 4. Different incidences of transmission of ALPV were obtained for *R. padi* aphids (29%) and *S. avenae* (16%). From the field survey conducted in the Swartland it appeared that alate aphids were mainly responsible for the spread of ALPV as virus diseased alates often produced offspring after arriving at a new destination.

Virus increase and pathogenicity of ALPV and RhPV were found to be very temperature dependent (Chapter 4). The fluctuation of temperature regimes experienced in nature may influence viral performence under natural conditions. Temperature influences virus increase and pathogenicity in the host which in turn predisposes the aphid to other external factors like parasitic fungi and attack by natural enemies. Diseased aphids are less agile and thus more prone to predation. Research done on latent infections of other insect picornaviruses influenced by temperature support this finding (Moore *et al*., 1981a & b; Podgwaite & Mazzone, 1986).

The results from this study indicates that an epizootic condition can exist in a natural aphid population infected by ALPV. With our knowledge of ALPV it is difficult to single out climate as the only major factor which can influence natural enemies like fungi, predators and parasitoids in natural aphid populations without taking into account the presence of aphid pathogenic viruses present, and the stage of development of such a population as shown in this study. Since ALPV can influence the development of aphid populations, it could influence management strategies in an integrated pest management system. The estimation of the developmental stage of an aphid population is very important

in decision making. This together with other climatic factors like rain, temperature and humidity can help to assist the decision maker. In future farmers will be influenced even more by market pressures like the rise in production costs, the ever rising cost of chemical pesticides and the global awareness for nature conservation pressure groups. This should lead to an increased use of integrated pest management practices in the small grain industry to reduce the use of harmfull chemicals.

APPENDIX A

CHEMICALS, BUFFERS AND SOLUTIONS

CHEMICALS, BUFFERS AND SOLUTIONS

A.1 Potassium phosphate buffer

Potassium phosphate buffer was made according to the specifications of Williams and Chase (1968)(A.1.1).

Unless otherwise stated all potassium phosphate buffers used were pH 7.0 and 0.1 M. When necessary a final adjustment was made to the pH.

A.1.1 Stock solutions

Solution A: KH_2PO_4, 0.5 M
Dissolve 68,04 g of $KH_2PO_4 . H_20$ in a final volume of 1 liter ddH_2O.

Solution B: K2HPO4, 0.5 M
Dissolve 87,09 g of $K_2HPO_4 .H_2O$ in a final volume of 1 liter ddH_2O.

A.1.2 0.1 M Potassium Phosphate (pH 7.0)

Mix 78 ml Solution A with 122 ml Solution B. Make up to 1 liter with ddH_2O. To obtain pH 7.2 mix 56 ml Solution A with 144 ml Solution B. Make up to 1 liter with ddH_2O.

A.1.3 Sodium azide stock solution 5% (w/v)

Dissolve 5 g sodium azide in a final volume of 100 ml ddH_2O. Add sodium azide to all the buffers to a final concentration of 0.02% (4 ml/litre of a 5% stock solution).

A.2 Saline

NaCl 0.15 M

A.3 Phosphate buffered saline (PBS)

Mix 1 part 0.1 M potassium phosphate buffer (pH 7.0) with 1 part 0.15 M NaCl.

A.4 Half strength PBS (1/2 PBS)

Mix 1 part (500 ml) PBS with 1 part (500 ml) ddH_2O.

A.5. Solutions required for the precipitation of IgG fractions

A.5.1 Ammonium sulphate (saturated)

Dissolve 132.15 g of $(NH_4)_2 SO_4$ in 250 ml ddH_2O. Bring this to the boil while stirring. Remove from heat and let cool. The solution is used

saturated at room temperature (RT).

A.6 **PBS - Tween 20 (PBS-T)**

PBS containing 0.05% (v/v) Tween 20 (Merck) (0.5 ml/l).

A.7 **PBS-T-Milk powder (PBS-T-MP)**

Add 0.2% (w/v) milk powder to PBS-T solution.

A.8 **ELISA substrate buffer (10% (v/v) diethanolamine (pH 9.8))**

Add 100 ml diethanolamine to ddH2O. pH to 9.8 with HCl. Make up to final volume.

A.9 **ELISA substrate**

Add 1 mg/ml p-nitrophenyl phosphate to 10% diethanolamine.

A.10 **10X Saline**

Dilute 0.15M NaCl 1 : 10 when using this solution.

A.11 **Knopps nutrient solution**

Nutrient solution was made according to the method of Riker and Riker (1936).

KNO_3	250 mg
$Ca(NO_3)_2$	1000 mg
$MgSO_4.7H_2O$	250 mg
KH_2PO_4	250 mg

Make up to 1 liter with ddH_2O and sterilise by autoclaving.

APPENDIX B

ISOLATION AND CHARACTERIZATION OF ALPV AND RhPV USED IN THIS STUDY

ISOLATION AND CHARACTARIZATION OF ALPV AND RhPV USED IN THIS STUDY

B.1 Introduction to virus purification

Essentially the aphid infecting viruses ALPV and RhPV were purified either from plants used to rear virus-infected aphids or directly from aphids. Rybicki and Von Wechmar (1982) previously extracted RhPV from plants co-infected with BYDV and BMV. This raised the question whether RhPV replicated or only accumulated in aphid infested plants. In follow-up work Williamson (1988) tested this hypothesis and showed that the virus accumulated in plants and that such plants could be regarded as virus "vectors". Gildow and D'Arcy (1988) confirmed these findings in a subsequent study.

In this section the purification of virus from plants colonized by virus-positive aphids under laboratory conditions, from field collected plants naturally infested by virus-positive aphids and from virus-positive aphids only, is presented.

B.1.1 Purification of virus from plants colonized with virus-positive aphids under laboratory conditions

Aphids were mass reared on barley seedlings at 24 - 26^{o}C (Appendix D.2.1; 2.4) and the plants harvested after one week (Appendix D.2.5). Virus was purified essentially as described by Rybicki and Von Wechmar (1982). Plant material was homogenized in 0.1M phosphate buffer pH 7.0 (A.1.1) in a 1 : 1 (w/v) ratio in a Waring blender. The homogenate was strained through cheese cloth. Clarification was carried out by addition of 20% (v/v) chloroform. The emulsion was mixed either by shaking or stirring at 4^{o}C for 15 min. The phases were separated by centrifugation at 7 000 rpm for 10 min in a Sorvall GSA rotor.

The organic phase was discarded, and the virus in the aqueous phase was precipitated by the addition of 9% (w/v) polyethylene glycol (PEG, MW 6 000) and 3% (w/v) NaCl. The suspension was stirred at 4^{o}C for 30 min, followed by centrifugation in a Sorvall GSA rotor at 10 000 rpm for 10 min. The precipitate was then resuspended gently with a rubber spatula in one tenth of the original volume of phosphate buffer. A final low speed centrifugation was performed in a Sorvall SS 34 rotor and the supernatant was centrifuged in a Beckman ultracentrifuge at 34 000 rpm in a Type 60Ti rotor for 90 min or in a Type 35 rotor for 120 min. The slightly brown sticky pellet was resuspended overnight in 0.1M phosphate buffer.

Separation of RhPV and ALPV was achieved by zone electrophoresis (Appendix B.2).

B.1.2 **Field plants naturally infested by aphids**

Oat plants exhibiting typical barley yellow dwarf virus (BYDV) symptoms of dwarfing and red discolouration were collected from a field at Riebeek West (33 21'S ; 18 52'E). These plants were severely infested with *R. padi* aphids and were extracted for virus (Appendix B.1.1). The virus product was fractionated by rate zonal centrifugation on a 10 - 40% (w/v) sucrose gradient in a 0.1M phosphate buffer.

The method described by Williamson (1988) was used. This was briefly as follows: Linear sucrose gradients were formed in "ultraclear" SW 28 centrifuge tubes by constant mixing of 40% and 10% sucrose stock solution in a gradient former. Pouring of gradients was assisted by a peristaltic pump. Samples (about 2 ml) were layered onto the gradients and the tubes centrifuged in a SW 28 Beckman rotor at 26 000 rpm for 150 min at 5^{o}C. Virus bands were located by light scattering using an overhead point light source. The virus was noted as a blue opalescent band at a distance of about two thirds from the top. A white zone near the top of the tube was assumed to be degraded protein. Gradients were scanned at A_{254} nm and fractionated on an Isco Model 640 gradient fractionator coupled to an UA 5 absorbance monitor.

The position of the virus peak in the gradient was at a position very similar (Fig. B.1) to peaks obtained with picorna-like insect viruses which had sedimentation coefficients of about 164S (Williamson *et al.*, 1988). BYDV has a sedimentation coefficient of 115S (Rybicki *et al.*, 1990) which under the conditions utilized should have banded at about half way down the tube.

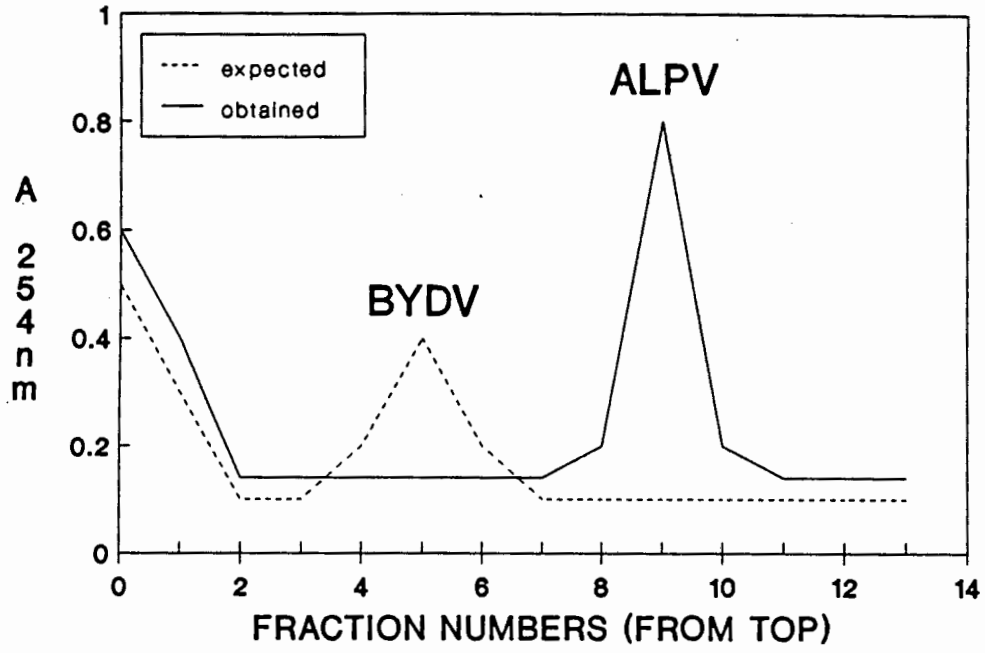


Fig. B.1. UV absorbance profile of sucrose density gradient fractionation of concentrated extracts of virus isolated from aphid infested field collected oat plants.

The virus peak was tested by Ouchterlony and DAS-ELISA with anti-ALPV serum. The tests were positive for ALPV and tested negative for RhPV. The remainder of the purified ALPV was used to immunize a rabbit (Appendix B.3). Aphid transmission with aphids from plants exhibiting the BYDV-like symptoms was negative (Von Wechmar & Laubscher, unpublished results).

The extraction of a significant quantity of ALPV from naturally infected plants exhibiting symptoms similar to BYDV was unexpected. It was the first incidence where ALPV was isolated from heavily aphid infested field grown plants. This finding was significant as it indicated the presence of large numbers of ALPV infected aphids in the region of Riebeek West in the Western Cape. To examine the effect of ALPV on natural aphid populations, this area was chosen for a further in depth study of ALPV as a growth limiting factor on aphid biology (Chapter 5).

B.1.3 **Virus purification from virus-positive aphids**

The method of D'Arcy *et al.* (1981b) with some changes was followed.

A known mass (usually 1 g to 5 g) of aphids was homogenized with an excess of 0.1M phosphate buffer in a mortar and pestle for 1 min. The homogenate was strained through cheese cloth and the residue was re-extracted with 10 ml of 0.1M

phosphate buffer and mixed with the first extraction. Extraction was done at room temperature.

The filtrate was clarified at 8 000 rpm for 10 min in a Sorvall SS 34 rotor. The supernatant fluid was concentrated by ultracentrifugation at 34 000 rpm for 90 min in a Beckman 60Ti rotor. The pellets were resuspended as described above (Appendix B.1) and the viruses separated by zone electrophoresis (Appendix B.2).

Virus concentrations of purified preparations (Appendix B.1.1, B.1.2 and B.1.3) were determined by ultraviolet spectroscopy (Appendix B.1) using an $E^{0.1\%}$ at 260 nm of 6 (Williamson, 1988).

B.2 Separation of ALPV and RhPV by sucrose density gradient zone electrophoresis

ALPV and RhPV often occur as mixed infections in aphid clones, but due to the similar biophysical properties the two viruses could not be separated by rate zonal or isopycnic gradient technique. However, due to a different coat protein surface net-charge, the two viruses could be separated by zone electrophoresis (Williamson *et al.*, 1988).

Zone electrophoresis was performed by the method described by Van Regenmortel (1972) using the same apparatus. The U-shaped electrophoresis apparatus was filled with electrophoresis buffer (0.035 M H_3BO_3, 0.0175 M NaOH, 0.0075 M HCl, 0.037 M NaCl, pH 8.6). A linear 10 - 40% (w/v) sucrose gradient was poured, by bottom displacement, onto a 50% (w/v) sucrose cushion, into the left arm of the apparatus. Gradients were poured with the aid of a two-flask gradient maker. The electrode vessels were filled with a saturated NaCl solution to cover two reversible Ag-AgCl electrodes. Approximately 1 mg (0.2 - 0.5 ml) of a mixed virus suspension was adjusted to 45% (w/v) sucrose concentration and phenol red was added as a standard reference marker. The sample was loaded by bottom displacement to lie between the 50% sucrose cushion and the sucrose gradient. Electrophoresis was for approximately 18 hr or overnight at 20 mA at room temperature. Virus bands were located by light-scattering with a fluorescent light source. When compared to phenol red, ALPV had an average relative electrophoretic mobility (Rø) of 0.26 ± 0.02 (three determinations) (Table B.1). RhPV bands were less visible and were located by screening the fractions by DAS-ELISA (Appendix B.3.4). The fractions were immediately diluted in 0.1 M phosphate buffer and concentrated by ultracentrifugation allowing extra centrifugation time to compensate for the

increased viscosity due to the sucrose.

The average Rø value was calculated for ALPV and RhPV purified from aphids collected from different aphid clones (Table B.1). These virus preparations were further checked by DAS-ELISA for purity and were shown to be either ALPV or RhPV. SDS-PAGE also indicated that the viruses had been successfully separated (Fig. B.5). Zone electrophoresis purified virus was used as positive controls in DAS-ELISA assays (Appendix B.3.4) (Chapter 2, 4 and 5).

Table B.1. $R_{ø}$ values from purified ALPV and RhPV obtained from different aphid clones as indicated (mean ± SE). Number of determinations are given in brackets.

CLONES	ALPV	RhPV
S. avenae-Rse(5)	0.3 ± 0.02	0.6 ± 0.02
P. *padi*-Stell(3)	0.23 ± 0.04	0.48 ± 0.09
R. padi-Beth(3)	0.26 ± 0.04	0.46 ± 0.02
*R. padi**	0.238 ± 0.03	0.495 ± 0.05

* Williamson (1988)

B.3 Introduction to immunological studies

The ALPV antiserum utilized for the majority of assays was prepared by Dr. C. Williamson against a fraction obtained from sucrose density gradient zone electrophoresis.

The available antiserum was specific for ALPV only and did not react with aphid body proteins when tested in DAS-ELISA and Western blots and in preliminary immunogold labelling studies (Williamson, 1988; A. Hackland, University of Cape Town, B.Sc(Hons) project, unpublished results). Anti-ALPV serum from a single bleeding collected seven weeks after immunization was used throughout this project (Williamson, 1988).

Antisera to RhPV were prepared by Von Wechmar (unpublished) and Rybicki (1984). The virus was purified from barley plants infested with an aphid clone infected with RhPV only. The particular antiserum used was a pooled product of IgG prepared from several bleedings. The antiserum was checked and found to be RhPV-specific by indirect ELISA and Western blotting (Williamson, 1988). To be able to detect ALPV and RhPV *in situ* with immunofluorescent technique and

immunogold labelling, antisera had to be very specific and pure to avoid nonspecific reactions with aphid host proteins. The products available were found to be suitable for the purpose.

B.3.1 Antiserum production

The antisera mentioned above were raised in rabbits according to the method described by Van Regenmortel (1982). Rabbits were immunized with an intramuscular injection consisting of 0.5 ml antigen emulsion (antigen + normal saline + Freund's incomplete adjuvant). The rabbit was then boosted at weekly intervals with 0.5 ml intramuscular injections for three weeks. After this, the rabbits were boosted again three weeks later.

Rabbits were bled from a marginal ear vein at weekly intervals, starting two weeks after the first injection of antigen. Ten to 20 ml of blood was collected in sterile containers at each bleeding. The blood was allowed to clot at room temperature and centrifuged at 10 000rpm for 10 min to separate the serum fraction from the remaining blood cells. Serum was stored at 4°C for immediate use or at -20°C.

A new anti-ALPV serum was prepared from zone electrophoresis purified ALPV isolated from field grown aphid infested oat plants (*Avena sativa*) (Appendix B.1.2).

B.3.2 Purification of gamma-globulin (IgG)

The procedure of Clark and Bar-Joseph (1984) was used for the purification of gamma-globulin serum fractions.

Antiserum was diluted with distilled water and mixed dropwise with an equal volume of saturated ammonium sulphate (Appendix A.3). The mixture was left at room temperature for 10 min. After low speed centrifugation the globulin fraction was resuspended in half strength PBS (1/2 PBS) (Appendix A.4). This procedure was repeated. The original starting volume was maintained. This suspension was then dialyzed overnight at 4°C against 2 liters of 1/2 PBS to remove the remaining ammonium sulphate. The gamma-globulin fraction was further purified by ion exchange chromatography by filtration through a diethylaminoethyl (DEAE) cellulose column (Whatman DE 52 anion-exchanger). The cellulose was equilibrated and the IgG eluted with 1/2 PBS. One milliliter fractions were collected and the eluent was scanned by UV absorption at 280 nm. All fractions

with an A_{280} nm > 1,4 were pooled, and the IgG fraction adjusted to a concentration of 1 mg/ml with 1/2 PBS (A_{280} = 1.4; Clark & Bar-Joseph, 1984). All spectrophotometer readings were taken on a twin-beam Beckman Model 25 (UV/visible) machine. One millilitre aliquots were frozen at -20°C.

B.3.3 Conjugation of alkaline phosphatase to IgG fraction

Alkaline phosphatase was conjugated to IgG using gluteraldehyde (EM grade) as described by Clark and Bar-Joseph (1984). Alkaline phosphatase from calf intestine (Seravac) was used with a specific activity at 1 000 units per mg protein.

Enzyme was dissolved directly in purified IgG at a concentration of 2 mg/ml. This suspension was dialyzed against 1 liter 1/2 PBS overnight at 4°C. Gluteraldehyde (25% gluteraldehyde stock, Merck) was added to give a final concentration of 0.05%. The mixture was left at room temperature and occasionally agitated for 4 hours, followed by dialysis overnight at 4°C in 1 liter of 1/2 PBS to remove gluteraldehyde.
Bovine serum albumin (BSA) (fraction V) (Seravac) was added to a concentration of 0.2% (w/v) as a stabilizer to the conjugated IgG and stored at 4°C in vials.

B.3.4 Enzyme linked immunosorbent assay (ELISA)

The double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and indirect ELISA were performed as described by Clark and Bar-Joseph (1984) and Rybicki & Von Wechmar (1981), with PBS-based pH 7.0 buffers throughout. DAS-ELISA was utilized to characterize, diagnose and quantify products. Indirect ELISA was used to estimate antiserum titre and to screen antisera for contaminating virus antibodies.

B.3.4.1 ELISA buffers

Buffers and solutions utilized in ELISAs are presented in Appendix A.1.

B.3.4.2 ELISA procedure

Nunc "micro ELISA" flat bottomed trays were used. Appropriate dilutions of IgG were made in PBS. Dilutions of coating antibodies, 200 µl per well, were incubated for 1 - 2 hr at 37°C in a humid box or overnight at 4°C.

Trays were washed (3 X 5 min) by flooding with PBS-Tween (PBS-T Appendix

A.6), and drained upside down on paper towelling. The wells were blocked by flooding the tray with PBS-T-MP (Appendix A.7) and incubated for 30 min at 37°C or overnight at 4°C.

Where antigen consisted of purified or semi-purified virus preparations it was diluted serially 2-, 5- or 10- fold. Antigen (200 µl/well) was incubated for 90 min at 37°C or overnight at 4°C. Wells were washed as before.

Preparation of single aphids for DAS-ELISA was as follows: single aphids were placed in wells of a roundbottom multiwell perspex plate (wells 1.5 cm wide and 1 cm deep) containing 200 to 400 µl PBS-T-MP. Aphids were crushed with the bottom end of empty Wasserman tubes (1 tube/well).

The conjugate (1 mg/ml) was diluted in PBS-T-MP. Conjugate was aliquoted as 200 µl/well and incubated for 90 min 37°C. Wells were washed as before.

Substrate (Appendix A.9) was freshly prepared for each assay, by dissolving p-nitrophenyl phosphate hexahydrate (1 mg/ml) in 10% (v/v) diethanolamine (pH 9.8). Substrate was added (300 µl/well) and incubated at room temperature for 30 to 60 min. Plates were then read in a Titertek Multiscan Spectrophotometer at A_{405} nm. The yellow colour of p-nitrophenol phosphate is an indication of a positive reaction.

Positive controls used in the detection of ALPV or RhPV included purified virus, aphids from positive clones tested by DAS-ELISA for the presence of ALPV and RhPV and clean aphid clones. Uninfected *Myzus persicae* aphids were used as negative controls. The optimal proportions of the antisera used in the assays were determined for each antiserum. This involved grid titrations of purified ALPV or RhPV (serial five fold dilutions) against coating concentrations (1 ml/mg) of 1/100, 1/300 and 1/600 and conjugate dilutions (l ml/mg) of 1/100, 1/300 and 1/600. The combination of coating IgG concentration and conjugate dilution with the widest range of A_{405} nm readings for ALPV and RhPV, and low background A_{405} nm reading for controls, were taken as the optimal proportions. For ALPV a coating concentration of 1/400 and a conjugate concentration 1/500 was found suitable for the antiserum used. The results of the calibration determination is given in Table B.2.

Viral preparations were screened with known ALPV and RhPV antiserum (Williamson, 1988) to positively identify the viruses used in this study. Fig. B.2 illustrates the reactions with anti-ALPV and anti-RhPV serum of virus

Table B.2. Absorbance readings (A_{405} nm) of single aphids tested by DAS-ELISA for the presence of ALPV and RhPV in a *R. padi* aphid clone.

	NUMBERS TESTED													
	ALPV	RhPV		ALPV	RhPV		ALPV	RhPV		ALPV	RhPV		ALPV	RhPV
1	0.5(+)	0.2(-)	11	0.4(-)	0.2(-)	21	0.7(+)	0.1(-)	31	0.6(+)	0.1(-)	41	0.9(+)	2.1(+)
2	1.7(+)	0.1(-)	12	0.5(+)	0.1(-)	22	>3.0(+)	2.0(+)	32	0.6(+)	0.1(-)	42	0.6(+)	0.1(+)
3	0.6(+)	1.4(+)	13	0.8(+)	2.7(+)	23	0.6(+)	0.1(-)	33	0.8(+)	0.1(-)	43	1.0(+)	1.4(+)
4	>3.0(+)	1.2(+)	14	1.1(+)	2.0(+)	24	1.0(+)	1.7(+)	34	0.8(+)	1.4(+)	44	0.8(+)	0.1(-)
5	0.9(+)	1.2(+)	15	1.0(+)	1.2(+)	25	0.7(+)	0.8(+)	35	0.6(+)	0.6(+)	45	1.1(+)	0.8(+)
6	1.2(+)	1.4(+)	16	1.2(+)	1.3(+)	26	0.4(-)	0.1(-)	36	0.8(+)	0.1(-)	46	>3.0(+)	0.9(+)
7	1.5(+)	0.2(-)	17	0.6(+)	0.3(-)	27	0.5(+)	0.1(-)	37	1.1(+)	0.1(-)	47	1.2(+)	0.1(-)
8	>3.0(+)	0.1(-)	18	1.0(+)	2.5(+)	28	0.8(+)	0.2(-)	38	1.8(+)	0.1(-)	48	>3.0(+)	1.8(+)
9	0.4(-)*	0.1(-)	19	0.7(+)	1.5(+)	29	1.1(+)	1.4(+)	39	1.3(+)	0.1(-)	49	1.0(+)	1.2(+)
10	0.4(-)	0.1(-)	20	0.7(+)	0.1(-)	30	1.5(+)	2.7(+)	40	0.8(+)	0.1(-)	50	0.7(+)	0.1(-)

* Absorbance readings $A_{405} \leq 0.4$ were considered to be negative. Sensitivity of detection by DAS-ELISA was approximately 23 ng/200 µl (Appendix B.3.4.2.).

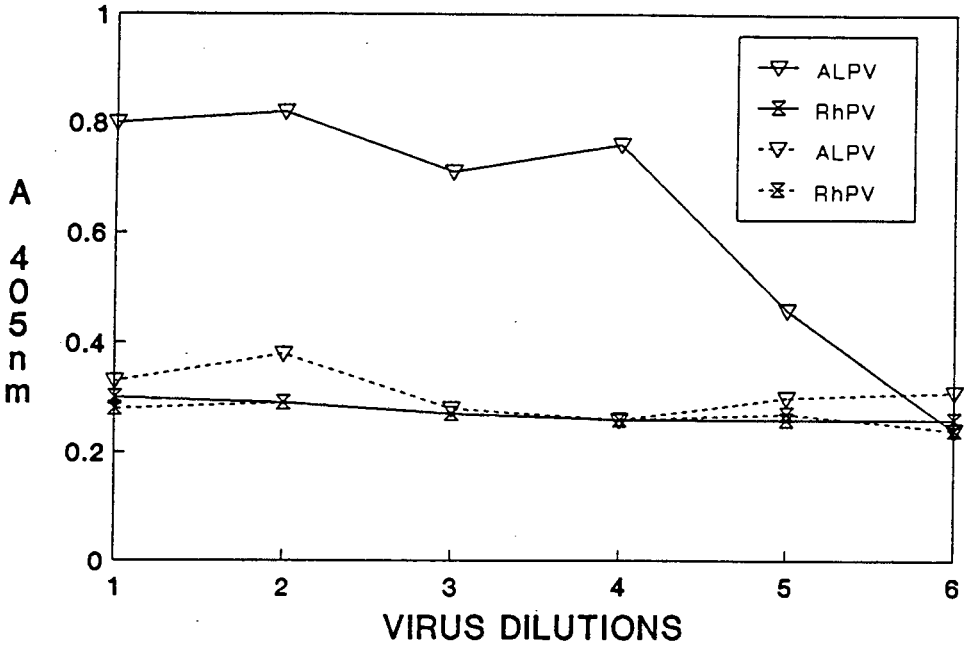


Fig. B.2. A typical DAS-ELISA result with purified virus preparations prepared from virus-infected *S. avenae*-Rse (——), and *R. padi*-Stell aphids (·····) utilizing anti-ALPV and anti-RhPV serum.

preparations from *S. avenae*-Rse aphids (Appendix D.1) taken from a clone maintained at 20°C and aphids taken from a *R. padi*-Stell clone maintained at 15°C (Appendix D.1). The *S. avenae*-Rse preparation was strongly positive for ALPV, but reacted negative with anti-RhPV serum indicating that ALPV was the dominant virus infection present in the *S. avenae*-Rse clone. This indicated that RhPV was not present or that the concentration was too low (inapparent infection) to detect with DAS-ELISA. ALPV and RhPV preparations separated by zone electrophoresis (Appendix B.2) were sufficiently pure to react only with their homologous antisera when screened by DAS-ELISA. Negative readings were obtained for both anti-ALPV and anti-RhPV serum for the *R. padi*-Stell preparation (Fig. B.2).

A typical dilution series of purified ALPV starting with 1 mg/ml virus is illustrated in Fig. B.3. The sensitivity of detection for RhPV and ALPV was approximately 23 ng/well.

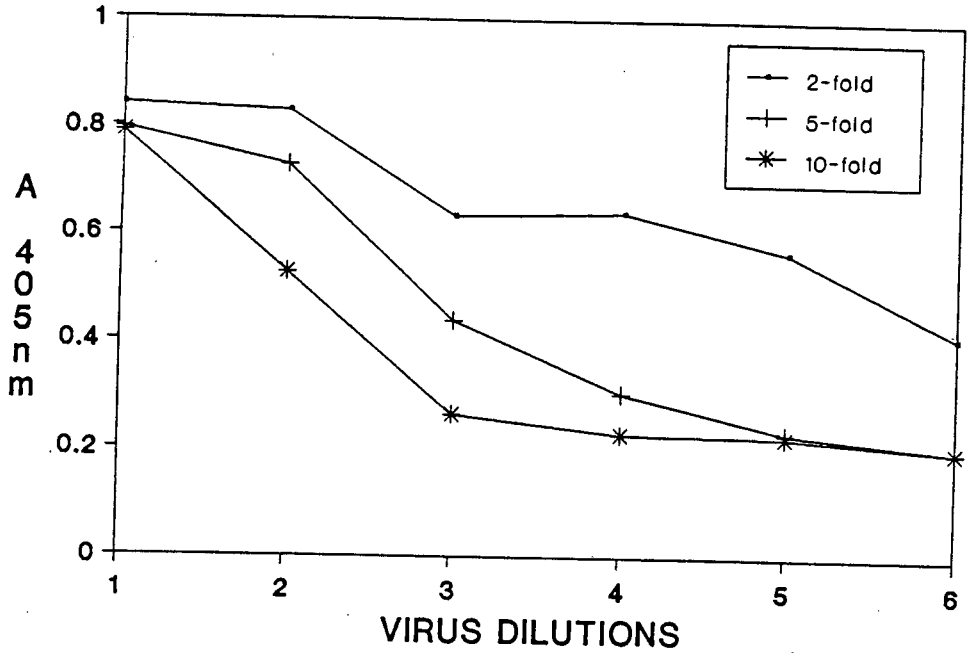


Fig. B.3. Dilution series of zone electrophoresis purified ALPV (1mg/ml).

B.3.5 Immuno Specific Electron Microscopy (ISEM)

ISEM was used to detect virus particles in single aphids and virus preparations purified from plants (Appendix B.1.1 and 1.3). ISEM was performed essentially as described by Milne and Lesemann (1984).

Purified virus preparations and virus taken from dead aphids were trapped and/or decorated with anti-ALPV-serum, negatively stained with 2% uranyl acetate pH 4.1 and examined in a Phillips 201 C electron microscope.

This work was done with the assistance of G. Kasdorf, Plant Protection Researsch Institute (PPRI), Department of Agricultural Development, Pretoria, previously PPRI Stellenbosch. Antiserum for trapping and decoration were used at a dilution of 1/1 000 and 1/10 respectively. Virus was diluted in saline/PBS pH 7.0 and stained.

ALPV and RhPV purified from *R. padi*-Beth and *S. avenae*-Rse showed that the virus particles were isometric with an approximate size of 25 nm. Similar results were obtained with virus from dead and paralysed *R. padi*-Beth and *S. avenae*- Rse aphids when analysed by ISEM (Fig. B.4). The decoration of anti-ALPV serum was

uniform and very specific.

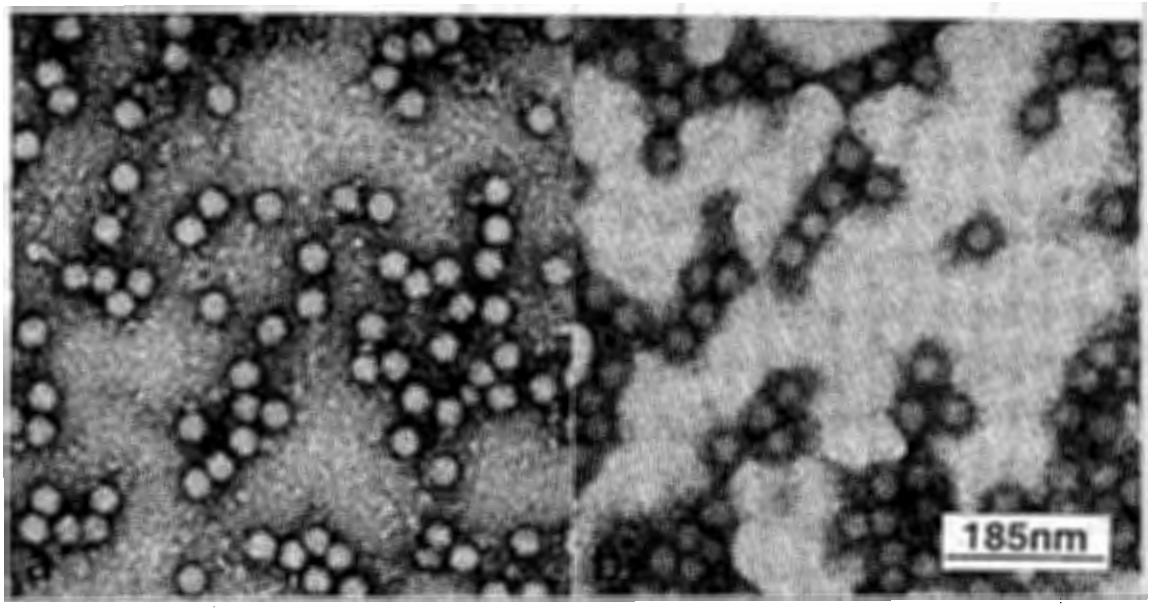


Fig. B.4. Immuno-electron micrograph of virions from one dead *R. padi* aphid (a) virions trapped with 1/1 000 dilution of anti-ALPV serum and (b) trapped and decorated with 1/10 dilution of anti-ALPV serum.

B.3.6 Conclusion

The results presented in this section provides evidence that the aphid clones used in this study were infected with the same viruses as described by Williamson (1988).

The antisera utilized in DAS-ELISA specifically bound to either ALPV or RhPV. Based on the evidence presented, the available antisera were considered virus specific and suitable for detecting ALPV and RhPV *in situ* in dissected aphids by the indirect immunofluorescent technique (Chapter 2). and by immunogold labelling on ultrathin sections of embedded aphids (Chapter 3).

Purification of ALPV from naturally *R. padi* infested diseased appearing plants confirmed the occurrence of ALPV in these aphids.

B.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were analysed by SDS-PAGE on 12.5% or 15% vertical polyacrylamide gels according to the method of Laemmli (1970). SDS-PAGE was used to determine virus protein molecular weights and the purity of virus isolates.

Stock solutions

Resolving gel buffer

1M Tris - HCl (pH 8.8); Dissolve 60.6 g Tris in ddH_2O and adjust to pH 8.8 with concentrate HCl.
Make up a final volume of 500 ml with ddH_2O.

Stacking gel buffer

1M Tris HCl (pH 6.8). Dissolve 60.0 g Tris in ddH_2O and adjust to pH 8.8 with concentrated HCl.

Acrylamide stock solution (40%)

200 g acrylamide monomer
5.3 g N,N'-methylene bisacrylamide
Made up to a final volume of 500 ml with ddH_2O.
The solution was stored at 4^oC in the dark.

Electrophoresis buffer (10X)

0.25 M Tris (30.3 g Tris)
1.92 M glycine (14.4 g glycine)
1% (w/v) SDS (pH 8.3) (10 g SDS)
Made up to a final volume of 1 litre with ddH_2O.

Disruption mixture

10% (w/v) SDS (10 g SDS)
10% (w/v) 2-mercaptoethanol (10 ml 2 - mercaptoethanol)
15% (w/v) glycerol (15 ml glycerol)
0.005% (w/v) bromophenol blue
in 0.125 M Tris - HCl (pH 6.8).

10% sodium dodecyl sulphate (SDS)

10 g SDS in a final volume of 100 ml ddH_2O.

2% ammonium peroxydisulphate (persulphate)

Dissolve 0.15 g in 10 ml ddH_2O: MAKE UP FRESH!

80% glycerol

80 ml glycerol in a final volume of 100 ml ddH_2O.

TEMED (N', N' - tetraethylmethylethylenediamine)

Keep bottle sealed and store at 4°C!

Gel solutions

Resolving gels:

	Volume required(ml)	
	12.5%	*15%*
40% acrylamide stock	25.0	30.0
ddH_2O	20.2	15.2
1 M Tris - HCL (pH 8.8)	30.0	30.0
1.5% (w/v) ammonium peroxydisulphate	4.0	4.0
10% (w/v) SDS	0.8	0.8

TEMED (Appendix B 6.1) (60 µl) was added immediately before pouring and the gel was overlayed with isopropanol.
STIR QUICKLY AND POUR!.

Stacking gels *(5%)*

	Volume required(ml)
40% acrylamide stock	3.8
ddH_2O	18.8
1 M Tris - HCl (pH 6.8)	3.8
1.5% (w/v) ammonium peroxydisulphate	1.4
80% (v/v) glycerol	2.0
10% (w/v) SDS	0.3

TEMED (80 µl) was added immediately before pouring.

Gel preparation

Electrophoresis was performed in Hoefer Mighty Small SE 200 apparatuses (Hoefer Scientific Instruments, San Francisco). A resolving gel solution was poured rapidly between the sealed glass and metal plates, separated by plastic spacers. The gel solution was poured to give a gel length of approximately 50 mm. The gel was overlaid with isopropanol to ensure a flat surface and to exclude air. After ±10 min polymerization, the isopropanol was poured off.

A 5% stacking gel solution was now poured on top of the polymerized gel, and a 10-slot comb inserted into the stacking gel. The sample wells were rinsed with electrophoresis buffer, and filled with this buffer. The apparatus can be kept overnight at 4°C with the comb left in place, or used immediately.

Electrophoresis

The samples to be electrophoresed were disrupted for 5 min at 95°C in a 1:1 mixture with disruption buffer. Pharmacia low molecular weight markers were used (Pharmacia Fine Chemicals, Sweden). The disrupted samples were loaded into the wells with a Hamilton syringe. Electrophoresis was performed at a constant current of 25mA (4 - 5 mA/cm) until the dye front had just run off the bottom of the gel approximately 3 hr. Proteins were detected by staining of gels.

Staining and destaining of gels

Staining solution

Dissolve 0.2% (w/v) PAGE Blue 83 (BDH, UK) in 45% (v/v) methanol : 10% (v/v) acetic acid in ddH_2O. PAGE blue 83 was dissolved in methanol before the addition of acetic acid and water. The solution was filtered before use.

Destaining solution

Make up a solution containing 25% (v/v) : 65% (v/v) : 10% (v/v) - methanol : water : acetic acid. Gels were destained in several changes of destain solution. Gels were stained for 2 - 16 hrs with gentle shaking and were phototgraphed immediately and stored in sealed plastic bags.

On PAGE all ALPV preparations analysed gave the typical protein profiles recorded by Williamson (1988) i.e. 34 000, 32 000 and 31 000 daltons for ALPV and

31 000, 30 000 and 28 000 daltons for RhPV. The typical profile of ALPV and RhPV proteins separated on SDS-PAGE is illustrated in Fig. B.5.

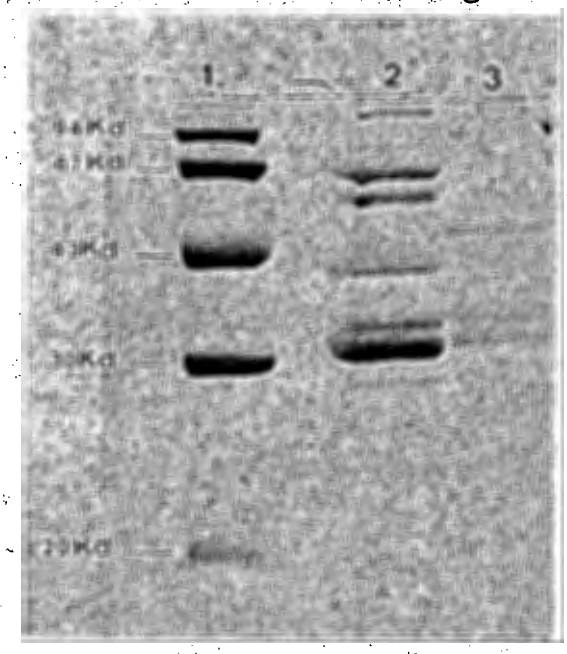


Fig. B.5. Protein profile of ALPV and RhPV proteins analysed by 12.5% SDS-PAGE. Lane 1 molecular weight markers; lane 2 ALPV and RhPV mixed sample and lane 3 zone electrophoresis purified ALPV.

APPENDIX C

EMBEDDING

EMBEDDING

C.1 Embedding of material for transmission electron microscopy (TEM)

The procedure for embedding of aphid bodies for transmission electron microscopy (TEM) was adapted from Kimmins (1987).

C.1.2 Embedding with ethanol and acetone

1. Dissect in or inject 2% glutaraldehyde in 0.02 M sodium cacodylate (NaCaC) (pH 7.2) into insect (use Tween 20 to reduce surface tension around body)
2. Fixing time 6-12 hr (2 hr - change 1X)
3. Rinse in 0.135 M NaCaC - 3 X 10 min. (keep specimen always wet - remove excess fluid with Pasteur pipette)
4. Fix in 1% OsO_4 in 0.1 M NaCaC for two hrs at $4^{o}C$. Cover with a box or cap tubes - change 1X - can leave overnight
5. Rinse with 0.135 M NaCaC - 2 X 6 min.
6. Rinse with ddH_2O - 3 X 6 min.
7. Leave in 2% uranyl acetate in ddH_2O for 2 hr
8. Rinse 2 X 6 min. with ddH_2O
9. Dehydrate with 30, 50, 70, 90, 96 and 100% (each 2X) EtOH for 20 min. each
10. Dehydrate in 100% EtOH : 100% acetone, 1 : 1
11. Dehydrate in 100% acetone 2 X 1hr
12. Infiltrate with Spurr : acetone, 1 : 1 - 2 hr or overnight
13. Infiltrate with Spurr 2 X 2 hr
14. Polymerise at 60 - 70 ^{o}C for 8 - 12 hr

Specimens were trimmed and cut with a Reichert-Jung ultramicrotome. Sections were lowered onto the grids by submerging the grid in the water in the boat attached to the glass knife by means of specially designed forceps.

C.2 Coating of grids with Formvar

For coating of grids, a 0.5% Formvar film was chosen. To obtain this 20 µl Formvar (v/v) was dissolved in 1.2- dichloroethane. Time was allowed for it to dissolve.

To layer the Formvar onto the grid, 20 μl of it was floated onto water held in a funnel with a tap. A sieve is fitted inside the funnel to support the grids. By removing H_2O from the tap the Formvar can be layered gently onto the grid.

All other grid and specimen preparations for ultrathin tissue sections are given in Chapter 3.

APPENDIX D

APHID PROPAGATION, MAINTENANCE, HANDLING AND SAMPLING

APHID PROPAGATION, MAINTEMANCE, HANDLING AND SAMPLING

All experiments were conducted in a temperature controlled environment. Ambient temperature was constant at 22°C. Aphid maintenance has been described by Von Wechmar (1990c). Conditions described below were for all aphid species used.

D.1 Aphid clones

Aphid clones were reared from single aphids. Their origins and year of collection are listed in Table D.1.

Table D.1. The origin of aphid clones.

CLONE	ORIGIN AND DATE COLLECTED
Rhopalosiphum padi (Beth)	Bethlehem, Orange Free State, 1988
R. padi (Leip)	Leipoldtville, Cape Province, 1987
R. padi (Stell)	Stellenbosch, Cape Province, 1986
Diuraphis noxia (Brits)	Brits, Transvaal, 1986
D. noxia (Beth)	Bethlehem, Orange Free State, 1988
D. noxia (Vent)	Ventersdorp, Transvaal, 1988
Sitobion avenae (Rse)	Riviersonderend,Cape Province, 1982
Myzus persicae	Cape Town, Cape Province, 1986
Brevicoryne brassicae	Malmesbury, Cape Province, 1988

D.2 Aphid maintenance

D.2.1 Maintenance conditions

Cages with aphids for virus propagation were kept at 24 - 26°C. Cages with new plants were prepared on a weekly basis. To safeguard aphid clones, stock colonies were kept in incubators at 7 - 10°C. Under these conditions aphids were transferred every six to eight weeks.

Aphids for virus preservation were kept at 4°C in canning jars (Appendix D.2.4) or cages. Under these conditions aphids were transferred every 12 and eight weeks respectively.

D.2.2 Surface sterilization of seeds

One homogeneous batch of Clipper barley seed was used throughout. The seed was free of seedborn plant virus disease (Von Wechmar, unpublished). To ensure that no infectious ALPV and RhPV was present on the seedsurface and to minimise fungal infections, seeds were surface sterilized by submersion in 2% formaldehyde for 1 min and rinsed for 20 min under running water followed by rapid drying. Seeds were then stored at 4°C. Surface sterilized seeds were pre-germinated in vermiculite before use.

D.2.3 Barley seedlings grown in soil

Seedlings were grown in heat-sterilized soil in growth rooms at controlled conditions of approximately 70% humidity, and a cycle of 14L:10D VHO Grolux fluorescent lighting with temperatures of 24°C and 21°C respectively.

D.2.4 Aphid containers

Cages

Wooden cages (45 X 45 X 50 cm) covered with gauze were used to accommodate a tray carrying nine pots of barley seedlings. One side of the cage consisted of a loose flap fitted for easy servicing of the cage. The cages were placed on two tier custom built trolleys (eight cages/trolley) fitted with eight feet long VHO Grolux fluorescent lighting and kept at 24 - 26°C on a 12L:12D cycle.

Incubators

Incubators with automatic temperature control, fitted with four shelves, each illuminated by 2 ft VHO Grolux fluorescent light tubes, were used. Air was circulated by an internal fan. Depending on requirements, 12 to 36 plantpots could be accommodated in each unit. Different temperature regimes could be selected. For routine maintenance this varied from 10 to 7°C (12L:12D). Maintenance at low temperature had the advantage that aphid reproduction was slowed down and barley plants lasted longer. In certain experiments the incubators were used with different temperature regimes. This will be specified in the relevant section. With

eight incubators available for aphid holding and/or experimental set-ups, it was possible to maintain several aphid species and clones.

Canning jars

Large canning jars (23 X 11 cm) were fitted with a double gauze lid. Germinated surface sterilized seeds (±20/jar) (Appendix D.2.2) were placed on 3 cm of sterile moist vermiculite in jars. The vermiculite was kept moist with Knopp's nutrient solution (Appendix A.5). Jars with pregerminated seeds were left on a sunny windowsill until coleoptiles were green before using them, or to keep a supply at 4°C under VHO Grolux fluorescent light (12L:12D).

Plastic vials

Plastic specimen vials (3.5 X 5.5 mm) were utilized to isolate different clones of field collected aphids. A hole was drilled in the plastic screw cap and this fitted with a layer of double gauze. Germinated surface sterilized seeds, about 5, were placed in moist vermiculite (1 cm deep) in vials. The vermiculite was kept moist with Knopp's nutrient solution (Riker & Riker, 1936) (Appendix A.6). The plastic vials were kept at 4°C under VHO Grolux light on a 12L:12D cycle before use.

Petri dishes

Petri dishes were utilized to maintain single aphids for short periods of time. Fresh Clipper coleoptile leaves were placed on moist filter paper in the Petri dish. Leaves were changed every two days and the filter paper moistened with water daily.

D.2.5 Harvesting of aphid infested plants and aphid transfer

Plants were cut off at the base of their stems and excess aphids were shaken off the plants onto newspaper covered with a dusting of talcum powder to prevent aphids from sticking together and sticking to the paper. A portion of the aphids were transferred to new plants for further propagation and the remainder frozen at -20°C. Harvested plants were stored in sealed plastic bags at 4°C and were processed for virus purification within three weeks of harvesting. Unwanted plant and aphid material was sprayed with Dazzel (containing Diazinon), and left for 12 hr in a nonventilated room before autoclaving and discarding.

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