Binding and toxicity of plant lectins to insects

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BINDING AND TOXICITY OF PLANT LECTINS TO INSECTS

A thesis submitted by Stephen David Woodhouse, B.Sc. MSc in accordance with the requirements of the University of Durham for the degree of Doctor of Philosophy.

Department of Biological Sciences
April 2002

DECLARATION

I declare that no part of this work has been submitted by me for any degree in this or any other university. All of the work presented was conducted by me, except where otherwise stated in the text.

Stephen D. Woodhouse

The road goes ever on and on

Down from the door where it began.

Now far ahead the road has gone,

And I must follow if I can,

Pursuing it with weary feet,

Until it joins some greater way

Where many paths and errands meet.

And whither then? I cannot say.

The Fellowship of the Ring

J.R.R Tolkien.

ABSTRACT

The toxicity of plant lectins to insects after oral ingestion was investigated by incorporating the proteins into insect diets. Bioassays carried out using an artificial diet system demonstrated that kidney bean (*Phaseolus vulgaris*) lectin (PHA) caused a significant decrease in survival of larvae of the tomato moth, *Lacanobia oleracea*. Jackfruit (*Artocarpus integrifolia*) lectin (jacalin) and black mulberry (*Morus nigra*) lectin both caused a significant decrease in growth of the peach potato aphid (*Myzus persicae*) when compared to controls in an artificial diet based bioassay.

Interactions of lectins with insect gut tissues *in vivo* were studied by immunolocalisation. Binding of the snowdrop lectin (*Galanthus nivalis* agglutinin;GNA) and jack bean (*Canavalia ensiformis*) lectin (Concanavalin A; Con-A) to the digestive tract of *L. oleracea* larvae was observed and localised at the electron microscope level after oral ingestion of the proteins. GNA was also observed to bind to the midgut of the two-spot ladybird *Adalia bipunctata*. No disruption of the brush border membrane of either *L. oleracea* or *A. bipunctata* was observed. Binding of GNA to the peritrophic membrane of *L. oleracea* was observed by fluorescence microscopy.

Histological evidence of lectin binding to insect guts *in vivo* was corroborated by *in vitro* studies, which showed that the lectins GNA and Con-A bind to sections of the digestive tract of *L. oleracea* larvae. Binding of Con-A to proteins from brush border membranes, solubilised brush border membranes and peritrophic membranes was also observed.

The use of confocal microscopy showed that GNA bound to the midgut and haemocytes of the peach potato aphid *Myzus persicae*, both when incubated with isolated tissues and cells and when fed orally to live insects, providing evidence for transport of GNA across the gut wall.

Larvae of *L.oleracea* fed the lectins GNA and PHA showed a significant increase in polyphenoloxidase levels within the haemolymph, suggesting that the lectins were causing systemic responses in the insects.

A partial sequence for leucine aminopeptidase a potential receptor for lectin binding was obtained from a cDNA library constructed from the midgut of the tomato moth larvae.

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ACKNOWLEDGEMENTS

I would like to acknowledge the financial support provided by the Scottish Office and the Rowett Research Institute in Aberdeen.

I gratefully acknowledge Dr Elaine Fitches for her patience and time in proof reading a great deal of this thesis.
Dr Beulah Leitch and Christine Richardson for their help and advice with the microscopy,

I would like to thank everybody in Lab 1 (past and present) for their help and advice during my thesis, Elaine, Rachel, Dave, Loc, Du, Martin, Xavier, Hillary, Tony, Louise, James, Jenny, Angharad, Gillian and Colin (who will not be forgotten). In particular Romaan for useful discussions over a pint or two.

I should also like to thank my supervisor John Gatehouse for his help, advice and support over the project.

Thanks also to the Fonteyn Court crew past and present, particularly Peter, Keith, Simon, Stuart and Prash.

Finally I should like to thank my Mum, Dad, Sister, and partner Sally for their patience and support.

Chapter One

Introduction

1.1 General introduction

Despite improvements in agricultural production during the latter half of the twentieth century, shortages of food remain a problem in many parts of the modern world. Although the causes of these shortages are complex, and include political and ethical considerations outside the realm of science, nevertheless science can still contribute to alleviating the problem. In many areas of the world farming practices achieve suboptimal yields, due to pests, weeds and diseases, or poor soil or growing conditions (Hobbs *et al.*, 1998). Even where these constraints do not apply, or have been largely overcome, production must increase to keep pace with population growth. For example, it has been pointed out that over the next 30 years global rice production must be raised by another 300million tons. Although at global level there is adequate unused potential farmland, in South Asia 94% of suitable land was being farmed in 1997-99 (http://www.fao.org/docrep 05/11/02). Therefore, as there is little scope to increase rice production in this area the demands needs to be met by increased yields from existing riceland. This requirement could be particularly significant in view of the fact that rice is the staple dietary constituent of at least half the worlds population (Tu *et al.*, 2000). In other areas of the world such as Latin America where there is adequate unused agricultural land, only a fraction is realistically available for agricultural expansion, as much is required to retain forest cover

and to support infrastructural development (http://www.fao.org/docrep 05/11/02). The increases in crop yields which have already been made through the scientific process of crop improvement have made a major contribution to global food supplies, and plant breeding has the potential to continue increasing yields in future.

Increased yields from existing agricultural land remain the highest priority for crop improvement, although a short term solution to suboptimal yields would be to increase the land available for cultivation. However, this could result in increased deforestation. The effects of deforestation can lead to erosion of topsoil, therefore increasing desertification, giving rise to famine, or conversely to floods (Secretariat for Convention to Combat Desertification 2000). In addition, gross deforestation is known to contribute to global warming, resulting in effects such as abnormal weather patterns (Harrison 2001) and changes in sea level (Wigley & Raper 2001). Through all these factors agriculture is adversely affected, with decreased crop yields being caused in a variety of ways, such as the poleward progression of insects and plants and associated diseases (Planton 1999).

New crop varieties which can give higher yields for lower inputs of fertilisers, pesticides and other exogenously applied materials are thus viewed by most relevant agencies as a central strategy in efforts to address the problem of world hunger. Previous work with conventional plant breeding methods has been successful in producing crops with many of the agronomically desirable qualities of high yield, disease resistance, etc., but has been necessarily limited in what

can be achieved by the available gene pool in the crop species. There is a need to be able to produce crop varieties with phenotypical characteristics not present in any naturally occurring variety, and for this purpose, plant genetic modification (GM) technology has been seen as a timely solution. GM technology, in theory, gives the scientist precise control of the genome of a crop species, and allows any phenotype to be transferred from one species to another.

While GM technology has the potential to improve all aspects of crop production, much of this promise is as yet unfulfilled, due to a lack of fundamental knowledge of the mechanisms by which many desirable phenotypes (such as high yield) are determined by the genes of an organism. When such knowledge is available, production of new crop varieties has already been undertaken. One area in which progress seems likely is improvement of the nutritional quality of crops. Currently 1 million children die every year as a result of vitamin A deficiency and many others suffer problems such as increased risk of infection. A further 2 billion people suffer from iron deficiency (mainly in underdeveloped nations). The development of a strain of rice with increased Vitamin A and iron content (known as "Golden Rice") could be a great step forward in easing these nutritional problems (Anon. 1999). Although previous GM crops have been highly priced, "Golden rice" is being offered at an affordable price (Wrong & Tait 2000), to allow its adoption by poor farmers. Although yield increases have been sought by increasing the production of harvestable plant matter, yields could also be increased by modification of crops to confer traits such as the ability to grow in

hostile environments, or to increase protection against pests and diseases (Gatehouse *et al.*, 1995). This thesis describes work directed towards the goal of protecting crops from yield losses due to insect pests through the use of a GM strategy. Such a strategy has advantages and drawbacks, as will be discussed, but must be evaluated in comparison with existing strategies of crop protection.

Currently, the main method for crop protection involves the use of chemical pesticides, since they significantly increase crop yields and thus provide many benefits to farmers and to consumers (Anon 2000). Oerke *et al.* (1994) estimate that yields of many crops could decrease by as much as 50% if they were not adequately protected from insect damage. Pesticide usage also provides direct economic benefits to consumers; for example in the USA, Zilberman *et al.*, (1991) estimated that for every $1.00 increase in pesticide expenditure, gross agricultural production increases by $3-6.50, the benefit to consumers being lower food prices. Although currently effective, there are a number of drawbacks to pesticide usage. A number of crop pests have developed resistance to pesticides as a direct consequence of their indiscriminate use. A classic example of this is the rice brown planthopper, *Nilarparvata lugens,* a major rice pest; the species has accrued resistance to a number of pesticides such as organophosphates, and carbamates (Hemingway *et al.*, 1999). In these instances resistance was shown to be due to increased levels of carboxylesterases within the insect (Karunaratne *et al.*, 1999). The same problem has occurred in other pest species with both similar and other types of

pesticides. In addition, pesticides can also be toxic to beneficial insects, e.g. some acaricides cause toxicity to the predatory bug *Dicyphus tamanini* (Castane *et al.*, 1996).

Another major problem encountered with pesticides is their inherent toxicity and their deleterious effects on both the environment and mankind. It has been shown that pesticides exert toxic effects on aquatic microorganisms via run off and drift; these compounds are then metabolised or bioaccumulated by the microorganisms (DeLorenzo *et al.*, 2001). Increased morbidity upon exposure to pesticides has been demonstrated with effects such as watery eyes, headaches and muscular pains (Gomes *et al.*, 1998). Furthermore, accidental organophosphate poisoning can lead to death (Singh *et al.*, 2001) and prolonged exposure to organophosphates is linked with variant Creutzfeldt-Jacob disease (Churchill *et al.*, 1999). The organochlorine pesticide DDT (1,1,1-trichloro-2.2-bis (p-chlorophenyl) ethane) has a half life in water of 22 years and even longer in soil (Howard *et al.*, 1991). Although its use is now restricted, it is still assimilated by aquatic species from coastal sediment (Green *et al.*, 1986). DDT is highly fat soluble and since it accumulates in an organism faster than the organism can get rid of it, accumulation of the pesticide is magnified through the food chain (Mellanby 1992). DDT has toxic effects to birds via egg shell thinning (Faber & Hickey 1973), and through immune suppression in mammals and (Vial *et al.*, 1996) (Bannerjee *et al.*, 1995). Some studies have found a correlation between breast cancer and DDT (Krieger *et al.*, 1994) (Wolff *et al.*, 1993). Problems with

pesticides are often exasperated by incorrect or inappropriate pesticide usage. For example, pesticides are often used at the wrong time, at the wrong concentration, or without following the correct safety procedures. It should be noted that these problems occur mainly in the third world, due to poor education (Harrington & Grace 1998), although cases of pesticide poisoning due to misuse or accidents regularly occur in the USA (Gladen *et al.*, 1998).

These drawbacks point to a clear role for plants with endogenous protection against pests being a valuable component of agricultural systems. Plant genetic engineering has provided the means to achieve this aim. The use of transgenic plants in the field of crop protection would be particularly useful if the protection offered could be directed against specific pests, and had no detrimental effect on beneficial or secondary organisms, or on the ultimate commercial viability of the product. Indeed, this has already been achieved with the commercialisation of transgenic crops expressing the entomocidal toxin from *Bacillus thuringiensis* since 1995. Such insect resistant crops show significant levels of resistance to pests such as cotton bollworms but are harmless to many beneficial insects such as ladybirds, which prey on crop pests (Hargrove 1999)

When adopting this strategy it is important to ensure that only those proteins with high insecticidal activity but also with no/low mammalian toxicity are used in transgenic crops. Other candidates for expression in transgenic crops to enhance endogenous resistance include endogenous plant defence proteins such as lectins.

1.2 Lectins

Lectins are proteins which are found widely in living systems. The term lectin is derived from the Latin verb "*legere*" which means to pick out, select or chose, and refers to the remarkable selectivity and specificity with which lectins recognise and bind to carbohydrate structures. These proteins have been defined as "proteins possessing at least one non-catalytic domain, which binds reversibly to a specific mono or oligosaccharide" (van Damme *et al.*, 1998), or as "a group of carbohydrate binding (glyco) proteins of non-immune origin capable of specific recognition of, and reversible binding, to carbohydrates without altering their covalent structure" (Kocourek & Horejsi 1983). Recognition of glyconjugates is an important event in biological systems, and is frequently mediated by carbohydrate-protein interactions (Mercedes Iglesias & Wolfenstein-Todel 1996). The ability of lectins to bind specific carbohydrates is a determining factor in their different proposed functions (Pusztai & Bardocz 1995).

1.3 Properties and binding specificities of selected plant lectins

1.3.1 Snowdrop (Galanthus nivalis) *agglutinin (GNA)*

Of the 18 species of Snowdrop in Europe and Western Asia the most common snowdrop in the UK is *Galanthus nivalis*. GNA is found in relatively high concentrations in the bulbs of the snowdrop, and can be isolated using affinity chromatography (Van Damme *et al.*, 1987). It is also present in ovaries and other floral tissues. The lectin is a tetrameric protein with a molecular weight of

approximately 50,000, containing four subunits of molecular weight approximately. 12,000. The protein has been fully sequenced and a three-dimensional structure obtained by X-ray crystallography is available. The lectin is not glycosylated and exhibits binding specificity for mannose (Van Damme *et al.*, 1998). GNA agglutinates rabbit but not human erythrocytes (Van Damme *et al.*, 1987) and is virtually non-mitogenic to human lymphocytes (Kilpatrick *et al.*,1990).

1.3.2 Jack bean (Canavalia ensiformis) lectin (Concanavalin A; Con A).

Concanavalin A is synthesised in the seeds of the Jack bean and was initially isolated in 1936 by conventional protein purification methods. (Sumner & Howel 1936). The structure of the molecule is pH dependent: at neutral pH it is composed of 4 non-glycosylated subunits of 26.5 kDa, but below pH 5.6 the molecules are dimeric. Con A exhibits a binding specificity for Glucose (Glc), Mannose (Man) & N-acetylgalactosamine (GalNAc), with its highest binding specificity being for terminal α-linked mannose residues (Goldstein & Poretez 1986). Con A is a metalloprotein with one Ca^{2+} and one Mn^{2+} ion per subunit and demetalisation leads to Con A losing its reactivity (Agrawal & Goldstein 1967). Con A agglutinates most erythrocytes and is a mitogen causing stimulation of T lymphocytes (Wang & Edelman 1978). Like GNA, Con A is highly resistant to gut proteolysis (Nakata & Kimura 1985).

*1.3.3 Jackfruit (*Artocarpus integrifolia*) lectin (jacalin)*

A lectin, jacalin, can be isolated from the seeds of the jackfruit, and was originally purified on a column of immobilised guar gum or galactosamine. Jacalin exhibits binding specificity for Galactose (Gal) and N-acetylgalactosamine (GalNAc) (Kabir 1994). The lectin agglutinates all human red blood cells as well as erythrocytes from sheep and rabbit. (Kabir 1994)

1.3.4 Black mulberry (Morus nigra) lectin

The lectin from black mulberry has a similar binding specificity to jacalin in that it binds to Galactose (Gal) and N-acetylgalactosamine (GalNAc); however, it also has a slight affinity for mannose. (Angel, C & Pickford, W. Pers. Comm.).

*1.3.5 Kidney bean (*Phaseolus vulgaris*) lectin (phytohaemagglutinin; PHA)*

PHA is perhaps the best documented plant lectin. It is found mainly in the seeds of kidney bean where it is an abundant protein available in gram quantities (Van Damme *et al.*, 1998). It is also present in all vegetative tissues of the plant (Borrebaeck & Mattiasson 1983). The protein is a tetramer of 118kDa composed of two different subunits (Allan & Crumpton 1971), an erythroagglutinating E type subunit, and a mitogenic leucoagglutinating L type subunit (Pusztai & Stewart 1978). PHA has a complex binding specificity recognising virtually all surface carbohydrates (Pusztai & Bardocz 1995). PHA is a stable protein, resistant to proteolysis, that can survive passage through the entire alimentary canal of the rat intact. A proportion of the ingested PHA binds to and exerts a mitogenic effect

on epithelial cells (Pusztai *et al.*, 1990). This in turn stimulates protein synthesis (Palmer *et al.*, 1987) and induces extensive, dose and polyamine-dependent, reversible hyperplastic growth of the gut, (Bardocz *et al.*, 1997, Bardocz *et al.*, 1990), causing some microvillar damage of enterocytes (King *et al.*, 1982). PHA is capable of affecting the immune system of mammals, inducing the synthesis of lymphokines interleukins-2 and IgE (Pusztai 1991) (Nowell 1960).

1.4 Roles of lectins within plants.

Plant lectins have been known to exist for over 100 years, since Stillmark first observed that castor bean extracts agglutinated red blood cells (Stillmark 1888). Subsequently a large number of lectins have been isolated and characterised (Van Damme *et al.*, 1998). However, although lectins appear to be ubiquitous in plants, their roles in plant physiology are not well understood. Lectins are known to be synthesised on the rough endoplasmic reticulum, from where they are translocated. Although it is not entirely clear how the lectin reaches the protein bodies for deposition; it appears that some lectins such as the PHA-L isolectin are first transported through the Golgi stacks, where they are fucosylated prior to deposition in protein bodies (Pusztai 1991). This appears to be true for a number of other lectins, including the seed lectin of *Bauhinia purpurea* (Herman & Shannon 1984).

Genes encoding lectins have been well conserved during evolution (especially in *Leguminosea*), and the encoded proteins form several families of homologous

proteins, This suggests that lectins play an important role in the life of the plant (Pusztai 1991). Lectins are present in many plant tissues, but occur primarily in seeds where they can constitute a large proportion of the total seed protein (Liener 1976). Lectins are thus present in a wide variety of foodstuffs consumed by humans and other species, although there is evidence that these proteins can play a role in defence against plant predators (see below). The accumulation of lectins to high levels in the seeds of certain plant species suggests their role in these cases is that of storage proteins. However, some plants contain lectins at very low levels (Van Damme *et al.*, 1998), and in these cases, the lectins must be playing a different role.

It is apparent that plant lectins can play more than one functional role, depending on the type of lectin in question and the plant species. It has been shown that lectins are involved in bacterial colonisation of legume roots, for the establishment of symbiosis (Sharon & Lis 1989). In roots lectins are an important determinant of host specificity (Diaz *et al.*, 1989) (Bolhool & Schmidt 1974); however, their role may not be in the actual species-specific recognition, which appears to be mediated by tetrasaccharides (Lerouge *et al.*, 1990). The lectin from the Jack bean *Canavalia ensiformis* (Concanavalin A;Con A) has been shown to induce phytoalexin production (Toyoda *et al.*, 1995), and to inhibit auxin induced elongation (Hoson & Masuda., 1995). The role of lectins which has received the most attention, however, is their involvement in plant defence against predators.

1.5 Lectins in plant defence and plant-insect interactions.

Unlike the animal kingdom, plants lack an active immune system to protect them from microbial or viral attack, and as they are sessile are unable to escape predation. Plants employ a variety of mechanisms to protect themselves from attack, including the production of secondary metabolites and defensive proteins. Although not initially recognised as defensive proteins, it has become apparent that lectins can and do play this role in plants, which is mediated by their ability to bind to specific carbohydrates. A role for lectins as defensive proteins in plants against insect predators was first proposed by Janzen *et al.* (1976), who suggested that the common bean (*P. vulgaris*) lectin was responsible for the resistance of these seeds to attack by coleopteran storage pests, although subsequent work showed that in this example the major resistance factor was the seed amylase inhibitor (Huesing *et al.*, 1991). Subsequent studies, involving the screening of purified lectins against insect pests of economically important crop plants in artificial diet bioassays (e.g. Murdoch *et. al.*, 1990) have confirmed that many lectins are insecticidal. Insecticidal lectins which have been identified in this way will be discussed by insect order.

1.5.1 Lectins active against insects of the order *Coleoptera*

Lectins toxic to the bruchid seed weevil *Callosobruchus maculatus*, a major storage pest of cowpeas in many parts of the developing world, have been identified in by several groups. Murdoch and co-workers screened 17

commercially available plant lectins for insecticidal activity against this pest (Murdoch *et. al.*, 1990). Five lectins were found to cause a significant delay in larval development at dietary levels of 0.2% and 1.0% (w/w) and all had carbohydrate binding specificity for N-acetylgalactosamine/galactose (GalNAc) or for N-acetylglucosamine (GlcNAc). The winged bean (*Psophocarpus tetragonolobus*) lectin (GlcNAc-specific) was also shown to be toxic to *C. maculatus* (Gatehouse *et al.*, 1989). The rational for using lectins specific for N-acetylglucosamine is based on the fact that the insect midgut contains chitin, a polymer of N-acetylglucosamine, in the peritrophic membrane (Richards and Richards, 1977). Of all the lectins tested against *C. maculatus* by Murdoch *et al.*, wheat germ agglutinin (WGA) was found to be the most potent. The same workers later identified rice and stinging nettle lectins (UDA) as being toxic to *C. maculatus*, exhibiting similar levels of toxicity to WGA; like WGA they are specific for GlcNAc (Huesing *et. al.*, 1991).

Czapla and Lang (1990) took a similar approach when they screened a range of lectins for activity against the Southern corn rootworm (*Diabrotica undecimpunctata*), a major economic pest of corn. Of the lectins tested three, from castor bean, pokeweed and green marine algae, were found to be toxic to the neonate larvae when applied topically (2%) to the artificial diet. Several others, including WGA, were found to inhibit larval growth by at least 40% when compared with larvae fed on control diet. As reported by Murdoch *et. al.*, (1990) for *C. maculatus*, all those lectins with insecticidal activity against corn root worm were either specific for GalNAc or GlcNAc.

1.5.2 Lectins active against insects of the order *Lepidoptera*

Comparatively few lectins have been tested by bioassay in artificial diet and found to be toxic to members of this economically important insect order. Czapla and Lang (1990) tested a range of lectins for insecticidal activity against the European cornborer, *Ostrinia nubilalis*. The lectins from castor bean (*Ricinus communis*), Camel's foot tree (*Bauhinia purpurea*) and wheatgerm (WGA), specific for GalNAc, GalNAc and GlcNAc respectively, were found to give 100% mortality after 7 days when administered to neonate larvae as a 2% topical application; WGA and the lectin from *R. communis* were also found to inhibit larval weight gain by >50% at 0.1% topical applications. The LC50 values for *R. communis*, WGA and *B. purpurea* lectins against cornborer were 0.29, 0.59 and 0.73 mg/g of diet respectively. Czalpa and Lang also reported that the soya bean lectin actually increased larval weights of *O. nubilalis* by >25% compared with control larvae, in contrast to earlier reports where addition of this particular lectin at the 1% level was found to be detrimental to the larval growth of *Manduca sexta*, the tomato hornworm (Shukle and Murdock, 1983). The snowdrop lectin, GNA, also exerts a detrimental effect on larvae of a lepidopteran species, *Lacanobia oleracea* (tomato moth). Larvae chronically exposed to GNA showed a weight reduction of 50-60% (Fitches *et al.*, 1997).

1.5.3 Lectins active against insects of the order *Homoptera*

The absence of Bt toxins with activity against Homoptera has led to insecticidal activity of lectins against homopteran pests receiving much attention. Powell *et al.* (1993) used an artificial diet bioassay system to test a series of lectins against the rice brown planthopper (*Nilaparvata lugens*), an important pest of rice in S.E. Asia, and although some lectins (e.g those from garden pea or potato) had no effect on insect survival, other lectins decreased insect survival significantly. The two most effective proteins tested were the lectins from snowdrop (GNA; mannose-specific) and wheat germ (WGA; GlcNAc-specific), each of which gave approximatelyimately 80% corrected mortality at a concentration of 0.1% w/v in the diet. The LC50 value for GNA against brown planthopper was found to be 0.02%, or approximately 6mM (Powell *et al.*, 1995). GNA was also found to be toxic to another sucking pest of rice, the rice green leafhopper, *Nephotettix cinciteps*. These findings, taken in conjunction with the absence of toxicity of GNA towards mammals (q.v.), point to a potential use for this lectin in crop protection.

Habibi *et. al.* (1993) carried out similar bio-assays in order to identify lectins which may be suitable in the control of the potato leafhopper (*Empoasca fabae*); the lectins tested were specific for glucose/mannose, GlcNAc or GalNAc. Of those tested, 6 were found to cause a significant reduction in insect survival at dietary levels of 0.2% to 1.5% (w/w). Those found to be effective were from jackfruit, pea, lentil and horse gram and also PHA and WGA. Rabhé and Febvay (1993) demonstrated that the lectin from *Canavalia ensiformis* (Con A)

was a potent toxin of the pea aphid *Acyrthosiphon pisum*, having a significant effect upon both survival and growth; in comparison, WGA was relatively ineffective. Chitin binding lectins from wheat germ (WGA), stinging nettle and Brassica spp. were also reported to cause high levels of mortality to the cabbage aphid *Brevicornye brassicae* when incorporated into artfificial diet (Cole, 1994). Subsequent experiments have shown that the snowdrop lectin, GNA is also inhibitory to aphid development. Artificial diet studies to evaluate the insecticidal properties of GNA demonstrated a 34 % reduction in nymph survival on the peach potato aphid *Myzus persicae,* at a level of 1500ug/ml (Sauvion *et al.*, 1996), and an effect on fecundity. This effect would be significant in preventing the build-up of an insect population.

1.5.4. Lectins in plant defence against pathogens

Lectins have also been implicated in resistance to pathogens. To be successful against bacteria and fungi the lectin will have to prevent their invasion at an early stage of infection before they can penetrate more deeply into the plant. This is usually achieved by lectin binding to the bacterial or fungal cell wall (Pusztai & Bardocz 1996). Activity of this type is shown, for example, by the stinging nettle (*Urtica dioca*) agglutinin (UDA), which effectively reduced the growth of the fungal pathogen *Botrytis cineava persoonia* by 85%, and, in combination with chitinase, completely stopped fungal growth (Broekaert *et al.*, 1989).

If the carbohydrate binding affinity of lectins is considered to be vital for their role as defensive molecules, it is unlikely that a single lectin would be capable of defending a plant against all potential predators, particularly since the glycosyl structures expressed on the surface of bacteria, fungi, insects and other animals are usually different. Fortunately as most pests are host specific to some extent, it is possible for a plant to evolve lectins with the correct carbohydrate specificity. The selectivity of lectin toxicity towards different predator species has potential applications in plant protection.

1.6 Mechanism(s) of lectin toxicity towards insects

The mechanism(s) by which some lectins are toxic to higher animals have been studied extensively (see section 1.7), although they are not yet fully understood. In contrast, mechanisms of lectin toxicity to insects are largely unexplored. In higher animals, lectin toxicity involves a number of factors, among which are:

(i) resistance of ingested lectin protein to proteolysis, i.e. its survival and activity in the gut;

(ii) binding of the lectin to glycoproteins on the surfaces of cells in the gut wall;

(iii) lectin-mediated interactions between the gut microflora and cells in the gut wall;

(iv) mitogenic effects of lectins on gut cells, i.e. the stimulation of cell division;

(v) passage of lectins across the gut wall, possibly by endocytosis, and migration of intact lectin to the circulatory system, causing systemic effects on other organs.

Of these factors, (ii) and (iii) are mediated by the carbohydrate-binding specificity of the lectin, but the others are not; there is thus no simple correlation between the specificity of a lectin and its toxicity. This is the case in insects also, and all the above factors can reasonably be expected to play a role in determining lectin toxicity.

A common feature of lectin toxicity in both lower and higher animals is a requirement that the protein binds to the surface of cells of the gut epithelium. The binding of PHA to midgut epithelial cells of the bruchid *C. maculatus* was demonstrated by immunofluorescence microscopy by Gatehouse *et al.* (1984). In contrast, there was no binding of the lectin molecules to midgut epithelial cells of a related bruchid species, *Acanthoscelides obtectus*; this insect is a storage pest of *P. vulgaris* seeds and can tolerate moderately high levels of PHA (Gatehouse *et al.*, 1989). The binding of GNA to the midgut epithelial cells of rice brown planthopper was investigated by Powell (1993), using immunological techniques. In agreement with other results, specific binding of the lectin to the gut wall was observed, although the lectin also bound to the yeast-like symbionts in this species. Rabhé *et al* (1995) have also demonstrated the binding of Con A to the midgut epithelium of another sap sucking insect, the pea aphid *A. pisum.*

Lectins may also bind to the peritrophic membrane, as opposed to the epithelium, in the midgut region of insects. This membrane exists in most phytophagous insects protecting the midgut epithelial cells from abrasive food particles. Since the membrane is composed primarily of chitin, certain lectins may exert their toxic effects through binding to this membrane, particularly as many of the insecticidal lectins are specific for N-acetylglucosamine (Huesing *et. al.*, 1991; Czapla and Lang, 1990). However, apart from the work of Eisemann and co-workers (q.v.) there has been little other direct evidence reported to support this hypothesis. The toxicity of the mannose-specific lectins such as GNA clearly cannot depend on binding to chitin polysaccharides.

The effects of WGA, Con A and lentil lectin on larvae of the blowfly, *Lucilia cuprina* have been thoroughly studied in experiments designed to elucidate the mechanism of lectin toxicity in insects (Eisemann *et. al.*, 1994). These authors propose that the insecticidal effects of lectins on blowfly larvae are caused by at least three different mechanisms of action; a reduced intake of diet due to feeding deterrence, a partial blockage of the pores of the peritrophic membrane, and the direct binding of specific lectins to the midgut epithelial cells. Blockage of pores in the peritrophic membrane could cause a restriction in the uptake of the products of digestion, and a subsequent shortfall of nutrients available to the larva. This conclusion is consistent with the observation that ingested wheat germ lectin, Con A or lentil lectin cause no obvious damage to the larvae, and that larvae can exhibit reductions in weight of up to 80-90% caused by these

lectins before there is substantial mortality. The large amount of undefined material observed on the gut lumen side of the peritrophic membrane after a larva has fed on the growth medium containing lectins suggests that an aggregation of ingested material has been induced, and thus the blocking effect is not simply due to lectin binding.

A feeding deterrent effect caused by lectins has been observed in several studies. Experiments measuring food intake and feeding behaviour in rice brown planthopper exposed to lectins have been reported by Powell *et. al.* (1995). When adults of this insect were fed a diet containing either GNA (0.1% w/v) for 24 h, the volume of honey dew produced was reduced to less than 10% of that collected from control insects. Since the volume of honey dew excreted is roughly proportional to the volume of fluid ingested it would appear the lectin, which is known to be toxic to brown planthopper (Powell 1993) has a marked feeding deterrent effect. The deterrent effect decreased with time, so that there was some recovery in the amount of honey dew produced over a time period of 48 h, but this never reached the levels produced by control insects. Similarly, blowfly larvae, when offered a free choice between pads containing either bovine serum albumin (5 mg/ml) in the presence of 50mM WGA or bovine serum albumin alone where nine times more likely to choose the latter than the lectin treated pad (Eisemann, *et. al.*, 1994). These preliminary observations suggest that some lectins can directly stimulate avoidance responses. The feeding deterrent effect, if mediated by gustation, may be due to the binding of lectins to

glycoproteins situated on dendrites of chemoreceptor neurones near the insect mouth parts. A consequent disruption of the normal functioning of these neurones may give rise to abnormal sensory input to the central nervous system, resulting in a partial inhibition of feeding.

The toxicity of certain lectins towards insects may involve several mechanisms of action, as is the case in higher animals. However, no causal relationship between the proposed mechanisms of action caused by lectins and the relative importance of their effect on insects has been determined.

1.7 Toxicity and immunological/mitogenic effects of lectins towards mammals

The resistance of lectins to degradation or inactivation in the harsh environment of the mammalian gut determines their ability to interact with cell surface glycoproteins in the digestive tract. Since many lectins will both survive extremes of pH and resist proteolysis, species that consume plant material containing lectins will be exposed to these proteins (Van Damme *et al.*,1998). From such interactions, toxic effects can follow. For example, uncooked kidney beans (*Phaseolus vulgaris*) have long been known to be toxic to mammals, due to the seed lectin, PHA. Toxicity due to the lectin is only abolished if the protein is denatured by thorough cooking of beans; it should be noted that in general plant lectins are more resistant to heat denaturation than other plant proteins (Pusztai & Bardocz 1996). PHA is toxic to mammals because, as a powerful mitogen, it

causes the cell turnover time in the intestine to decrease from 72 to 12 hours leading to an increased proportion of immature cells, the ability to digest and absorb nutrients is reduced as a consequence (Puzstai *et al.*, 1990). Experiments in rats have demonstrated that various dietary lectins not only act as powerful mitogens for the small intestine, but can survive the passage across the intestinal epithelium and affect remote organs such as the pancreas. (Pusztai 1991).

Some lectins can also affect constituents of the mammalian immune system acting either as mitogens or anti-mitogens (see fig 1.1). Mitogenic lectins, such as PHA and Con A, act as polyclonal activators because they will activate most B and/or T cells, not just those bearing a specific receptor. PHA and Con A can cause an increase in lymphocyte population of up to 20%. It is thought that lectins bind to a receptor on T cells and associated molecules (T-cell receptor complex), causing signals to be released, resulting in the synthesis of interleukin 2 (IL-2) and interleukin 2 receptors (IL-2R) (Kilpatrick 1999). Anti-mitogenic lectins act by binding to accessory molecules disrupting signals that would otherwise lead to the production of immunological agents; for example wheat germ agglutinin causes anti-mitogenic activity in chickens (Greene & Waldmann 1980).

It has also been stated that a high intake of antinutrient lectin in mammals affects both thymus & spleen by causing atrophic changes, some of which are

Fig 1.1 Model for mitogenic and antimitogenic activity of lectins

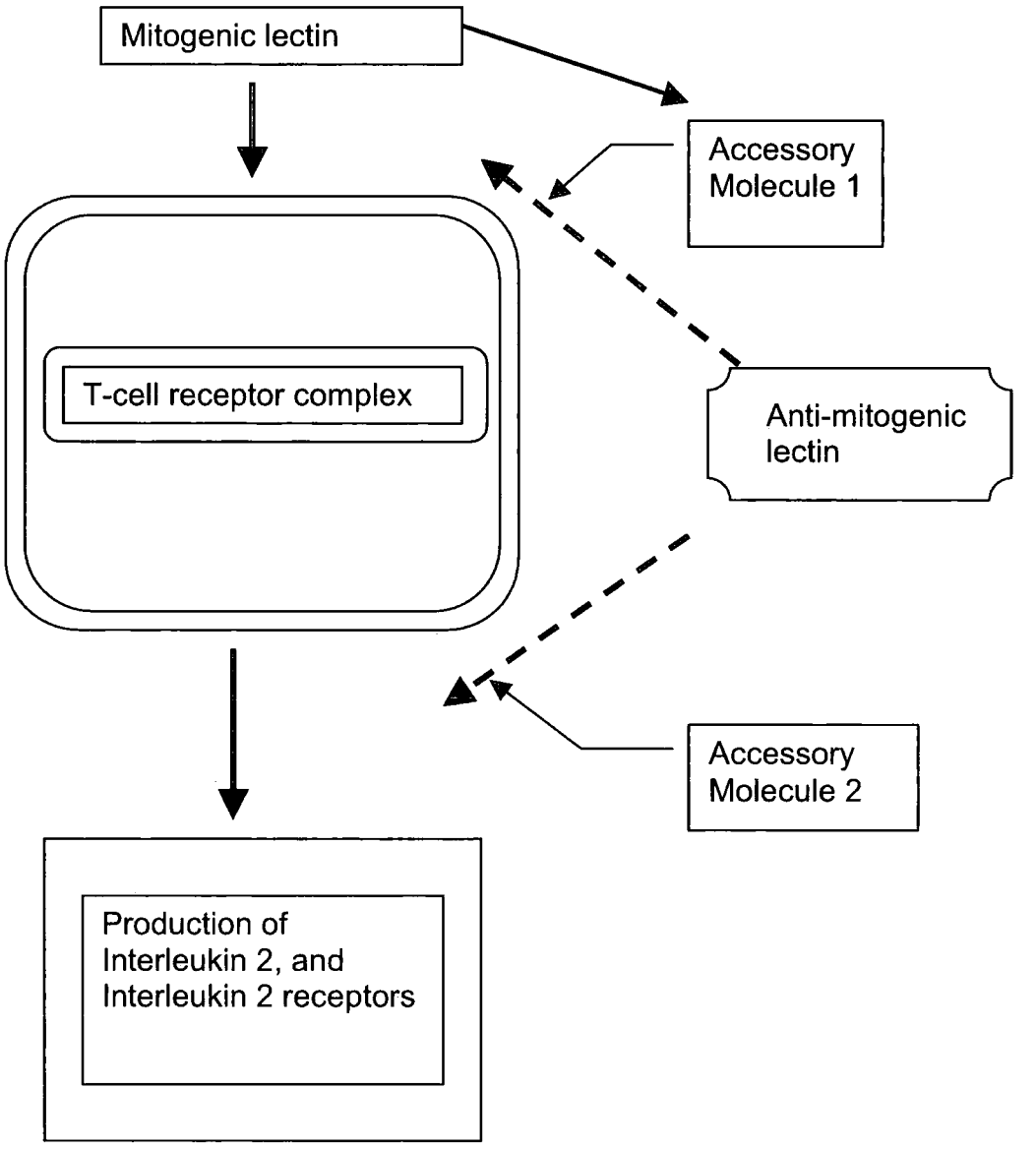


Fig 1.1 Model of the mitogenic and antimitogenic action of lectins. Leading to the production of Interleukin 2, and Interleukin 2 receptors

The simplest model is that mitogenic lectins bind to the T-cell receptor complex, to which accessory molecules bind. This leads to the transmission of activation signals by accessory molecules, activating the production of interleukin 2 and interleukin 2 receptors.

Antimitogenic lectins act by blocking the normal transmission pathways, and can therefore act to prevent mitogenic activity.
(Kilpatrick, 1999)

irreversible, with potentially serious effects for the immune system, especially T cell mediated immunity. In the gut Type-1 hypersensitivity reactions may occur even to highly degradable proteins; however such reactions are more extensive with stable lectins such as PHA, which remain bound to the brush border cells in the gut (Pusztai & Bardocz 1996).

In vertebrate species the activation of the complement system is an important part of the immune response. Within humans a mannose binding lectin (MBL) recognises carbohydrates on the surface of pathogens. This results in the activation of a mannose associated serine protease (MASP) from its inactive proenzyme form to its active form, thus resulting in the proteolytic activation of the complement components C4 C2 and C3, and finally in the killing of the pathogen (Endo *et al.*, 1998). Therefore the MBL-MASP complex plays a primary role in host defense during the lag period prior to the onset of adaptive immunity, through the generation of specific antibodies (Endo *et al.*, 1998). The action of lectins could disrupt this response.

Other lectins seem to be non-toxic to mammals. When administered orally, GNA was found to be non-toxic to rats (Puzstai *et al.*, 1990). This was not due to the protein being degraded in the gut, since in acute studies GNA remained 90% immunochemically active in the intestine. Initially, GNA was not detected bound to the villi in the rat intestine, but after chronic exposure (>10 days) bound GNA could be detected on the villi. It has been suggested that GNA can have a beneficial effect to mammals as it strongly reduces the bacterial overgrowth in

the rat gut by mannose-sensitive fimbriated *E. coli*. (Pusztai 1993). Although much confusion has been subsequently thrown over the toxicity of GNA towards mammals by not distinguishing this protein from other lectins in the "popular" press, no subsequent data to suggest that this lectin is toxic has been produced.

A paper by Ewen and Pusztai (2000) claimed that transgenic potatoes expressing GNA had toxic effects on rats, but careful reading of this paper shows that the authors believed that the potatoes were toxic because they were transgenic, and not because they were expressing GNA. As a lectin, GNA would be expected to bind to cell surface glycoproteins which contain mannose residues in their carbohydrate side chains. Such cell types in mammals include those of the gut epithelial surface (see above) and lymphocytes (Fenton *et al.*, 2000). However, binding of a lectin to a cell surface, while necessary for the protein to show toxicity, does not in itself mean that a given lectin will cause any toxic effect. Since lectins with similar carbohydrate-binding specificities to those of plant lectins are found in animals, the interaction of a carbohydrate-binding protein with cell surface glycoproteins alone cannot result in toxicity.

Many mammals (including humans) feed on plant foodstuffs which contain lectins as a significant proportion (>1%) of total protein, and thus an assumption that these proteins as a class are necessarily toxic to mammals is erroneous. In those cases where lectins are shown to be toxic, the mechanism of toxicity must involve processes other than binding to cell surfaces.

1.8 Lectins in medicine

Because of their differing binding specificities, lectins have a diverse and expanding use in medicine and medical research, some of which are briefly described below.

1.8.1 Lectins and HIV

Mannose specific lectins such as the snowdrop (*Galanthus nivalis*) bulb lectin (GNA) have been shown to prevent infection of $CD4^+$ T lymphocytes by HIV-1 (type 1 human immunodeficiency virus). GNA can also inhibit syncytium formation between virus infected and uninfected cells (Mahmood & Hay 1992). Since this cell type is a known receptor for HIV-1, and is the major target for the virus in preventing immune responses to its presence, GNA and similar lectins have been suggested to have potential as anti-HIV agents. This conclusion is in conflict with other work by Balzarini *et al.* (1991) who state that GNA does not inhibit HIV binding to $CD4^+$ cells, although both agree that GNA suppresses syncytium formation between persistently HIV infected HUT-78 cells and uninfected MOLT-4 cells. GNA also inhibits the infection of MT-4 cells by HIV types 1 & 2 (50% effective concentration being 0.3-0.8μg/ml), at concentrations comparable to those of dextran sulphate inhibition of these viruses. It is suggested that GNA interferes with an event in the HIV replication cycle prior to the attachment of the virions to the cells (Hammar *et al.*, 1995 Balzarini *et al.*, 1991). As GNA is highly selective for the glycoproteins of both HIV and SIV

(Simian immunodeficiency virus), it has been adopted as the basis of a simple broad-specificity ELISA for the quantitative determination of the viral glycoproteins in serum containing culture fluids (Mahmood & Hay 1992).

The lectin jacalin also has the ability to interact with lymphocytes, binding to the cell surface CD4+ antigen, and can block HIV-1 infection of CD4+ lymphoblastoid cells. However, jacalin does not inhibit syncytium formation due to its failure to prevent the interaction between gp120 and CD4 (Corbeau *et al.*, 1994).

1.8.2 Lectins and *Salmonella* infection.

Infection by *Salmonella typhimurium* and *Salmonella enteritidis* generally induces gut growth, and the effects of the infection on the metabolism and morphology of the small bowel resemble the changes induced by some plant lectins. This is not surprising as under some conditions most *Salmonella* serotypes express a number of lectin like components (Naughton *et al.*, 1995). Interference of gut colonisation by *Salmonella* by means of lectin treatments has been suggested (Naughton *et al.*, 2000).

1.8.3 Lectins and drug delivery

There are many problems associated with drug delivery, such as dilution by luminal fluids, or degradation prior to transfer across epithelial membranes. In nasal application the use of mucoadesic formulations composed of hydrophillic polymers has been successful. However, in oral applications mucoadesic drug

delivery systems are less successful due to the short turnover time of the mucus layer, leading to shedding of the mucus layer with the drug attached prior to contact with the mucosal epithelium. Due to this, development of a bioadhesive that will adhere directly to the absorptive enterocyte would be more successful. In this context lectins which are capable of binding to specific oligosaccharides, are of nonimmune plant origin, and are resistant to proteolytic degradation (increasing the transit time of drugs coupled to lectins) appear to be attractive carriers for oral drug delivery (Gabor *et al* 1998). The bioadhesive and endocytic potential of the tomato lectin has been demonstrated by the observation that 23% of orally administered tomato lectin-conjugated nanoparticles were taken up into the systemic circulation, as opposed to 0.5% of hapten blocked nanospheres (Hussain *et al.*, 1997).

1.9 Lectins as markers

In cells, glycoproteins and glycolipids are often considered to be components of the cell membrane, where they are present as integral membrane components, oriented so that the carbohydrate portion of the molecule projects into the extracellular space. Particular carbohydrate chains are often markers for specific cell types, e.g. the blood group antigens on erythrocytes. However, glycoproteins and glycolipids can also be found ininside cells, or in a soluble form in extracellular tissue fluid or serum. Many conjugated carbohydrates play roles in cell communication and signaling events. Since lectins bind to specific sugar residues, they have been much used as markers for cell types, and to investigate

communication processes. The main limitation to their use as tools to investigate cell communication and signaling is that the structures that they recognise on cells and tissues are heterogeneous and largely uncharacterised (Brooks *et al.*, 1997); but this limitation does not affect their utility as markers for cell types or for differentiation of cells into specific types. Of the commercially available lectins some are available conjugated to readily detectable markers such as biotin, gold or fluorochromes, or can be conjugated with relative simplicity. This gives the user the choice of whether to use a conjugated lectin, or a non-conjugated lectin and a specific labelled antibody.

Lectins are used in the field of histology at both the light and electron microscope level to localise specific carbohydrate structures. Indeed, the specificity of lectins for tissue carbohydrates is only equaled by monoclonal antibodies because of their ability to detect subtle differences in carbohydrate specificity (Stanley 1998). Due to this characteristic, lectins have become valuable probes for studying the expression and distribution of cell surface carbohydrates (Sharon & Lis 1989). Gipson & Tisdale (1997) have used fluorescent lectin probes to visualise goblet cells in conjunctival tissue by confocal microscopy. As lectins are able to distinguish glycosylation changes they are utilised in a number of fields such as relating changes to cell behaviour, or in pathology detecting early changes in disease.

Lectins are also used to visualise glycoproteins separated by SDS-PAGE gel electrophoresis and transferred to nylon or nitrocellulose membranes; commercial kits are available containing a selection of digoxigenin (DIG) conjugated lectins.

1.10 The insect haemolymph and immune system.

The potential for lectins to have both probiotic and antibiotic effects on mammals, depending on their sugar-binding specificities and other factors, suggests that multiple mechanisms must also be taken into account when their effects on insects are considered. Not only is there a potential for interference with gut functions, but systemic effects on insect defence mechanisms (which themselves are known to involve endogenous lectins) must be considered. Although insects have no immune system in the sense in which this is understood in higher animals, they do have a range of mechanisms for combating pathogens, and distinguishing "self" from "non-self". These mechanisms will be described in the following section.

Due to the nature of their environment insects are constantly exposed to a large number of microorganisms and eukaryotic parasites. However, relatively few encounters result in infection, due to insect systems that prevent such infections. The mechanisms by which infection is prevented are complex and generally poorly understood. Gillespie *et al.*, (1997) broadly divide the mechanisms preventing infection into three areas:

1) Physical barriers of the insects integument and gut.

The insect's exoskeleton, a thick chitinous layer, prevents movement of microorganisms and eukaryotic parasites into the insect. Additionally the lining of the digestive tract can prevent or cut down movement of microorganisms and eukaryotic parasites; the foregut and hindgut are lined with chitin, and the midgut is protected by a macroporous chitin structure, the peritrophic membrane, in most insects.

2) Induced synthesis of antimicrobial peptides and proteins by the fat body.

Upon induction, fat bodies produce antibacterial proteins such as lysozymes and hemolin. Induction is caused after recognition of nonself by receptors on the fat body. Soluble peptidoglycans (unique for bacterial cell walls) can induce protein synthesis in isolated fat bodies incubated in vitro, suggesting that a receptor for peptidoglycans may exist on fat body cells (Iketani & Morishima 1993).

3) The response of haemocytes when the physical barriers are breached.

Haemocytes are the insect's equivalent to mammalian blood cells and as such play an important role in the insect defence mechanism. Unfortunately the role of haemocytes in insects is not as well understood as in mammals. Indeed, there much ambiguity, and there appears to be differences in function and role of haemocytes depending on the insect in question. Confusion is so great that there is even disagreement about the number of insect haemocytes and their classification. The most consistent classification of haemocytes indicates that there are 5 main types (Gillespie *et al.*, 1997) these being prohaemocyte, granulocyte, plasmatocyte, spherulocyte and oencytoid. The granulocyte and

plasmatocyte provide cellular immunity against foreign antigens (being able to recognise non-self); it has been suggested that the insect granulocyte is a hybrid of mammalian B & T lymphocytes, and that it is functionally similar to the macrophage (Gupta 1986).

In 1991 Chadwick & Aston proposed an alternative schematic model of the insect immune system comprising three distinct phases (fig 1.2):

1) Induction phase

During this phase an inducer such as bacteria or a lipopolysaccharide breaches the structural barriers of the insect, and comes into contact with the appropriate cellular or soluble components of the immune system.

2) Mediation Phase

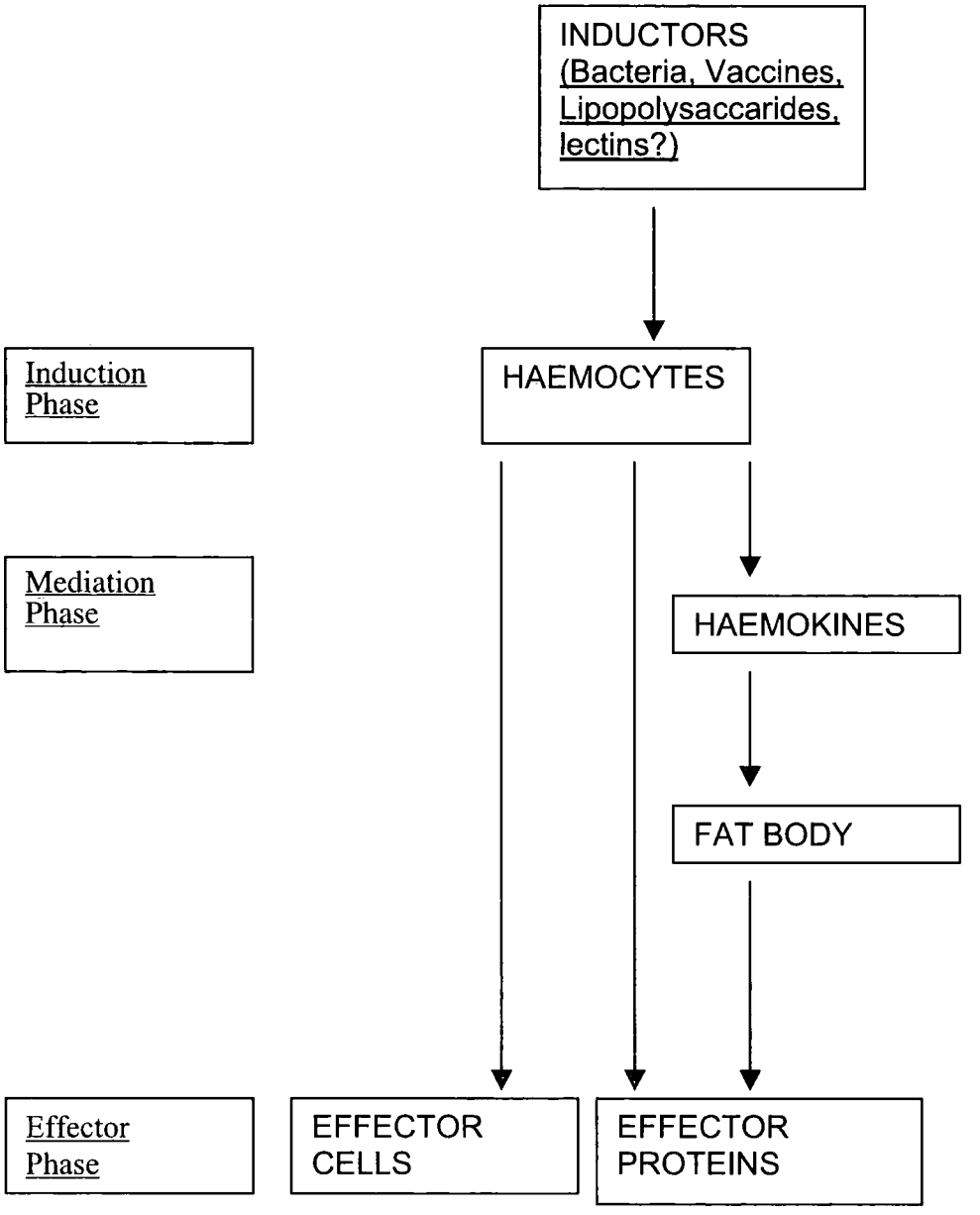
This phase involves the complex events between the recognition of the non-self inducer and the generation of active effector cells and substances.

3) Effector phase

The third and final phase includes containment, destruction, and elimination of the inducer by the generated effectors. Mechanisms such as phagocytosis, cytolysis or the phenoloxidase system are known to be involved in this phase.

It should be noted that both models agree that haemocytes play an important role in the insect immune system; furthermore the haemocytic immunity in insects is accomplished directly by phagocytosis, encapsulation, and nodule formation, and indirectly by immunologic factors, coagulation, prophenoloxidase-activating system and poison detoxification mechanisms. These processes are triggered as

Fig 1.2 Model of the insect immune system



defence reactions against foreign antigens and toxins. Some artificially introduced animate and inanimate objects can also induce some of these reactions. (Gupta 1991).

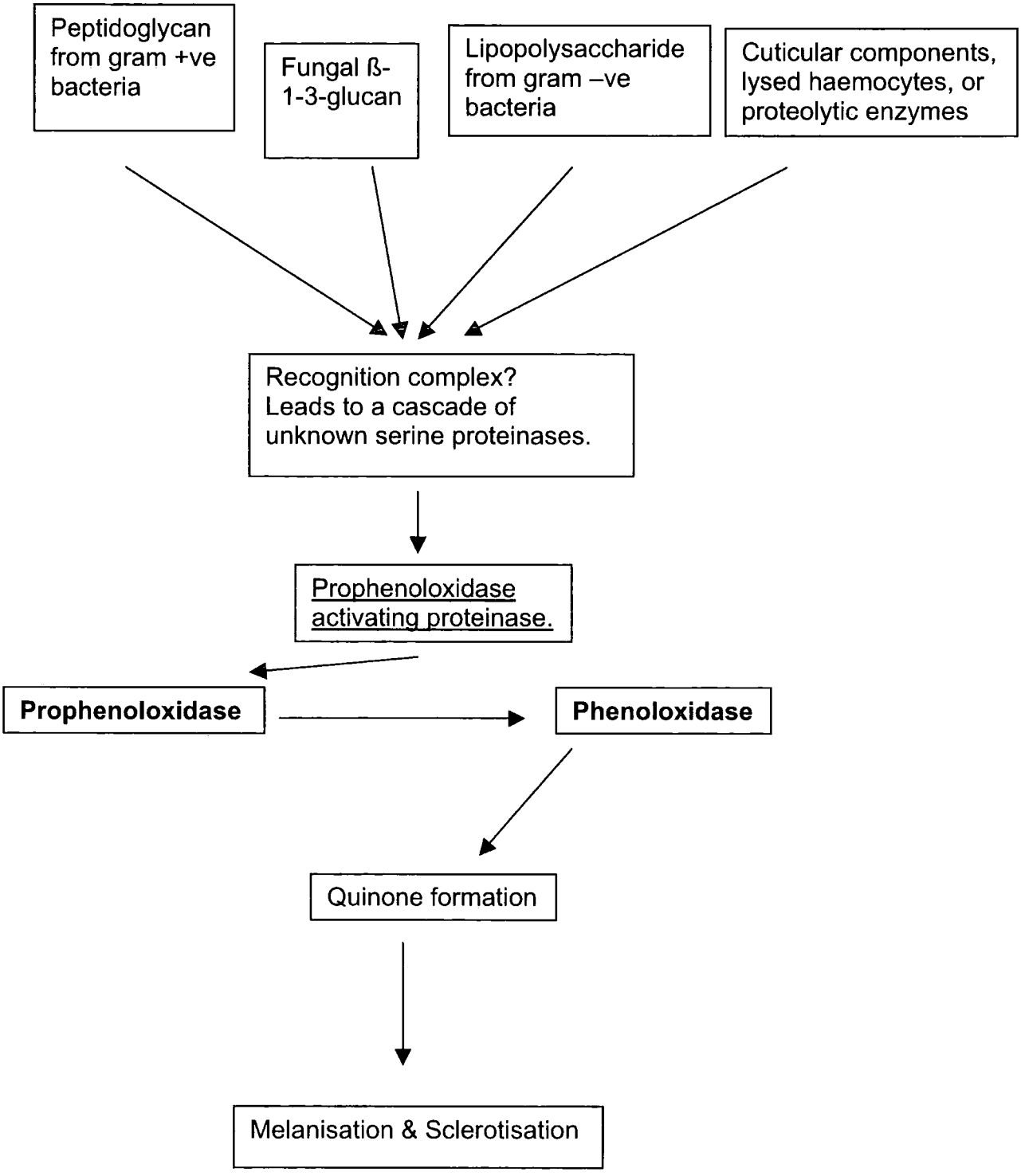
The effectiveness of the main haemocyte mediated defence (phagocytosis, nodule and capsule formation) is influenced by several parameters such as number and type of haemocyte present, and their "fitness". It must be hypothesised that the insect's suite of defensive mechanisms, to a large extent, act synergistically (Gupta 1991). If the functionality, the status of a response, or the number of haemocytes, are altered or have been impaired then the insect may be compromised. Lectins have the potential to interfere with these responses.

1.11 Insect defence: the prophenoloxidase/phenoloxidase system

The phenoloxidase system plays an important role in the insect's defence system, and is thought to be the insect equivalent to the complement system found in higher species (Armstrong 1991). The precise pathways by which this system is initiated are as yet unknown. Depending on the species, prophenoloxidase (PPO) is found in haemolymph, plasma and haemocytes (Gillespie 1997), as well as in the cuticle (Ashida & Brey 1995).

By taking information from a number of sources (Gillespie 1997; Ashida & Brey 1995; Soderhall *et al.*, 1994; Stadtman 1993; Felton *et al.*, 1992; Armstrong 1991; O'Brien 1991; Bidochka *et al.*, 1989), a model for the PPO pathway can be drawn up (fig 1.3). It is known that cuticular components, lysed haemocytes,

Fig 1.3 Phenoloxidase cascade



or proteolytic enzymes can initiate activity of the system causing the conversion of the inactive zymogen PPO to phenoloxidase.

The initiation is thought to be due to one or more serine proteinases (Soderhall *et al.*, 1994). The phenoloxidase enzyme oxidises tyrosine derivatives to ortho-quinones, which cross link terminal amines of proteins resulting in melanisation. This cross-linked protein polyphenol matrix is insoluble and is implicated in the encapsulation of pathogens (Bidochka *et al.*, 1989). In addition, the polyphenol matrix can lead to protein damage by polymerisation and/or fragmentation (Stadtman 1993), (O'Brien 1991). The effect of quinone formation can also lead to irreversible binding of quinones to nucleophilic side chains of proteins (-SH $-NH_2$ –NH). This in turn can lead to loss of lysine, histidine and thiol amino acids with an unknown loss of nutritive value (Felton *et al.*, 1992).

In crayfish blood, activation of the PPO can be achieved by fungal ß-1-3-glucans (receptors for which have been isolated on haemocytes) (Armstrong 1991). Microbial products including peptidoglucans and lipopolysaccarides can act as elicitors this cascade in invertebrates (Gillespie 1997). Initiation of the polyphenoloxidase cascade by lectins is possible due to their interaction with glycoproteins, mediated by their specific carbohydrate binding properties.

1.12 Target pests

Two insect pests were selected as subjects for work in this thesis. These came from two different Orders of phytophagous insects (Lepidoptera and Homoptera) with different feeding habits (chewing of foliage and fruits, and sap-sucking).

1.12.1 Tomato moth (*Lacanobia oleracea)*

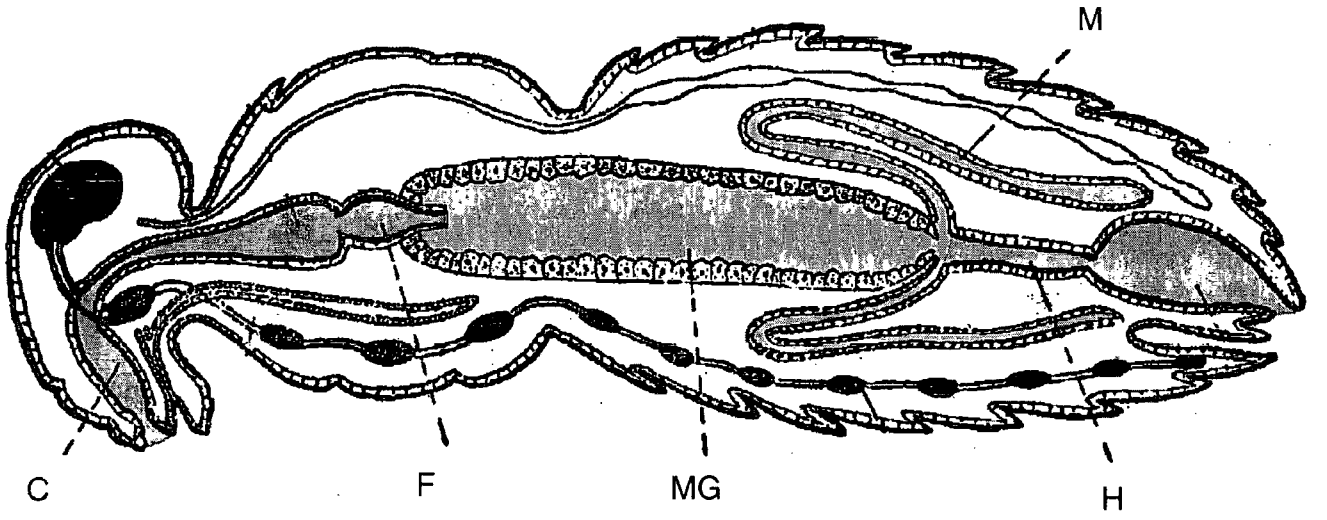
The lepidopteran pest tomato moth (*L. oleracea*) is widespread and common throughout most of Europe, including the UK; it can also be found in parts of Asia. It is a serious pest of a number of crops including peas and turnips as well as tomatoes. In tomatoes the larvae cause damage by consumption of tomato foliage as well as by biting into the fruit and stems of the plant. Currently the most effective method of control of *L. oleracea* is by the use of chemical insecticides such as Dipel with *Bacillus thuringiensis kurstaki* added. (Jarrett & Burgess 1982). However a resurgence of this pest is likely due to the trend of lower pesticide usage together with current growing techniques (peat bags) which means that sterilisation of soil (where pupae overwinter) killing the *L. oleracea* pupae is no longer carried out.

L. oleracea reared under optimal artificial conditions, progress through 6 larval instars over a period of 35-38 days. The pupal period is 25-30 days and neonates are produced within 2-3 days of egg laying. The larvae spend the majority of their time feeding with brief pauses for ecdysis and pupal formation (Fitches *et al.*, 1998). The larval digestive tract is the main target tissue for lectin

action in this species, and thus will be described in more detail. The intestine of *Lacanobia oleracea* is basically a long continual tube, which starts at the mouth and continues to the rectum. It can be divided into three distinct regions; foregut, midgut, and hindgut (fig 1.4). The foregut is short, lined with cuticle and relatively impermeable. This region is thought to play an important role in digestion (enzymes are passed forward from the midgut), and storage (by holding food prior to passage into the midgut). The midgut is the most permeable and largest region of the digestive tract, and therefore plays a central role in the transport and digestive process. The midgut is lined with an epithelial membrane made up of goblet and columnar cells (fig 1.5). Both cell types are lined with microvilli and are active areas for the transport of nutrients from the lumen to the cytoplasm, for subsequent transport throughout the larvae (Dow 1986). Although the midgut is the largest and most active part of the intestine, it must be remembered that it does not function in isolation and therefore both the fore and hind gut, peritrophic membrane, and malpighian tubules complement the midgut to a greater or lesser extent. Although the hindgut has a cuticular lining it is permeable to a certain extent and serves as final reclamation (Dow 1986). The midgut is lined by the peritrophic membrane, a permeable membranous sac surrounding food in the midgut lumen. The membrane is made up of chitin, proteins and proteoglycans (Spence 1991). The digestive sytem and the midgut lumen is also continuous with the Malpighian tubules; these tubules emerge from the alimentary canal at the junction between the mid and hindgut. They then loop up to and insert into the middle of the midgut, returning again to insert into the hindgut. The function

of the tubules is thought to be to act as an area for water and soluble nutrient reclamation (including potassium) from the hindgut, to be returned to the midgut (Dow 1986).

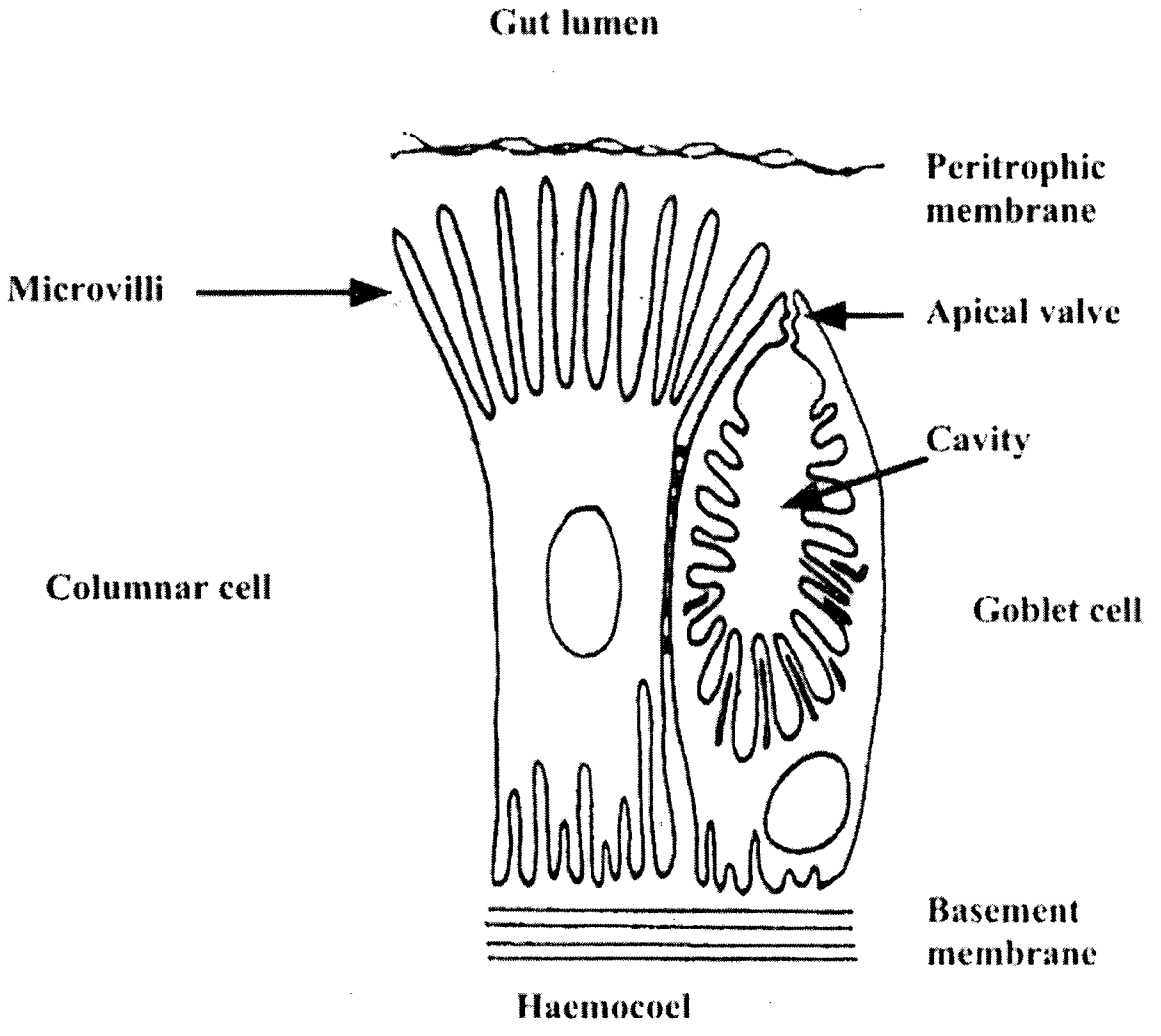
Fig. 1.4 Schematic diagram of the insect digestive system, (Gatehouse, J. A. & Gatehouse A. M. R. 1998).



Legend

C= Mouthparts
MG = Midgut
M = Malpighian tubules
F = Foregut
H = Hindgut

Fig 1.5 Schematic diagram of lepidoptera midgut epithelial layer (Dow, J. A. T. 1986).



1.12.2 Peach/Green Potato Aphid *(Myzus persicae)*

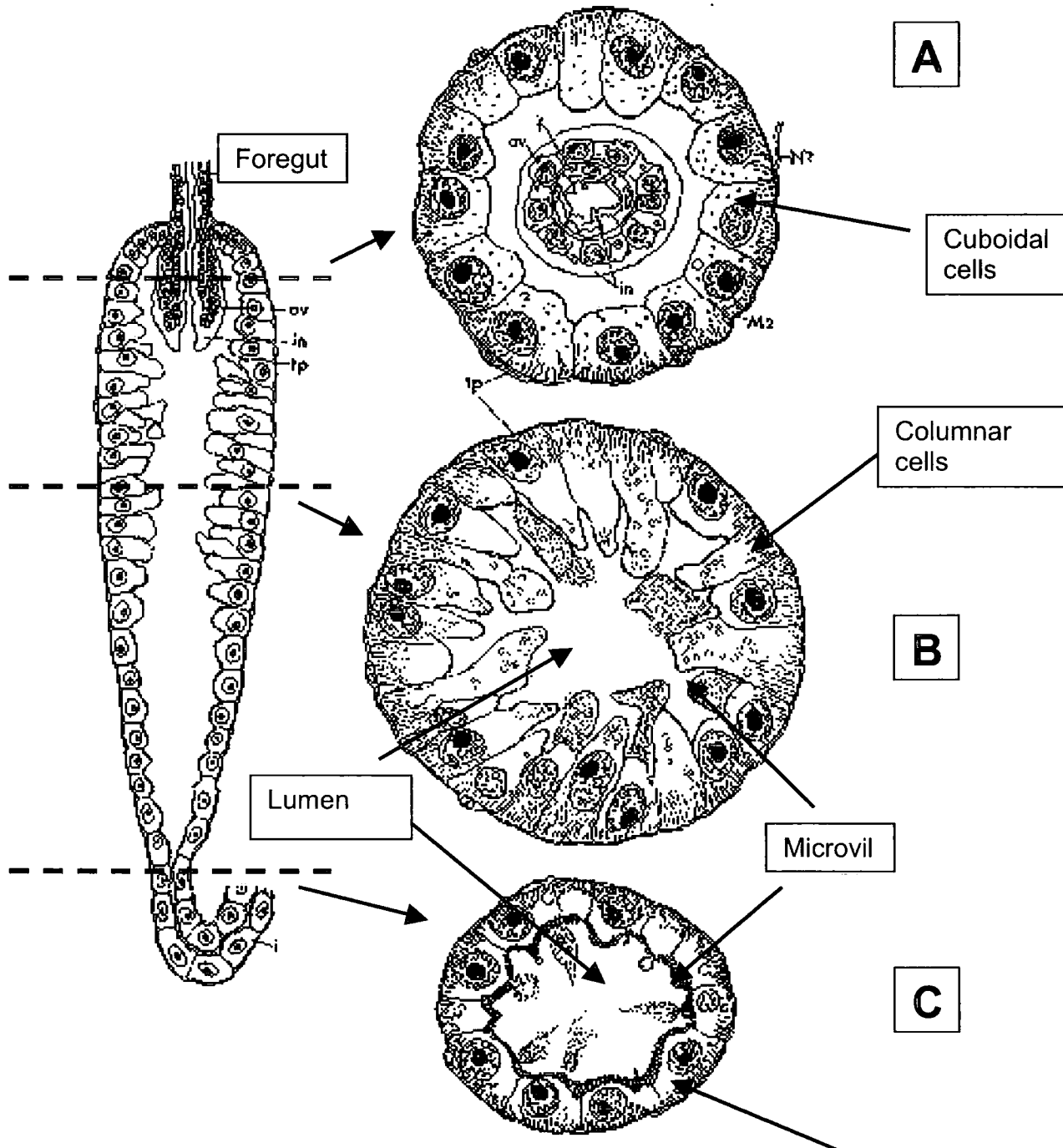
The aphid *Myzus persicae* is known by two common names, peach or green potato aphid; it is distributed worldwide and has many host plants. Agricultural crops include: broccoli, cabbage, carrot, cauliflower, green beans, lettuce, macadamia, papaya, peppers, sweet potato. This aphid also attacks many ornamental crops such as carnation, chrysanthemum, poinsettia and rose.

Aphids feed by sucking sap from their hosts. Seedlings or transplants of lettuce and other crops can be stunted by the attack of large populations of this aphid leading to economic damage. Green/peach potato aphids rarely cause economic losses on older cabbage and related crops. On fruit crops, extensive feeding causes distortion of young leaves and shoots and premature dropping of fruit. Aphids also act as vectors for many plant viruses, which is potentially the greatest consequence of aphid infestations, with *M. persicae* being responsible for the transmission of over 200 plant diseases (Toba 1963). The average life span of this aphid species is about 18 days, representing the period from birth of the nymphs to the death of the adult.

As with the lepidopteran target pest, the digestive tract of the aphid forms the primary target for lectin action. Its anatomy differs significantly from that of a lepidopteran species, as a result of its specialised feeding habits. The alimentary tract commences with the food canal situated in the interior of the maxillary

stylets, which eventually joins the foregut. The foregut is a thin tube made up of squamous epithelial cells, which secrete a chitinous intima, of which the basal cell membrane has a few invaginations, and the apical cell membrane microvilli (Forbes 1977). The foregut runs into the midgut/stomach section of the alimentary tract, which is divided into three cellular regions (fig 1.6). All of the midgut regions are lined with a striated border with microvilli and an elaborately enfolded basal membrane.

Fig 1.6 Structure of the foregut and midgut of *Myzus persicae*
(Ponsen, 1987)



KEY

F = Foregut
ov = Oesophageal valve
in = Intima
I = Intestine
tp = Tunica propria
M2 = Circular muscle fibres
N2 = Branch of medial dorsal nerve
A = Anterior stomach region
B = Middle stomach region
C = Posterior stomach region

1.13 Aims of Thesis

The work described in this thesis formed part of a larger programme to produce and evaluate transgenic crop plants with resistance to insect pests. As part of that programme, this project aimed to characterise lectin binding to insect guts and other tissues, and to test hypotheses about the mechanisms of lectin toxicity towards target insect pests.

The initial aim was to utilise known lectins to establish and test protocols for the detection of lectin binding, utilising rapid molecular biological and histological techniques, in conjunction with established methods such as bioassays to determine lectin toxicity towards insect species. The reason for detecting lectin binding to the insect gut is that such binding is a prerequisite for toxicity, (Powell *et al.*, 1998; Pusztai & Bardocz 1996; Harper *et al.*, 1995; Pusztai 1991). Screening a selection of lectins by determining their capacity to bind to insect guts gives an indication of a potential toxic effect, although it does not necessarily show which of the lectins that bind are toxic. This study was extended to include a number of novel lectins, produced and purified by the Rowett Research Institute, one of the collaborating members of the project, the aim being to identify lectins with sufficient toxicity towards insect pests for use in crop protection.

A second aim was to investigate lectin binding to insect guts at the cellular level through the use of ultrastructural studies. Since both GNA and Con A had been

shown to have toxic effects on *L. oleracea* larvae, characterisation of their binding was intended to elucidate mechanisms for their toxicity. This work was extended to the molecular characterisation of a major gut receptor for both lectins (particularly Con A), the glycoprotein aminopeptidase N. Additionally, a similar study was carried out with GNA binding to the midgut of a beneficial insect predator, the two-spot ladybird *Adalia bipunctata*, to see whether the lack of lectin toxicity towards this insect could be explained by differences in binding.

Finally, experiments to test whether lectins could have systemic effects on insect defensive mechanisms were carried out. The aims were to show that lectins could be taken up into the insect haemolymph after oral ingestion, and could then alter the insect's defensive responses.

Chapter 2

Materials and Methods

2.1 Chemical and biological materials

GNA was obtained from Drs. W. Peumans & E. Van Damme (Catholic University, Leuven, Belgium). Antibodies against recombinant GNA in rabbit were supplied by Dr. Christine Newell of Pestax, Cambridge, U.K. Antibodies against GNA purified from snowdrop bulbs were raised in rabbits using standard procedures (supplied by Dr. L. N. Gatehouse and Dr. R. D. D. Croy, University of Durham). LR white resin, nickel grids (150 mesh, hexagonal), goat anti-rabbit 10nm gold-conjugated IgG and goat serum were all obtained from Agar Scientific, Stansted, Essex. Black mulberry lectin, *Morus nigra,* was supplied by the Rowett Research Institute, Aberdeen. The VECTASTAIN elite ABC kit, Biotin (long arm) NHS, Biotin blocking kit and all other lectins were obtained from VECTOR Laboratories (Peterborough PE2 6XS). The ECL (enhanced chemiluminescence) kit from Amersham, Bucks, UK. The DIG (digoxigenin-labelled) Glycan Differentiation Kit and the DIG labelling kit were from Roche (previously Boeringher Mannheim). Primers from Oswel DNA Service (Southampton SO16 7PX). All other chemicals were from either the Sigma Chemical Company or BDH (Poole, Dorset) and were of analytical grade unless otherwise stated.

Details for common reagents can be found in the appendix.

2.2 Insect Cultures

Peach-potato aphid (*M. persicae*)

Stocks of the peach-potato aphid (*M. persicae*) were continuously reared on Chinese cabbage plants in environmentally controlled incubators at 21°C±2°C under a 16 hour light and 8 hour dark lighting regime.

Tomato moth (*L. oleracea*)

Larval cultures of *L. oleracea* originally obtained from Central Science Laboratories, MAFF, York, UK were reared continuously on optimal artificial diet (Bown *et al.*, 1997) at 25°C again under a 16 hour light: 8 hour dark regime.

2.3 *L. oleracea* artificial insect diet.

The components of the artificial diet are presented in the appendix. The freeze-dried potato leaf and casein was mixed with 2ml of distilled water (1ml for test diet) (note additional casein contained in control diet to compensate for test protein), to which a dissolved mixture of agar in 3ml of distilled water was added and mixed well with a spatula. The mixture was left to cool slightly (but not set) prior to addition of the remaining constituents of the diet. The diet was then mixed well and kept at 4°C. The diet was made up fresh and fed to *L. oleracea* larvae after being warmed to room temperature.

2.4 SDS-PAGE Gel Electrophoresis

Electrophoresis was conducted on proteins in a dissociating SDS-PAGE discontinuous buffer system (Laemmli 1970), and run in an ATTO AE-6450 gel tank. Concentration of Acrylamide Bis-Acrylamide in the gel is dependent on the protein being analysed. Unless otherwise mentioned a 15% Acrylamide Bis-Acrylamide gel was used. Volumes required for two 15% gels of size 9x10 cm are in table 2.4.1 and 2.4.2 (Hames 1981). The resolving gel is prepared first and poured between two glass plates once this is set, the stacking gel is prepared and poured on top of the resolving gel, into which loading lanes are made by the addition of a comb. Samples were prepared by the addition of 2XSDS sample buffer (see appendix) and 10% ß-mercaptoethanol. Samples were boiled for 5 minutes and kept on ice prior to loading. Using the ATTO electrophoresis tank gels, were run at 80v past the stacker gel and at 120V through the resolving gel, normally until the dye front reached the bottom of the gel, allowing separation of protein bands.

Table 2.4.1 Stacking gel constituents

Acrylamide Bis-Acrylamide (40%) (33:1)	0.937ml
3M Tris-HCl pH 8.8	1.875ml
Double distilled H_2O	5.963ml
10% SDS	100µl
2% Ammonium persulphate	500µl
N,N,N,N Tetramethyl-ethylenediamine (TEMED).	7.5µl

Table 2.4.2 Resolving gel constituents

Acrylamide Bis-Acrylamide (40%) (33:1)	5.625ml
3M Tris-HCl pH 8.8	1.875ml
Double distilled H_2O	6.79ml
10% SDS	150µl
2% Ammonium persulphate	560µl
N,N,N,N Tetramethyl-ethylenediamine (TEMED).	7.5µl

2.5 Detection of protein in polyacrylamide gels.

Following electrophoresis, gels were placed in the Coomassie blue stain solution (see appendix) for approximatelyimately 3 hours (staining time is dependent on the protein concentration) and then placed in destain solution (see appendix) until the protein bands can easily be distinguished.

2.6 Western blotting

To transfer separated protein bands from the polyacrylamide gel to a nitrocellulose membrane a semi dry western blotting technique was employed (Khyse-Anderson 1994). Transfer was carried out using an ATTO semi-dry western blotter. The transfer stack was set up in the following order (composition of solutions are set out in the appendix):

ANODE PLATE; 2 sheets of 3MM paper soaked in anode buffer 1, 1 sheet of 3 MM paper soaked in anode buffer 2, 1 Sheet of nitrocellulose (Schleicher & Schuell BA85) soaked in distilled H_20, 1 acrylamide gel to be blotted, 1 sheet of dialysis membrane (cellophane) soaked in distilled H_20, 2 sheets of 3MM paper soaked in cathode buffer; CATHODE PLATE.

Electrotransfer of proteins was carried out at 2.5mA cm^2 for 1 hour, after which continued work can be carried out on the proteins attached to the nitrocellulose membrane.

Chapter 3

Assays of Toxicity of Lectins to Insects

3.1 Introduction

The use of bioassays in which lectins are incorporated into artificial or semi-artificial diets for insects is viewed as a "best estimate" of the toxicity of the proteins when produced in a plant (transgenic or otherwise). Although evidence of toxicity in artificial diet does not always correlate with what is observed *in planta*, a given lectin, if possible, must eventually be tested in feeding trials both to verify its insecticidal effects upon a target pest species and to determine dietary levels require to achieve crop protection in the field. Prior to undertaking studies on lectin binding in this thesis, the toxicity of selected lectins towards the chosen target pest insect species was estimated by diet bioassays.

Aphids due to their size survive on a relatively small quantity of food and have a relatively high metabolic rate making them ideal for feeding trials, and the peach potato aphid *M. persicae* is a suitable test species as it infests a broad range of plants and is an economically important pest (see chapter 1), The tomato moth *L.oleracea* (see chapter 1) is also a good test species because it is relatively easy to culture, has a relatively high metabolic turnover and is representative of a second important group of crop pests (Lepidoptera).

The aim of this chapter is to establish bioassay methods to allow the effects of the lectins PHA, jacalin and the lectin from *Morus nigra* on *L. oleracea* and *M. persicae* to be estimated. A further aim is to identify lectins which have detrimental effects on the insect species.

3.2 Materials and Methods

Materials

Insect cultures were supplied and raised as described in chapter 2

3.2.1 *L. oleracea* PHA bioassay.

An artificial diet was made up as per section 2.3 with PHA as the test protein. On day 1 of the trial, first instar *L. oleracea* were placed into 6 feeding chambers (circular plastic pots 6cm diameter, 5cm depth), with 10 larvae in each chamber. Three chambers contained control diet and 3 contained the test diet with PHA at 2% of total protein (larvae were fed *ad libitum*). A piece of moist filter paper was placed in each chamber to maintain moisture in diet. Forty eight hours later the diet was changed. All remaining diet from day 1 and all the frass was collected and collated for each of the test groups and flash frozen in liquid nitrogen.
On day 5 of the trial the *L. oleracea* larvae were segregated into individual feeding chambers and the insect instar and weight recorded. Frass was collected and a recorded weight of diet was added to each chamber. On a daily basis from day 6 to the end of the trial on day 21 the diet was changed and a record made of the weight of diet given to each larva, weight of remaining diet, larval instar, and insect weight. In addition any remaining diet was collected, freeze dried and

weighed, and all frass produced by the larvae from the two feeding regimes was collected and collated, flash frozen in liquid nitrogen and then freeze dried and weighed.

3.2.2 *M. persicae* bioassays with the Jackfruit lectin *Artocarpus integrifolia* (Jacalin)

Artificial diet, capable of sustaining the growth and parthenogenetic reproduction of *M. persicae* was prepared according to Febvay (1988). Aphid feeding chambers were used for artificial diet studies, prepared according to Down (1996). Diet sachets were replaced every 48 hours (200µl diet per sachet). Ten aphid feeding chambers, each containing 10 newly mature, apterous, adult *M. persicae* feeding on artificial diet were set up. The following day 60 neonates were collected and placed in feeding chambers (5 per chamber). Thirty neonates were fed on artificial diet alone, and the remaining aphids were fed on artificial diet containing jacalin at 0.1% w/v.

To assess the effect of jacalin, aphid mortality was noted every 48 hours for 14 days. Aphid size was measured on day eight of the trial, aphids were subjected to image analysis to measure length (from the tip of the head to the end of the cauda) and width (across the metathorax). Image analysis was carried out using a Nikon-Type 104 microscope (x4 lens) fitted with a standard video camera; images were acquired by connecting the camera to the S-video input of an Apple Power Macintosh 7600/120 computer, and Apple Video Player software utilised to capture freeze-frame image. Images were analysed using the NIH Image

software package and the system was calibrated using an image of a millimetre scale.

3.2.3 *M. persicae* bioassays with the Black Mulberry lectin *Morus nigra*

This was completed utilising the same method described above in 3.2.2, the only difference being the substitution of the *Morus nigra* lectin for jacalin.

3.2.4 *L. oleracea* larvae bioassays with the Black Mulberry lectin *Morus nigra.*

This trial was carried out in the same fashion as described in 3.2.1. Except that PHA was replaced by the *Morus nigra* lectin.

3.2.5 Statistical analysis

The logrank test and subsequent Mantel-Cox analysis were carried out using the Statview package (Apple computers). All other analysis was carried out with the Microsoft excel package, using standard ANOVA analysis, simplifying to a student t-test when pairwise comparisons with a single variable were considered.

3.3 Results

Bioassays of lectins against tomato moth larvae (*L. oleraeca*)

3.3.1 Bioassay of *Phaseolus vulgaris* (french bean) lectin (PHA) against tomato moth larvae

L. oleracea larvae were fed from neonates on a potato leaf-based artificial diet containing PHA at 2% of total dietary protein. The control diet contained an equivalent amount of casein replacing PHA. The insect parameters measured in the experiment were larval survival, growth, development, and feeding.

3.3.1a Survival of *L. oleracea* after exposure to PHA.

PHA had a detrimental effect on the survival of *L.oleracea* larvae (fig 3.1) in this bioassay. Survival of control diet-fed larvae fell to approximately. 70% over the first two instars, but then remained almost stable, falling to approximately. 60% by day 21, when most larvae were in the 4^{th} and 5^{th} instars. Larvae fed PHA-containing diet showed a similar trend in survival with time, but the decrease in survival was greater over the first seven days, with survival falling to 50%, and continued to decline to day 10, by which time only 30% of the larvae were still alive. The survival then remained almost constant for the rest of the trial. The greatest effect of PHA on survival of the lectin-fed group relative to the control group was observed during the first instar, as indicated by the initial high mortality rate of the larvae in the feeding trial. The lectin continued to have an

effect through the second instar, but had no effect on survival of more developed larvae.

Data were subjected to survival analysis, which confirmed the conclusions drawn above. A logrank test showed that the two survival curves were significantly different ($p = 0.021$, Mantel-Cox analysis). The cumulative hazard plots confirmed that the treatment had no significant effect after day 10 of the assay.

3.3.1b Effect of PHA on growth of *L. oleracea.*

The growth of *L. oleracea* larvae was estimated from the mean weight of surviving insects. The PHA-fed larvae had a slightly higher rate of weight gain over the 21 day period (fig 3.2) than those on the control diet. After day 7 of the assay the PHA-fed larvae were consistently of higher mean weight than the control larvae, so that by day 21 the difference between the two treatments was approximately 20%. Statistical analysis (ANOVA) showed that differences were not significant at the 5% level either across the entire data set ($F = 0.23$, df 1,32, $p = >0.5$), or on day 21 of the trial ($F = 1.41$, df 1,14, $p = >0.1$).

3.3.1c Effect of PHA on development.

On days 7, 14 & 21 of the trial the numbers of larvae at each instar were noted (fig 3.3-5). On day 7 a slightly higher proportion of PHA-fed larvae had entered the 3rd instar compared to controls, whereas at day 14 the development of the PHA-fed insects had fallen slightly behind the controls, with higher proportions of control insects in the 3rd and 4th instars compared to PHA-fed insects. At day 21, the end of the trial, the PHA-fed insects were once again ahead of the control

insects in development, with some control insects still remaining in the 3rd instar, while all PHA-fed insects were either in the 4th or 5th instars. However, statistical analysis of the data (Chi squared) showed that none of the differences observed were significant at the p = 0.05 levels (7 days, p = >0.5; 14 days, p = >0.5; 21 days, p = >0.1).

3.3.1c Larval feeding

Metabolic measurements were taken by recording the intake of diet and production of frass by larvae on a daily basis. Intake of diet varied from day to day, with depressed feeding occurring at the time of instar change. Subsequently no curve can be seen (fig 3.6) and feeding patterns appear erratic. An ANOVA test indicates that there was no significant difference at the 5% level between control and treated samples with an (F = 0.39, df 1,30, p = >0.5).

Frass production also exhibited blips in the general trend, due to the effects of instar change. However, as samples were pooled there is a smooth increase in frass production with time (fig 3.7). An ANOVA test indicated no significant difference in frass production over the trial period (F = 2.02, df 1,30, p = >0.1). It was however noteworthy that the larvae fed on PHA had a higher output of frass even though there was no significant difference in food intake between the two treatments.

3.3.2 Bioassay of *Morus nigra* (black mulberry) lectin (MNA) against tomato moth larvae

As described in section 2.2, larvae of tomato moth (*L. oleracea)* were exposed to MNA at 2% of dietary protein from hatch to 16 days post-hatch, at which stage they were entering the 4^{th} instar. An ANOVA test indicated no significant differences between the mean weights of control and lectin-fed insects (fig 3.8) at any stage during the trial ($F = 0.72$, df 1,20, $p = > 0.1$).

An ANOVA test indicated no statistically significant effect on larval mortality ($F = 0.103$, df 1,30, $p = >0.5$), and there were no effects on development times between the control and MNA-fed *L. oleracea.*

Bioassays of lectins against aphids

3.3.3 Bioassay of *Artocarpus integrifolia* (jackfruit) lectin (jacalin) on the peach potato aphid.

As described in section 2.2.1, neonate *Myzus persicae* aphids were fed on an artificial diet in a feeding chamber for 15 days. Test insects were challenged with jacalin at a dietary concentration of 0.1% w/v. No significant difference in mortality between control and lectin fed insects was observed, both treatments showing a decline in survival of approximately. 20% over the first 7 days of the assay, with no change thereafter. Survival analysis confirmed this conclusion. However, the lectin treatment did have an effect on aphid development. On day six an ANOVA test indicated that jacalin fed aphids were significantly smaller

than the control aphids (fig 3. 9). Aphid length ($F = 11.6$, df 1,40, $p = <0.001$), and width ($F = 8.316$, df 1,4, $p = <0.001$), were decreased by approximately 14%.

3.3.4 Bioassay of *Morus nigra* (black mulberry) lectin (MNA) against the peach potato aphid.

As described in section 2.2.2, neonate *Myzus persicae* aphids were fed on an artificial diet in a feeding chamber for 15 days. Test insects were challenged with MNA at a dietary concentration of 0.1% w/v. As for jacalin no significant difference in mortality was exhibited by lectin-fed insects when compared with controls (fig 3.10), but the MNA-fed insects show a retardation in growth. However, this lectin also had an effect on aphid growth. On day six MNA-fed aphids were reduced by approximately 22% in length, and by approximately. 19% in width (fig 3.11).

An ANOVA test indicated a significant difference in aphid length ($F = 0.57.74$, df 1,40, $p = < 0.001$), and width ($F = 0.56.11$, df 1,40, $p = < 0.001$).

3.4 Discussion

PHA exerts a toxic effect on *L. oleracea* larvae, as demonstrated by the 60% increase in mortality compared to the control treatment. The difference in the mortality rate is only shown during the first 10 days of the trial after which the difference in cumulative hazard is the same for both treatments. The assay shows that PHA is most toxic to neonate larvae, and that the toxicity decreases as the larvae develop, so that by the 3rd instar the lectin no longer has any toxic effect. This effect is directly comparable with the effects of snowdrop lectin (GNA) on *L. oleracea*, which is most toxic to the first two larval instars (Fitches *et al.*, 1997). The toxicity of PHA can also be compared to the effects of other lectins on other lepidopteran species. It is obviously less toxic than *Ricinus communis* agglutinin, which causes 100% mortality to neonate European corn borer *Ostrinia nubialis*, but more toxic than other lectins, for example the lectin from *Phytolacca americana*, which was shown to have no detrimental effect on the same species (Czapla & Lang 1990).

Although PHA can reduce larval survival, it does not appear to retard growth or survival, with no statistically significant differences are shown in this study. Subsequent work has shown that PHA can accelerate *L. oleracea* larval development (Fitches *et al.*, 2001), but that this effect is specific to one of the PHA isoforms, PHA-E.

This is an unusual result as lectins are normally thought to cause inhibition of development rate, as is the case with GNA on *L. oleracea,* GNA caused a 32% drop in the biomass of the *L. oleracea* larvae at the same dietary concentration as PHA in the present assay (Fitches *et al.*, 1997). The present bioassay fails to show statistically significant differences in larval weight over the trial, although the PHA-fed insects consistently weigh more than the control once the insects have gone into the third instar. Once again, subsequent work has shown that this is a real effect, and is due to PHA-E. One possibility for the failure to observe effects on growth and development in this bioassay between PHA and control fed Lepidoptera is possibly due to the PHA sample used being a mixture of the different isoforms, and thus containing 50% of the inactive PHA-L form. The data also suggests that since PHA is having an effect on larval nutrition since larvae fed on PHA produced 18% more frass than control fed larvae. It is possible that PHA affects the normal absorption of nutrients.

The bioassay of MNA with the *L. oleracea* larvae, indicating no significant toxic effects when fed at 2% of total dietary protein, show that the bioassay does not give "false positive" effects.

The effects of *MNA* and Jacalin on the peach potato aphid *M. persicae* were similar. Both lectins were tested at the same concentration, and neither lectin had a detrimental effect upon aphid survival. However, both lectins exerted a significant effect on aphid size, MNA fed aphids were 26% shorter and 22% narrower than control fed aphids, indicating an inhibitory effect of the lectin on the

development of the aphid nymphs. This inhibitory effect on aphid development is also exhibited when the aphid *M. persicae* is fed mannose binding lectins such as GNA (Sauvion *et al.*, 1996). Both *Morus nigra* and Jacalin exhibit the same Galactose (Gal) and N-acetylgalactosamine (GalNAc) binding specificity. Therefore, a hypothesis can be made that the effect on aphid size is due to the binding specificity of the lectin. A correlation between effect on *M. persicae* and lectin binding specificity, has previously been identified by Sauvion *et al* 1996 who noted negative effects of the mannose binding lectins GNA, NPA (daffodil lectin *Narcissus pseudonarcissus*), and ASA (garlic lectin *Allium sativum*) on aphid growth.

Although neither jacalin nor MNA causes an increase in aphid mortality they may still have a potential use in the field of crop protection as part of an integrated system, due to its ability to restrict aphid development. In contrast, MNA does not have any potential in control of *L. oleracea* larvae and provides a further example of the selective toxicity of lectins. This of course indicates the need for toxicity testing of any product to be carried out against a suite of different insects and indeed different species in general. Differences in toxicity of lectins are exhibited in other species. For example the Camels foot tree (*Bauhinia purpurea)* lectin will exert 100% mortality to the European corn borer *Ostrinia nubialis,* but causes only 10% mortality to the Southern corn rootworm *Diabrotica undecimpunctata* (Czapla & Lang 1990). The reason for the difference between species with regard to the toxicity of MNA or *Bauhinia purpurea* lectin requires further

investigation. Toxicity differences may be due to differing roles or abundance of binding receptors in the gut (or indeed elsewhere within the insects) of the two insects. However, until the mechanism by which aphid growth is retarded is determined, it will be difficult to determine the characteristic or mechanism that is either not present or not affected in the *L. oleracea* larvae.

Fig. 3.1 Survival curve and cumulative hazard curve for bioassay of PHA fed to larvae of *L. oleracea* (n = 30) in artificial diet (2% of total protein).

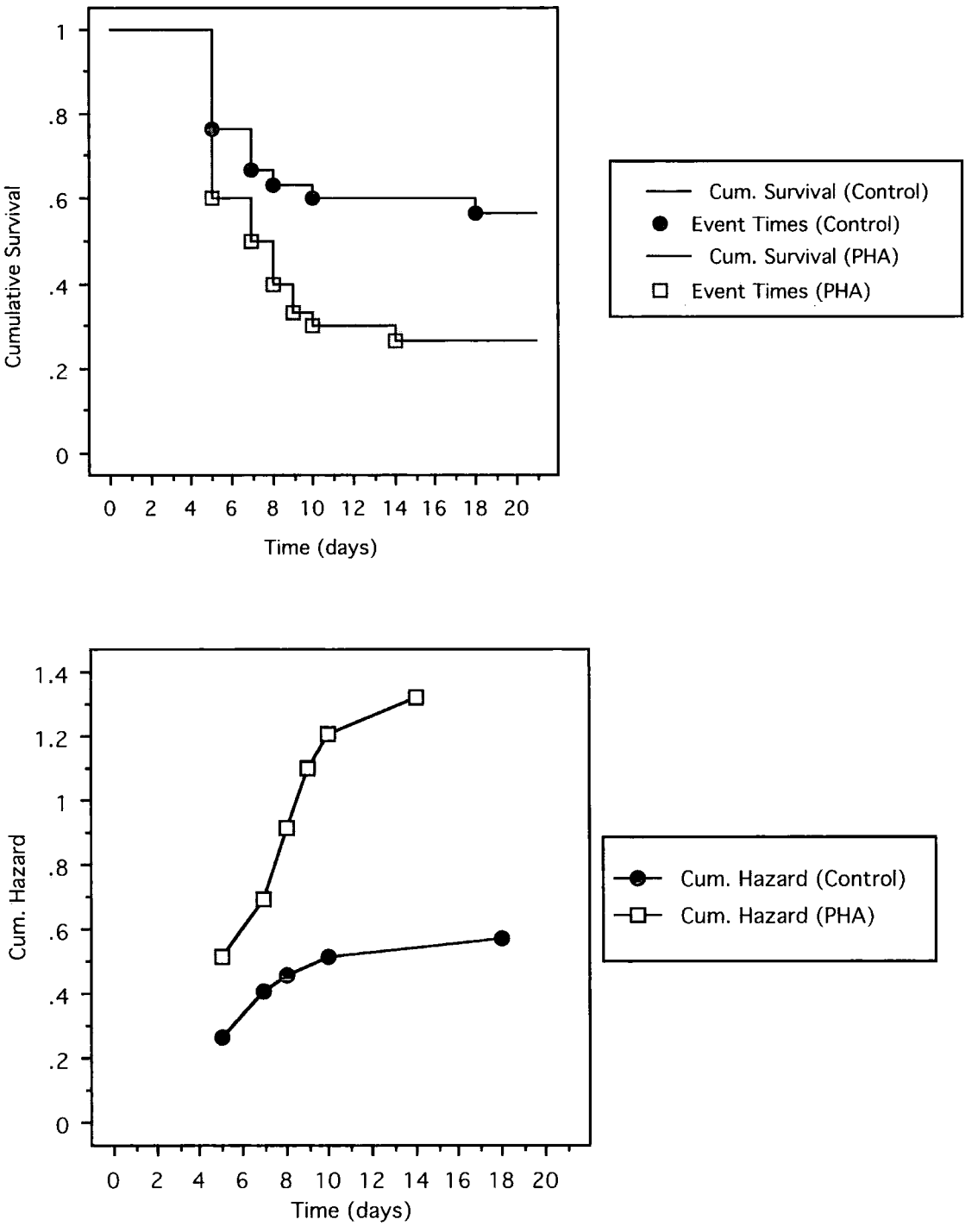


Fig 3.2 Mean weight increase of *L. oleracea* larvae over a 21 day feeding trial. Larvae were fed on either a control artifical diet or an artificial diet containing PHA Bars indicate the standard error within the data set (30 replicates) .

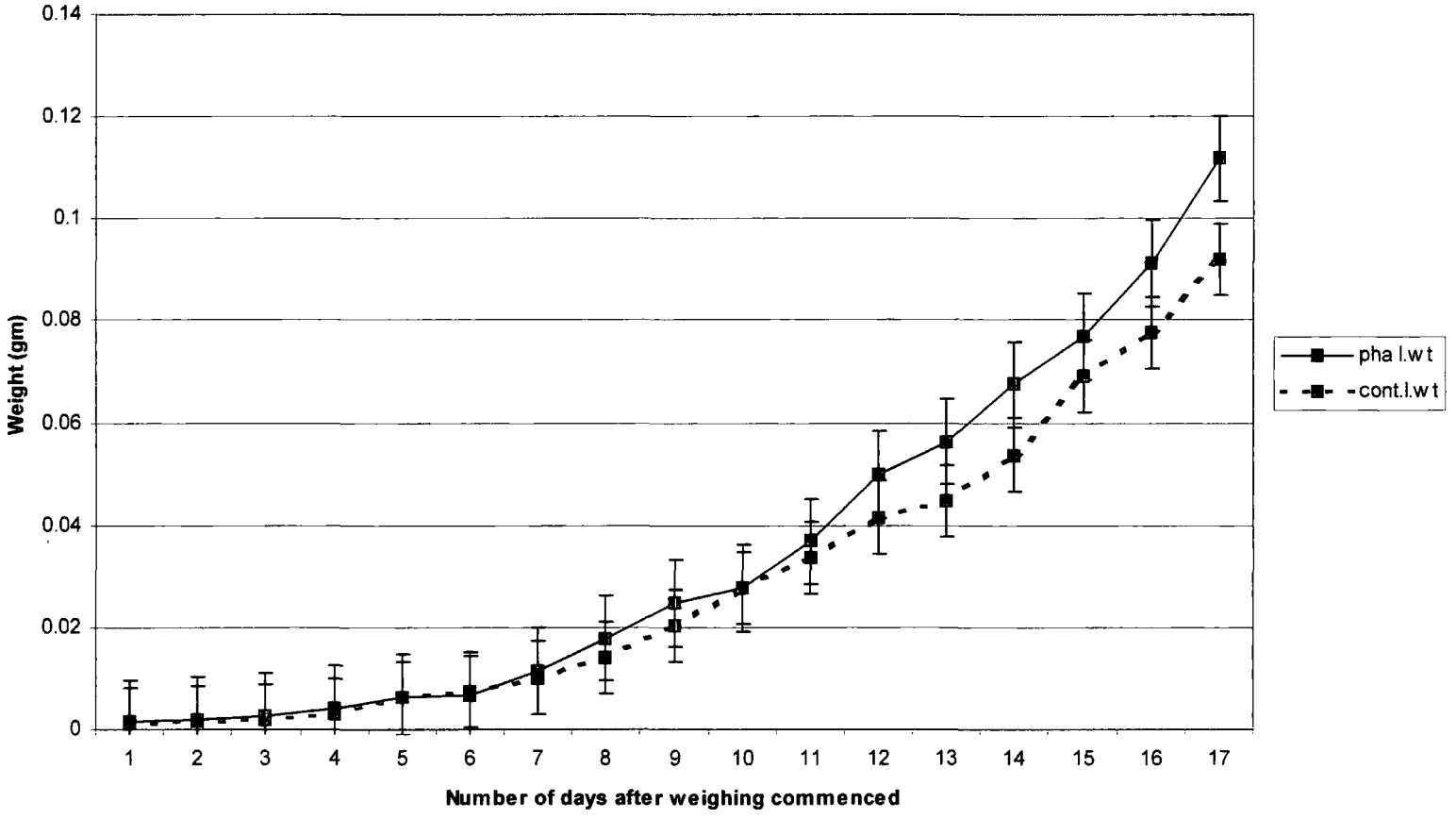


Fig 3.3 The effect of an artificial diet containing PHA on larval development of *L. oleracea* on day 7 of a feeding trial, measured by recording instar stage

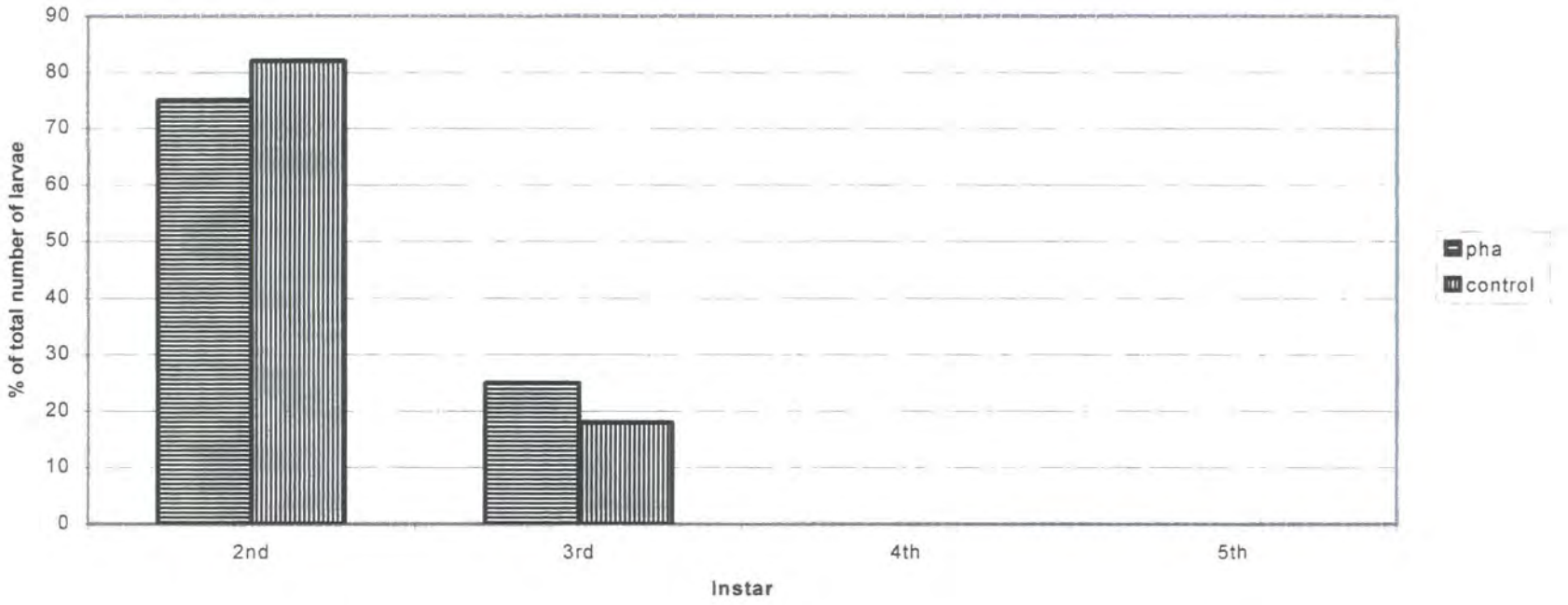


Fig 3.4 The effect of an artificial diet containing PHA on larval development of *L. oleracea* on day 14 of a feeding trial, measured by recording instar stage

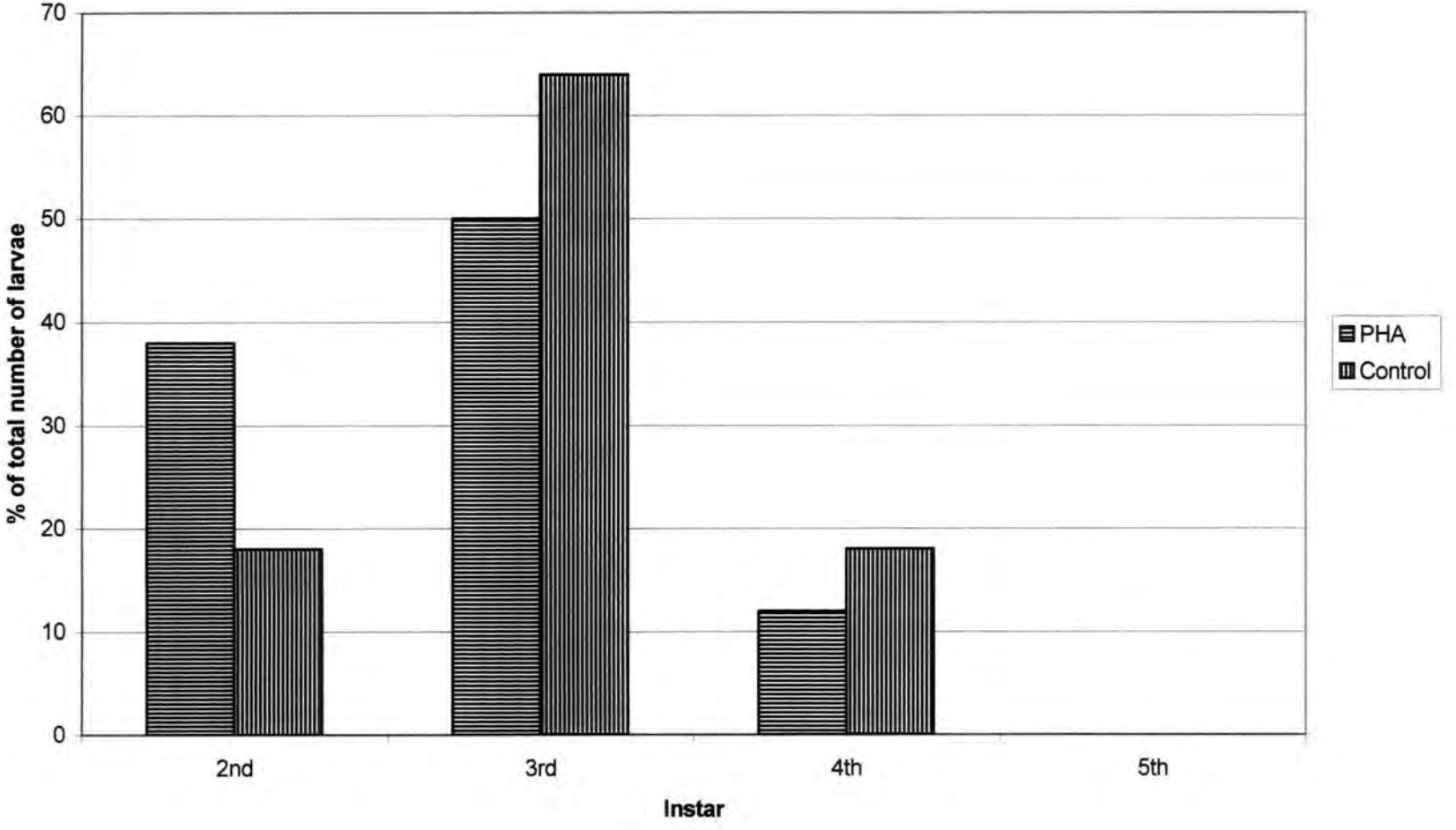


Fig 3.5 The effect of an artificial diet containing PHA on larval development of *L. oleracea* on day 21 of a feeding trial, measured by recording instar stage

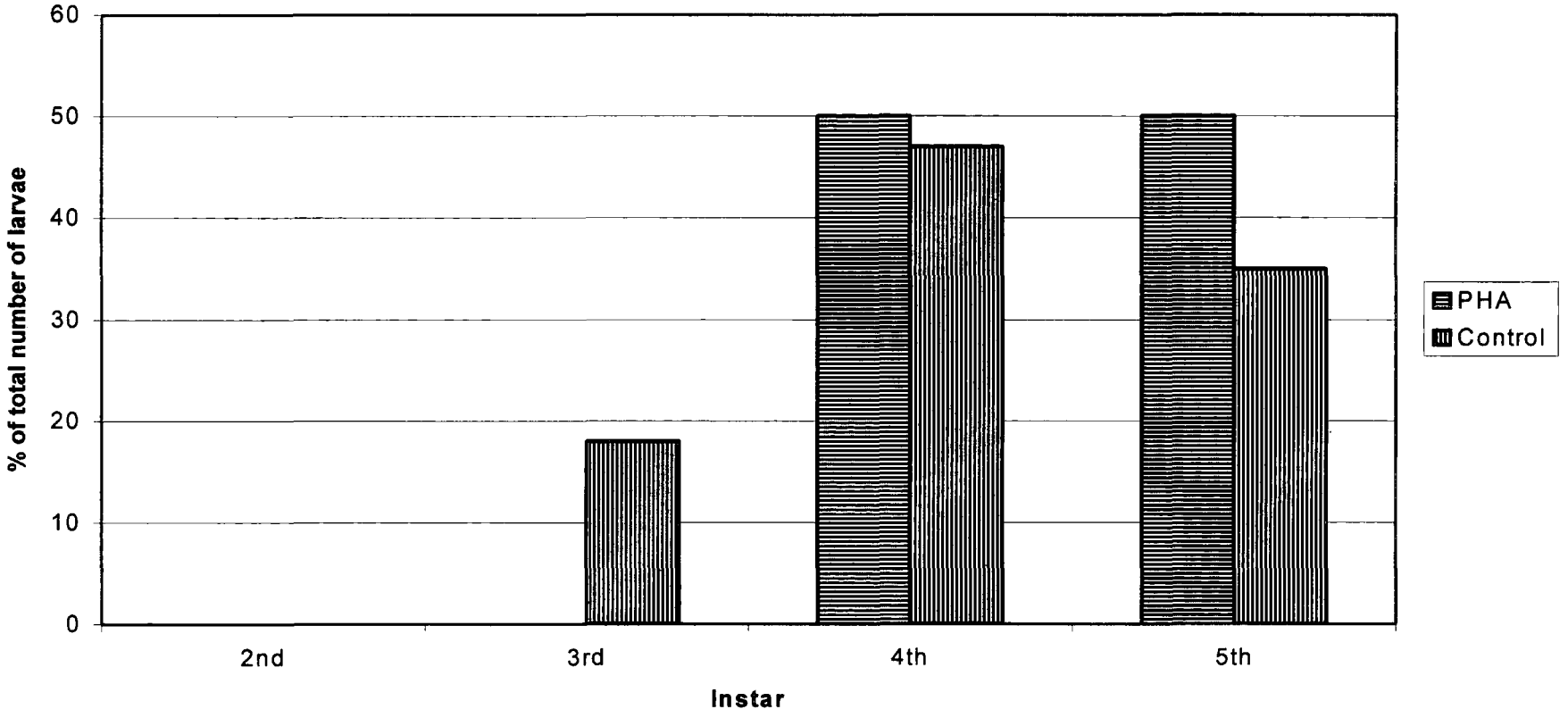


Fig 3.6 Dry weight of control and PHA artificial diet consumed by *L. oleracea* larvae during a 21 day feeding trial, with bars indicating the standard error between measurements on each day (30 replicates).

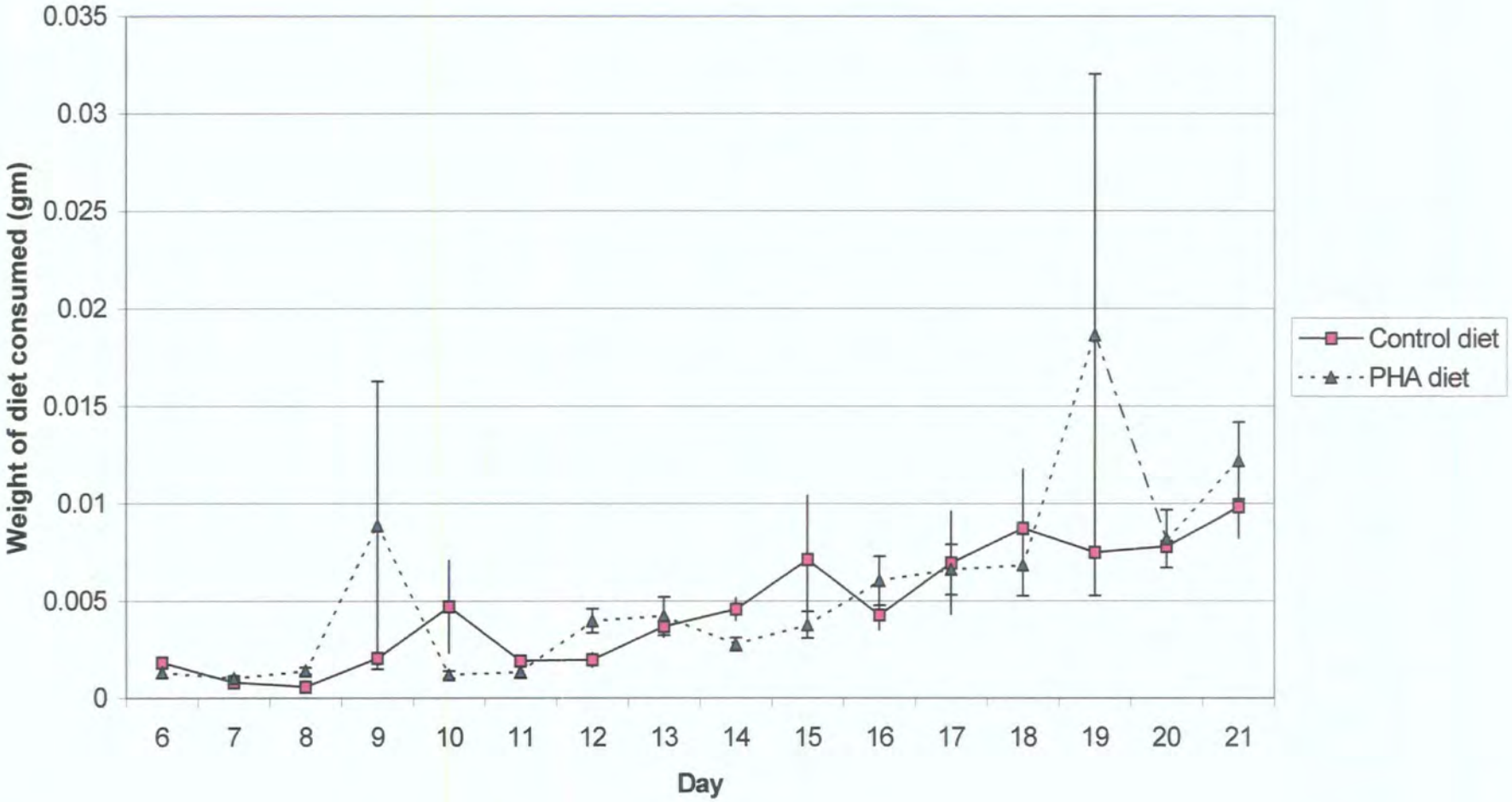


Fig 3.7 Weight of frass produced by *L. oleracea* over a 21 day feeding trial. Larvae were fed on either a control artificial diet or an artificial diet containing PHA at 2% of total protein.

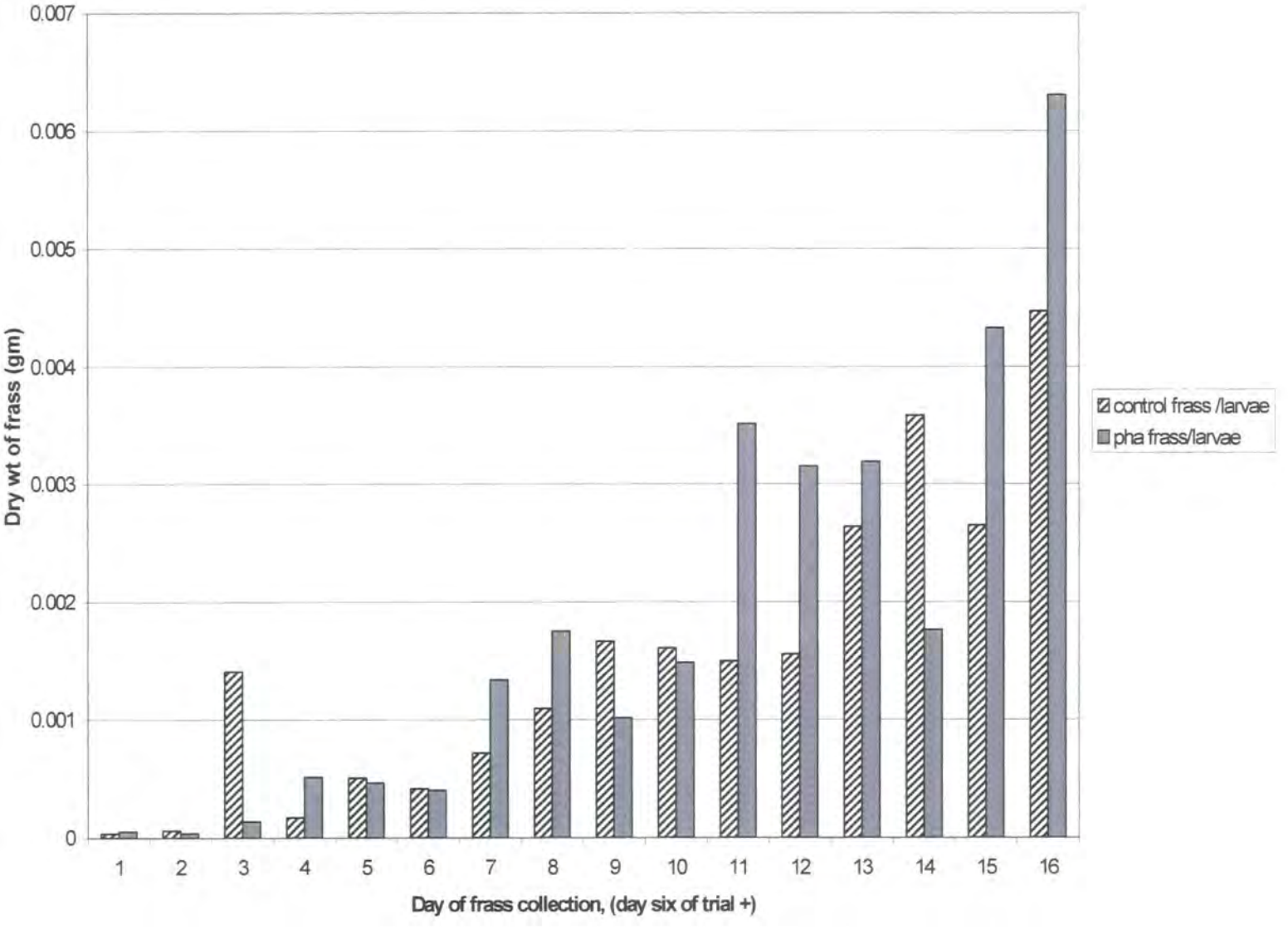


Fig 3.8 The effect of an artificial diet containing *M. nigra* on the weight of *L. oleracea* larvae with bars indicating the standard error at each data point (30 replicates).

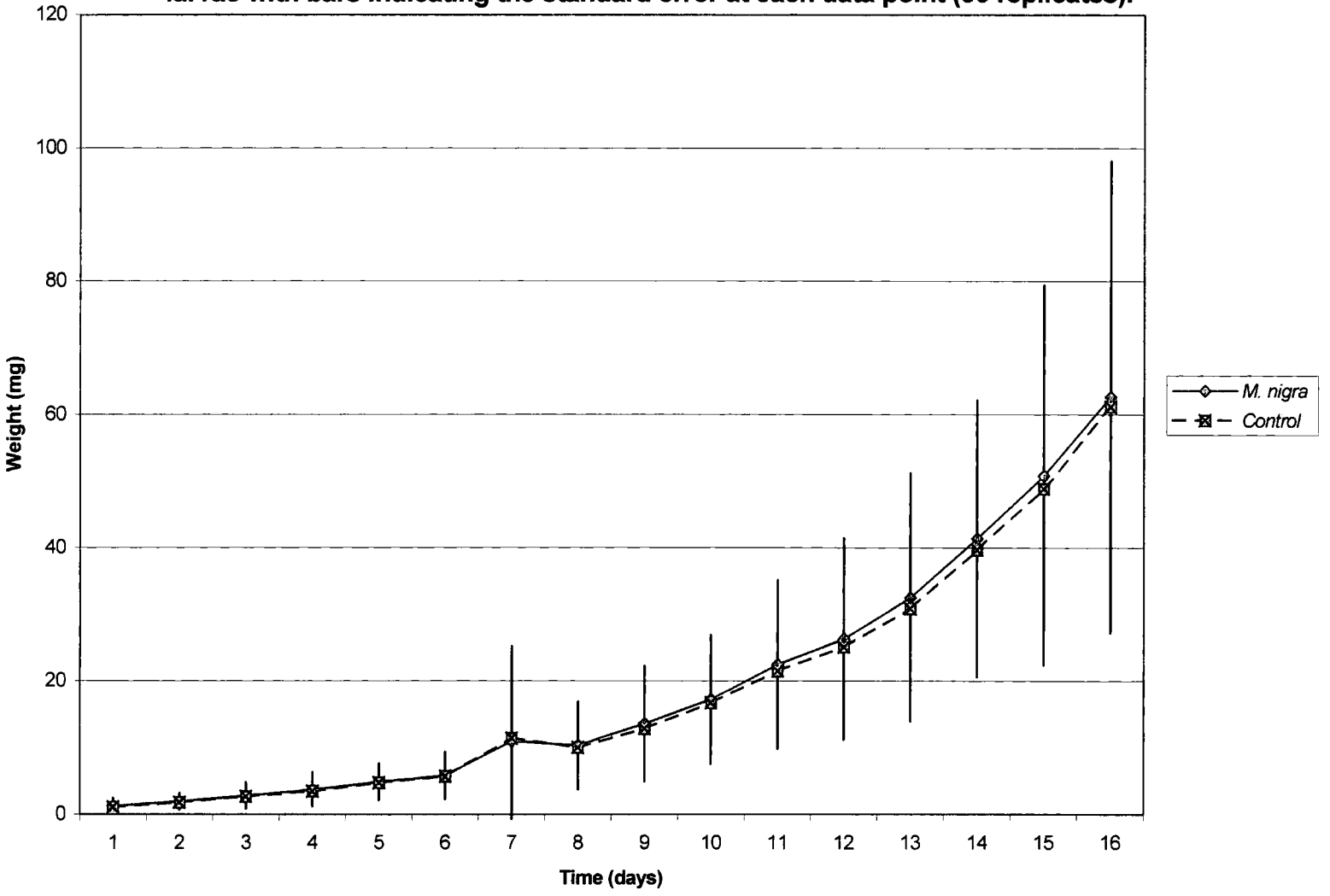


Fig 3.9 Effect of an artificial diet containing Jacalin on the dimensions of *M. persicae* on day 8 of a feeding trial, with bars indicating the standard error at each data point (30 replicates).

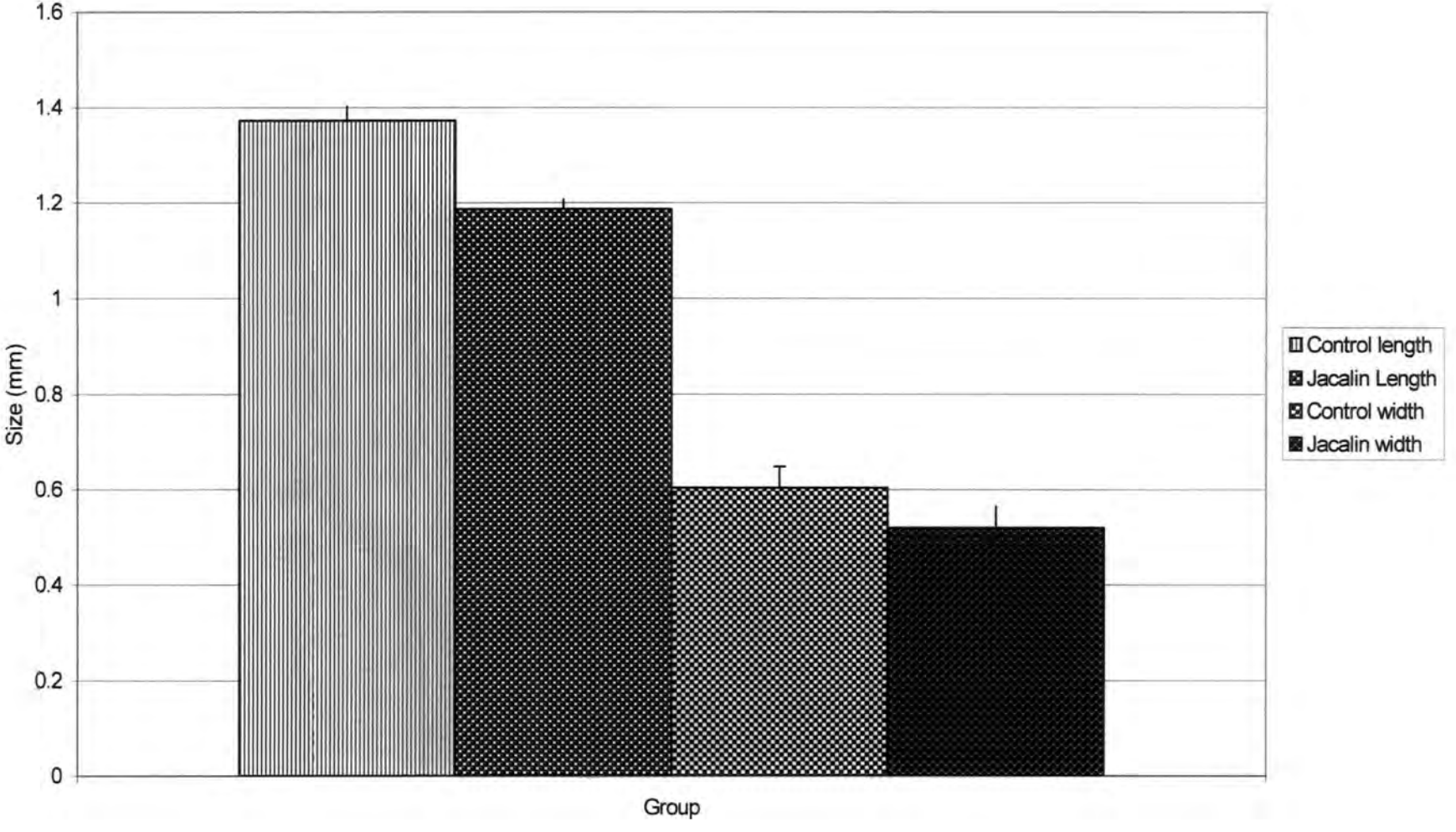


Fig 3.10 Effect of an artificial diet containing *M. nigra* on the survival of *M. persicae*

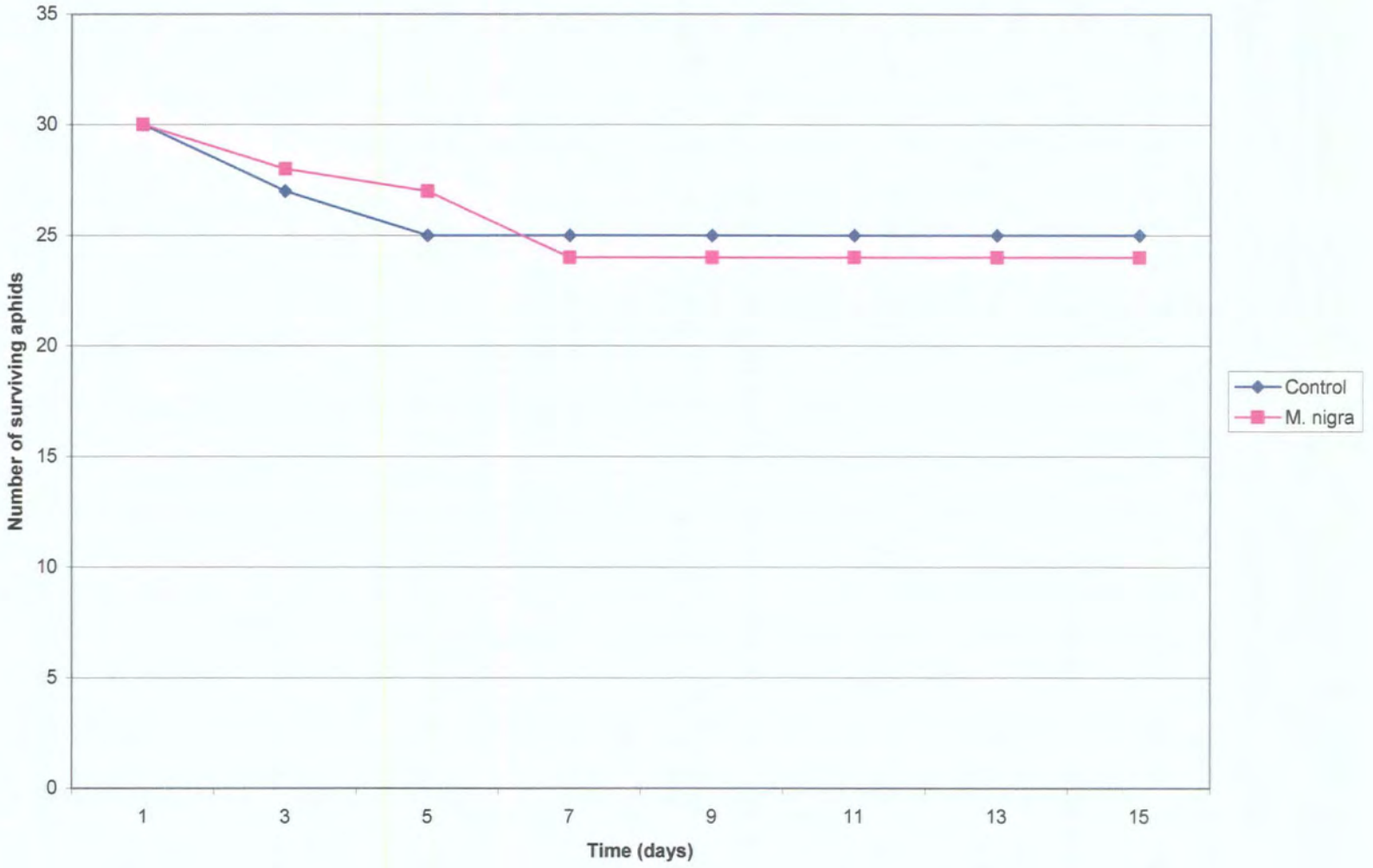
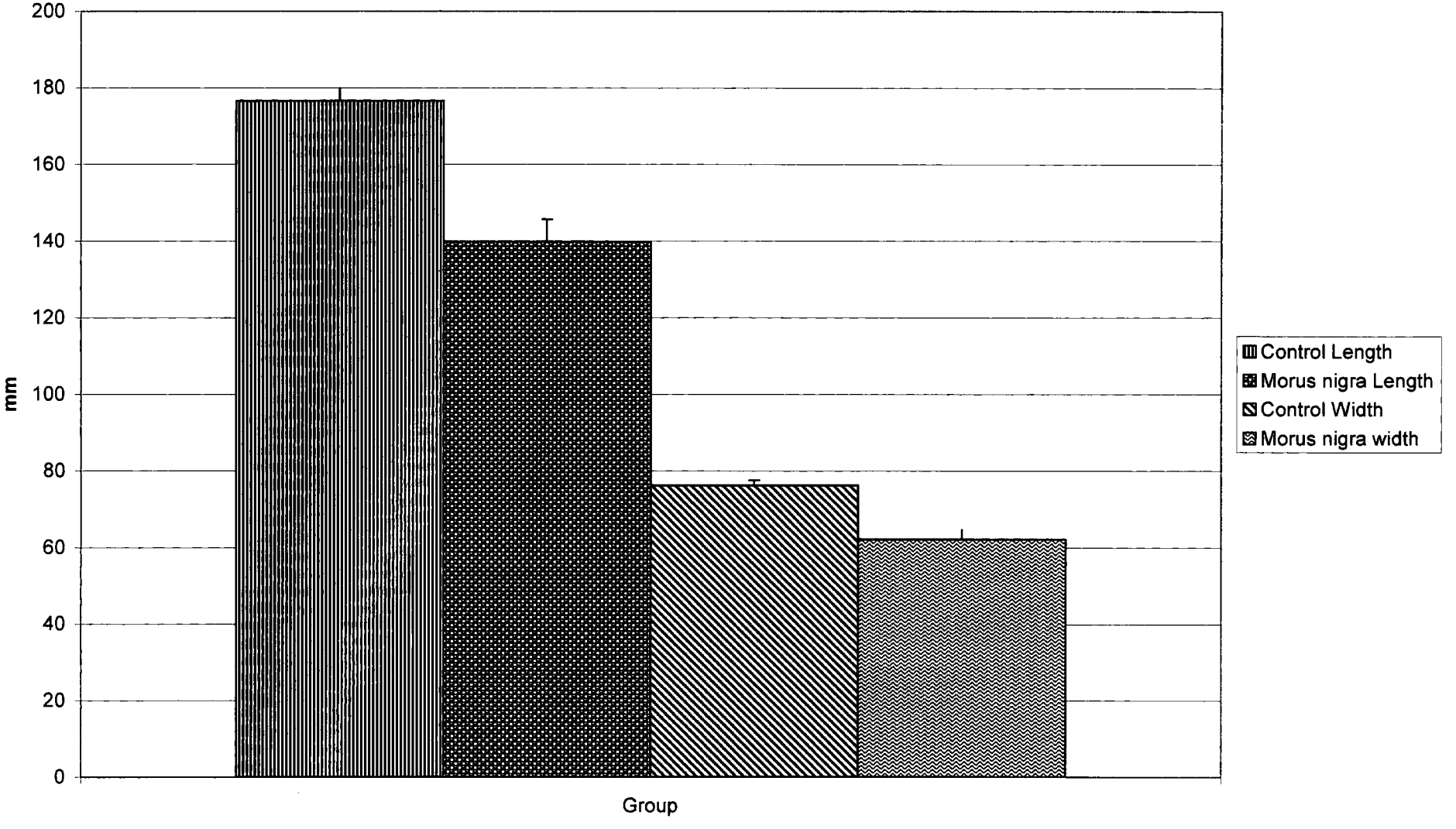


Fig 3.11 The effect of an artificial diet containing *M. nigra* on the size of *M. persicae*, with bars indicating the standard error at each data point (30 replicates) .



Chapter 4

Immunological Studies of Lectin Binding to Gut Tissues and Glycoproteins in Tomato Moth (*L. oleracea*) Larvae

4.1 Introduction

Lectin binding is thought to be a prerequisite for potential toxicity. Determination of lectin binding allows an informed decision with regard to potential toxicity of lectins and hence potential for use in insect control. This chapter describes protocols that were used to demonstrate lectin binding to insect gut tissues, and to perform preliminary characterisation of the glycoproteins in insect gut tissues which act as "receptors" for lectins. The aim of this chapter was to develop methods which could be used to screen novel lectins for their binding activity, and thus potential toxicity, using *L. oleracea* as a model.

4.2 Materials and methods

4.2.1 Immunolocalisation of GNA at the light microscope level using the Vector Elite ABC procedure.

4th instar larvae of *L. oleracea* were fed for 48 hours on artificial diet, either control diet or two test diets containing GNA or Con A at 2% of total protein. Larval midguts were dissected, on ice, flushed with water and immediately placed in fixative (3% paraformaldehyde, 1.25% glutaraldehyde in 0.05M PBS saturated with heptane), and cut into approximatelyimately 1cm pieces. After 30 minutes in the heptane loaded fixative the pieces and placed in fixative (without

heptane) and incubated overnight at room temperature. The gut pieces were dehydrated by incubation for one hour in each of a graded series of ethanol dilutions (12%, 25%, 50%, 75% and 100%). Gut pieces were then placed in a 100% ethanol/LR white resin mix 50:50 for 3 hours. Gut sections were then incubated for 2 days at room temperature in pure LR white resin. The resin was changed every 3 –4 hours during the day. Gut fragments were embedded in capsules containing LR white resin at 50-55°C for 24hours. All incubation steps were carried out on a rotating wheel.

Semi-thick sections (120nm) were cut using a microtome. Sections were mounted onto polysine slides and dried on a hotplate. Slides were incubated with GNA antibody at dilutions of either 1:5000 or 1:10000 overnight at 4°C in a moist environment. Detection of bound GNA on the slides was carried out using the Vector Elite ABC kit according to the manufacturers instructions. The final colour development gave a brown stain, indicating presence of GNA.

4.2.2 Localisation of lectin binding in *L. oleracea* at the electron microscope level.

Larvae were fed control diets, or lectin containing diets as described above (section 4.2.1), except that the peritrophic membrane was removed, and foregut, midgut, hindgut and malpighian tubule tissue was harvested and fixed as described. Ultra thin sections (60-80nm thick) were cut on a microtome (Leica Ultracut) and mounted on formvar and carbon coated nickel grids (150 mesh, hexagonal). For immunohistochemical labelling, sections were incubated for 10

minutes in heat inactivated goat serum before incubating overnight at 4°C in a 1:100 dilution of antibody raised against recombinant GNA in Tris-HCl buffer pH 7.5.

Sections were washed four times each for five minutes in Tris-HCl buffer pH 7.5, and once for five minutes in Tris-HCl buffer pH 8.2, before incubating for 1 hour in anti-rabbit 10nm gold-conjugated IgG (1:20 dilution in Tris-HCl buffer pH 8.2). The above Tris-buffers were prepared according to the guidelines sent with the gold conjugate (Agar Scientific, Stansted), except that the sodium azide was omitted. Sections were washed once for five minutes in Tris-HCl pH 8.2 and four times each for five minutes in distilled water, prior to staining for 10 minutes in 1% aqueous uranyl acetate and 10 minutes in lead citrate (Reynolds, 1963); sections were washed between and after staining five times each for five minutes, in distilled water. Sections were viewed with a transmission electron microscope (Philips EM400T).

4.2.3 Fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC) labelling of proteins.

Lectins were labelled with fluorochromes according to the methods set out by Haaijmen (1985) with the following modifications: the starting concentration of the lectin dissolved in distilled water was 2mg/ml giving a fluorochrome concentration in the conjugation reaction of 50μg FITC/RITC per mg protein. The conjugated protein was purified by gel filtration on a Sephadex G50 column, using PBS as solvent; protein eluted in the first fluorescent peak.

4.2.4 Detection of GNA-RITC on tissue blots of *L. oleracea*.

Fourth instar *L. oleracea* larvae were cut horizontally with a scalpel across the body providing a circular surface which was briefly blotted to remove excess moisture, and then pressed onto a polysine coated slide to make a small imprint. Slides were left to air dry and then incubated for 30 min in GNA-FITC, GNA-RITC or BSA-FITC/RITC. After 30 minutes the slides were washed with distilled water to remove unbound protein and viewed with a Nikon diaphot UV microscope.

4.2.5 Localisation of GNA on the peritrophic membrane of *L. oleracea*

The peritrophic membrane from fourth instar *L. oleracea* larva was dissected out and flushed with distilled water. The peritrophic membranes were incubated for 30 min in GNA-FITC 1mg/ml in PBS, or BSA-FITC 1mg/ml as a control. Unbound protein was washed off the sections with distilled water. The slides were viewed with a Nikon diaphot UV microscope.

4.2.6 GUT fresh tissue: GNA-FITC localisation.

Midgut were dissected from fourth instar *L. oleracea* larvae, the contents were flushed out with distilled water and the peritrophic membrane removed. The sections were placed in fixative overnight at room temperature, dissected ventrally to "splay" open the gut and mounted on slides. Sections were incubated in sudan black for 30min, and washed with distilled water to remove excess stain. The sections were incubated in either GNA-FITC, GNA-RITC, BSA-RITC or BSA-

FITC for 30 minutes. After washing off excess protein, sections were viewed with a Nikon diaphot UV microscope.

4.2.7 Localisation of GNA binding sites using GNA-FITC on embedded sections of *L. oleracea*

The feeding fixation and embedding procedure are the same as described for immunolocalisation at the LM level (section 4.2.1). Sections taken (120µm thick) were mounted onto polysine slides. The LR White sections were incubated in either GNA-FITC; distilled water or BSA-FITC for 30 min and washed with distilled water. Additional sections were incubated in 1mM α- methyl mannopyranoside for 30 minutes, washed with distilled water and incubated in GNA-FITC for 30min before washing again with distilled water. Sections were viewed using a Nikon diaphot UV microscope (using blue, and neutral density filters).

4.2.8 Detection of lectins on gut sections from *L. oleracea.*

Guts were dissected from fourth instar *L. oleracea* larvae, flushed with distilled water and the peritrophic membrane was removed. Gut sections were cut into 2cm pieces and incubated overnight 100% dry ethanol. Sections were filled with a solution of GNA-FITC/GNA-RITC or PBS, and incubated for 30 minutes at room temperature.

Unbound GNA-FITC/RITC was washed of and the sections were cut into parts, placed end down onto glass slides and viewed with a Nikon diaphot UV microscope.

4.2.9 Crude brush border membrane vesicle preparation (BBMV) preparation from Rat intestine

The rat was killed according to regulations and the small intestine was dissected out. Sections from the top and bottom end of the small intestine were removed and gut contents were flushed out using distilled water. One end of the small intestine was sealed with a dialysis clip, and the intestine was filled with 2× SDS sample buffer and sealed with a second dialysis clip. The gut section was incubated for 2hours at 4°C with occasional agitation after which the SDS-sample buffer (containing the BBMV) was removed and stored at -20°C.

4.2.10 Crude brush border membrane vesicle preparation (BBMV) preparation from *L. oleracea*

Larvae were chilled on ice to immobilise and then guts were dissected out and flushed with an ice cold solution of buffer A (store at 4°C) (300mM mannitol, 5mM EDTA, 17mM Tris –Hcl pH 7.5, 50μg/ml leupeptin). Guts were collated into groups of 10 in an eppendorf with 500μl buffer A, homogenised and frozen in liquid nitrogen.

4.2.11 Brush border membrane vesicle preparation (BBMV) of *L. oleracea* midgut

Guts were collected as described in section 4.2.10. 60 guts were homogenated in 3ml of buffer A, using a polytron homogeniser for 3x5 sec bursts at speed 8. The sample was left to stand on ice for 15 min and then centrifuged at 4500rpm (2500g) for 15min at 4°C. The supernatant was decanted off and centrifuged at 16,000rpm (30,000g) for 30min, this time keeping the pellet and storing the supernatant on ice.

The pellet was resuspended in0.5 volumes (relative to homogenate of ice cold buffer A and 1.5ml MgCl, and centrifuged as before, The pellet from the 2nd 16,000rpm spin was collected as BBMV. It was resuspended with aid of the polytron in 0.5x buffer A 4°C. The first and second 4,500rpm pellets were then combined and resuspended in original homogenate volume of 0.5 strength buffer A. This is the cell debris fraction (B). The first and second 16,000rpm supernatant fractions (C & D) constitute cytoplasmic soluble proteins. To create the soluble BBMV fraction, the BBMV was resuspended in 3ml of 0.5x buffer A, and 960µl of solubilisation buffer and 40µl 2% Triton X-100 were added.

4.2.12 Binding of GNA and Con A to gut proteins in *L. oleracea* larvae.

Binding of GNA and Con A to crude gut protein extraction

Crude extracts of protein were taken from the intestine of a rat and from the midgut of *L. oleracea* as described in section 4.2.9 and 4.2.10 respectively. Protein samples was boiled for 5 minutes in the presence of 2xSDS sample

buffer and 10% ß-mercaptoethanol. 10µg of each protein sample was separated on duplicate 12.5% polyacrylamide gels by SDS-PAGE (SDS-polyacrylamide gel electrophoresis) as described in section 2.4. Proteins on one gel were visualised by Coomassie blue staining, and proteins on the second gel were transferred to a nitrocellulose filter (Schleicher and Schuell BA85) by electroblotting (as described in section 2.5-6). The filter was probed with either Con A or GNA at 0.1mg/ml; bound lectin was subsequently detected using anti Con A or anti GNA antibodies. Bound antibodies were detected using peroxidase labelled secondary antibodies, followed by treatment with ECL reagents (according to manufacturers instructions) and exposure to X-ray film.

Binding of Con A to BBMV protein fraction

10µg samples of the BBMV fractions (prepared as in section 4.2.11) sample were separated on duplicate 12.5% polyacrylamide gels by SDS-PAGE (SDS-polyacrylamide gel electrophoresis) as described in section 2.4. Proteins on one gel were visualised by Coomassie blue staining, and proteins on the second gel were transferred to a nitrocellulose filter (Schleicher and Schuell BA85) by electroblotting (as described in section 2.5-6). The filter was probed with Con A at 0.1mg/ml; bound lectin was subsequently detected using anti Con A

antibodies. Bound antibodies were detected using peroxidase labelled secondary antibodies, followed by treatment with ECL reagents and exposure to X-ray film.

4.2.13 Detection of PHA binding to the midgut of the *L. oleracea* larvae.

Fourth instar *L. oleracea* larvae were fed on an artificial diet containing digoxygenin (DIG) - labelled PHA at 2% of total protein for 3 days and then fed with control artificial diet for 1 day as a "chase" to remove unbound PHA from the gut (diet information in section 3.2.1). Larvae were sacrificed and dissected on ice, the peritrophic membrane removed and the guts flushed with water. Gut sections were homogenised in Tris-HCl pH 8. Each sample was boiled for 10 min in 4x SDS-sample buffer in the presence of 10% ß-mercaptoethanol. 20µg aliquots of protein were separated on duplicate SDS-PAGE gels (see section 2.5-6). Proteins on one gel were visualised by Coomassie blue staining, and proteins on the second gel were transferred to a nitrocellulose filter (Schleicher and Schuell BA85) by electroblotting (see section 2.7). The filter was probed with anti-DIG antibodies and bound antibodies were detected using the DIG Glycan Differentiation Kit (Roche).

4.2.14 Detection of PHA binding to the gut of *M. persicae*.

Two sets of 20 mature *M. persicae* aphids were fed on an artificial diet for 48 hours, one diet contained DIG-labelled PHA and the other diet was a control, as set out in section 3.2.2. After the initial 48 hours aphids were fed with a control diet alone for a further 24 hours to "chase" labelled antibody which was not

bound to gut components from the gut. Guts were dissected from the nymphs, homogenised in Tris-HCL pH 8 and the presence of PHA was determined as set out in section 4.2.13.

4.3 RESULTS

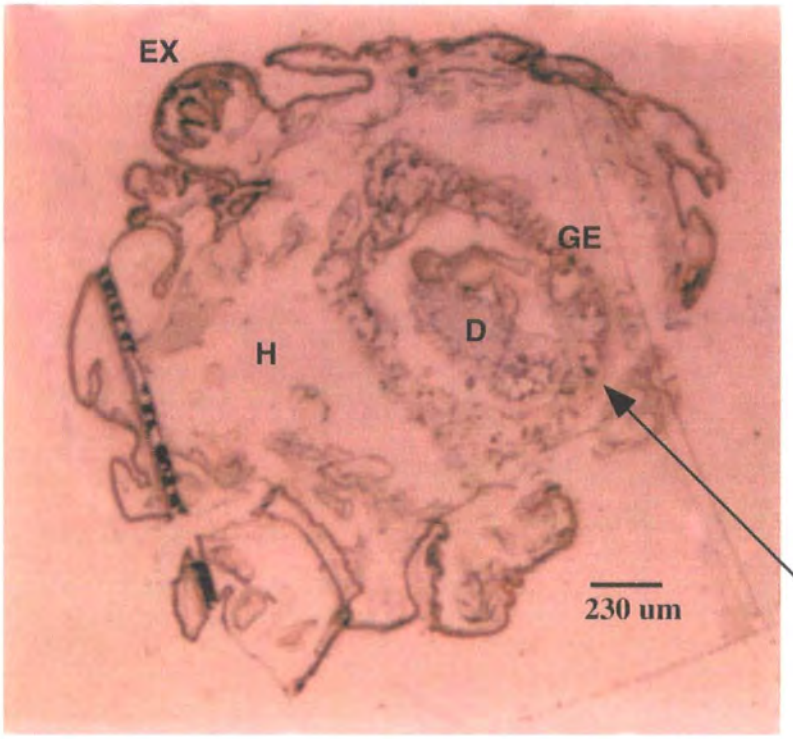
All sections shown are representative samples from ten different individuals exposed to each treatment.

4.3.1 Immunolocalisation of GNA fed to insects *in vivo* at the light microscope level.

Fourth instar larvae of tomato moth were fed on lectin-containing diets (with appropriate controls) and the presence of lectin in tissue sections after fixing and washing was detected immunologically. Plate 4.1.1 shows a whole transverse section (TS) of a midgut region from larvae fed on diet containing GNA at low power (×4 objective lens). A magnification of the section is shown in plate 4.1.2 (x10 objective lens). Dark brown colouration (indicating the presence of GNA) can be seen across the section, but is concentrated in three main areas. Looking at the section from centre out, the presence of GNA (brown staining) in the centre is that present within the undigested diet, which has been fixed by the processing technique. A circular structure lies outside the central region of the section, which has stained for the presence of GNA relatively weakly in most areas, although one or two patches of more intense staining are visible. This

structure is the gut epithelium and the surrounding musculature, and the staining pattern suggests that GNA has bound to this tissue, and has been either

Plate 4.1.1 T.S of the midgut of *L. oleracea* larvae fed on artificial diet containing GNA at 2% of total protein.
GNA was detected immunologically using peroxidase-linked secondary antibodies.



Legend:
D = Diet
GE = Gut Epithelium
H = Haemolymph
EX = Exoskelton

Dark brown colouration indicating presence of GNA

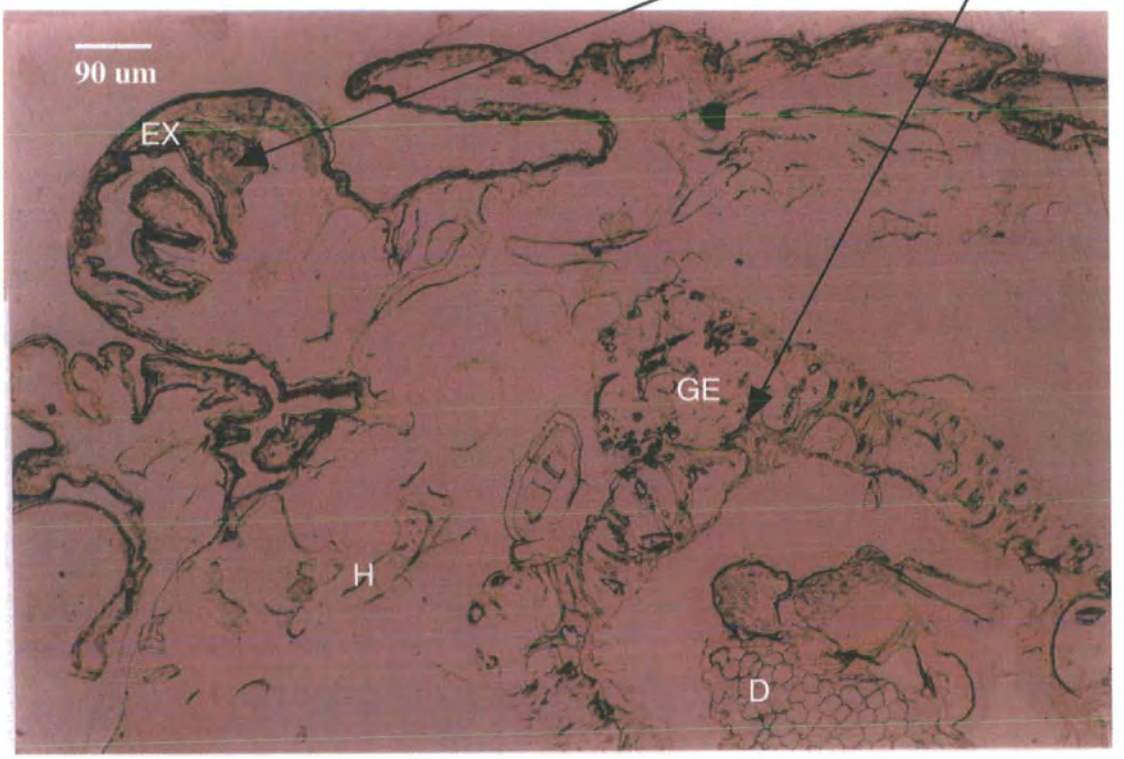


Plate 4.1.2 part of the T.S in plate 4.1.1 (midgut of *L. oleracea* larvae fed on GNA artificial diet).

transported across it, or has been able to pass through the tissue passively, resulting in staining throughout this gut tissue. Little staining is evident in the region between the gut and the outer exoskeleton; most of this region is haemolymph. The outer exoskeleton is strongly stained, but much of this brown colouration is probably an artefact of the staining system, since control sections also showed staining in this region, even when the primary and secondary antibodies were omitted from the procedure.

Plate 4.1.2 is the same section as plate 4.1.1 but at an increased magnification (x10 objective lens) in which the brown colouration (indicating the GNA), seen in plate 4.1.1 can be seen more clearly. Plate 4.1.3 shows a section from the midgut of a non GNA fed *L. oleracea* larvae. The section was incubated for 4 hours at room temperature in a 1mg/ml solution of GNA and then subjected to the same staining procedure as the sections in plates 4.1.1 and 4.1.2. As before in plates 4.1.1 and 4.1.2, brown colouration indicating the presence of GNA is exhibited across the section.

Plate 4.1.4 shows a section of midgut from a control fed *L. oleracea* larvae, subjected to the same staining procedure as the sections in plates 4.1.1 and 4.1.2. The lack of the development of a brown colouration in plate 4.1.4 acts as a control verifying the specificity of the GNA antibody.

Plate 4.1.5 is a gut section from GNA fed *L. oleracea* at high magnification (×40 objective lens) not incubated in primary antibody. As no brown colouration is exhibited one can conclude that the colouration in previous plates is attributable to the specificity of the anti GNA antibody.

Plate 4.1.3 section of midgut of *L. oleracea* larvae fed on control artificial diet. Section was incubated in GNA at 1mg/ml. GNA was detected using the ABC kit from Vector labs

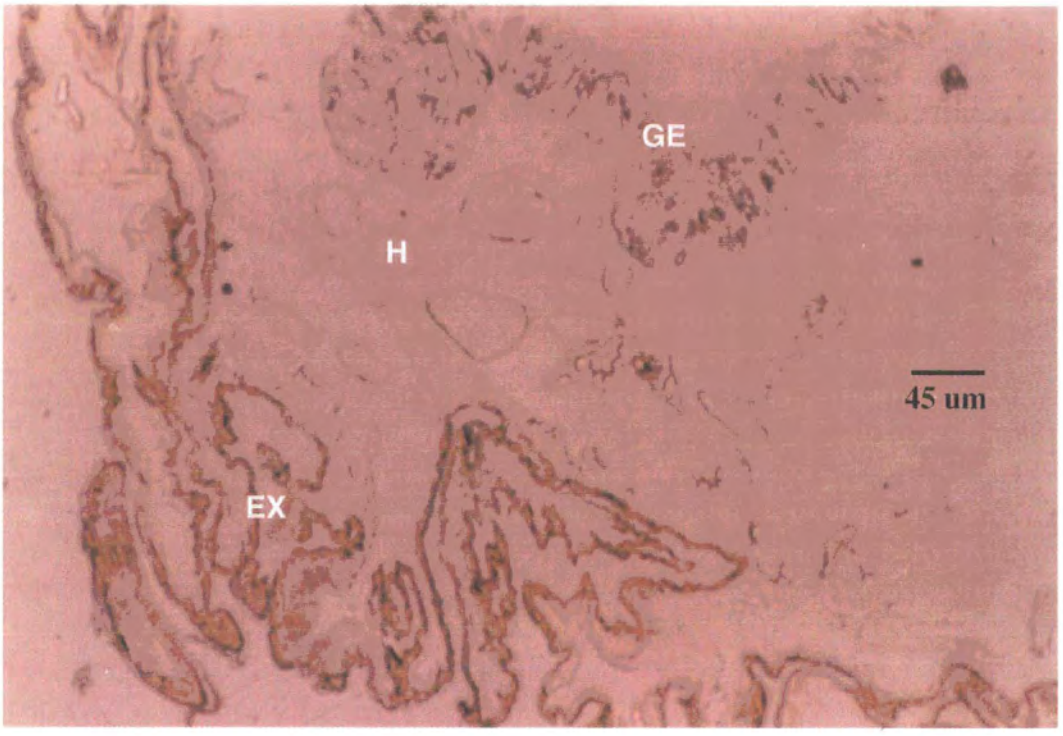


Plate 4.1.4 section of midgut of *L. oleracea* larvae fed on control artificial diet and subjected to the same staining procedure as section from GNA fed larvae (plate 4.1.1).

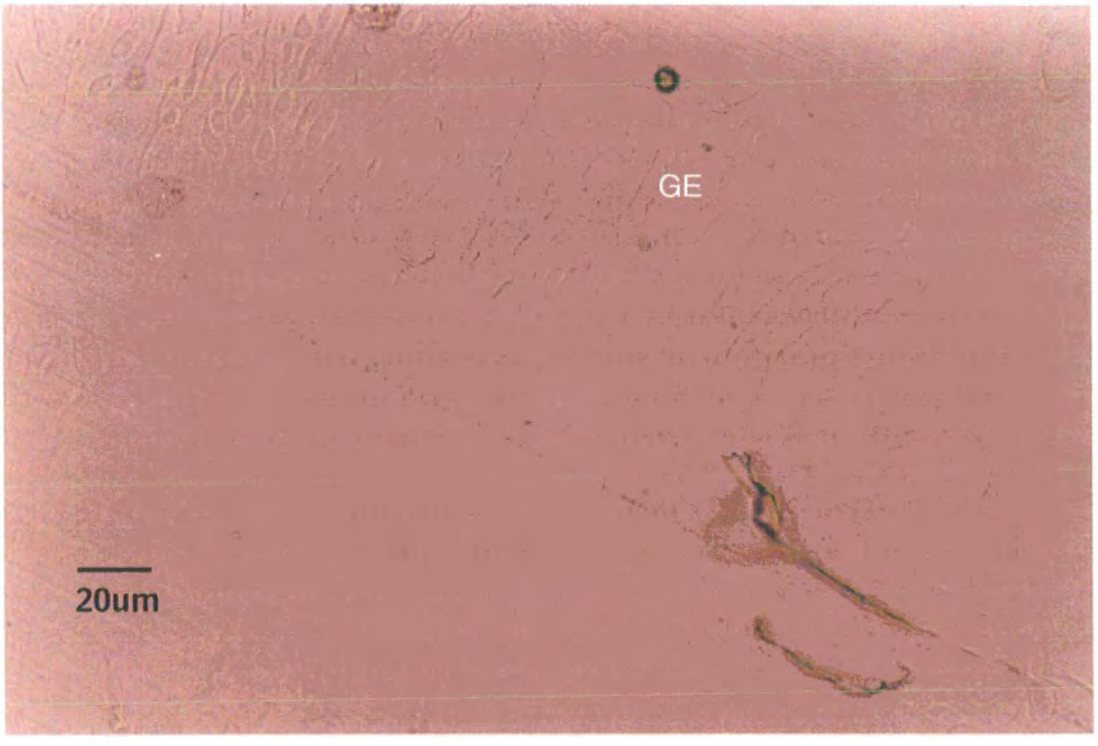
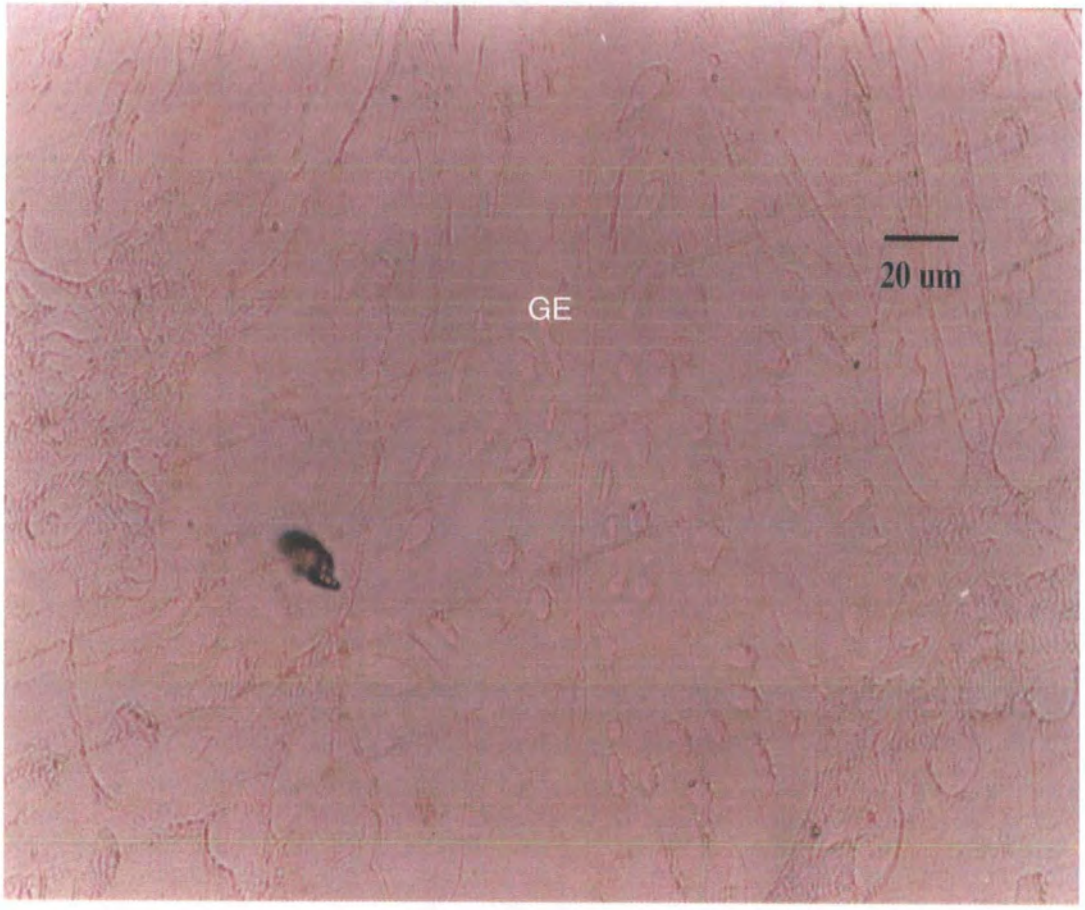


Plate 4.1.5 section of midgut from *L. oleracea* larvae fed on GNA at 2% of total protein in an artificial diet, the section followed the same staining procedure as plate 4.1.1 using the ABC kit from Vector labs except the primary antibody was omitted.



4.3.2 *In vitro* Localisation of GNA and Con A Binding, by Immunohistochemistry at the Electron Microscope Level.

As described in section 4.2.2 *L. oleracea* larvae were fed on an artificial diet containing either Con A, GNA at 2% of total protein or control diet (containing additional casein). The larval gut was dissected into its constituent parts i.e. foregut, midgut, hindgut and malpighian tubules and then fixed and embedded in LR-white resin. Following this sections were taken from different areas along the length of the gut and subjected to the immuno-gold staining procedure described in section 4.2.2. The resultant sections were viewed on a Phillips transmission electron microscope.

Effect of GNA and Con A on gut ultrastructure

Plate 4.2.1 shows sections from the midgut area of the *L. oleracea* larvae fed on either GNA, Con A or control diet. It is clear that neither Con A or GNA causes disruption of the epithelial membrane of *L. oleracea* larvae in the short term.

In vitro localisation of GNA in *L. oleracea* larvae.

Foregut sections from the GNA fed larvae show gold particles (indicating lectin binding) along the microvilli as well as across the other side of the gut epithelium in the cytosol (plate 4.2.2). No gold particles were apparent in the control sections (plate 4.2.2).

In midgut sections from GNA fed larvae gold particles, indicating the presence of GNA, were found along the microvilli, as well as in the cytosol on the other side

of the midgut epithelium (plate 4.2.3). No gold particles were observed on the control section (plate 4.2.3).

Fig 4.2.1 Low power electron micrographs of sections from the midgut of *L. oleracea* larvae fed on an artificial diet containing GNA, Con-A or a casein control diet.

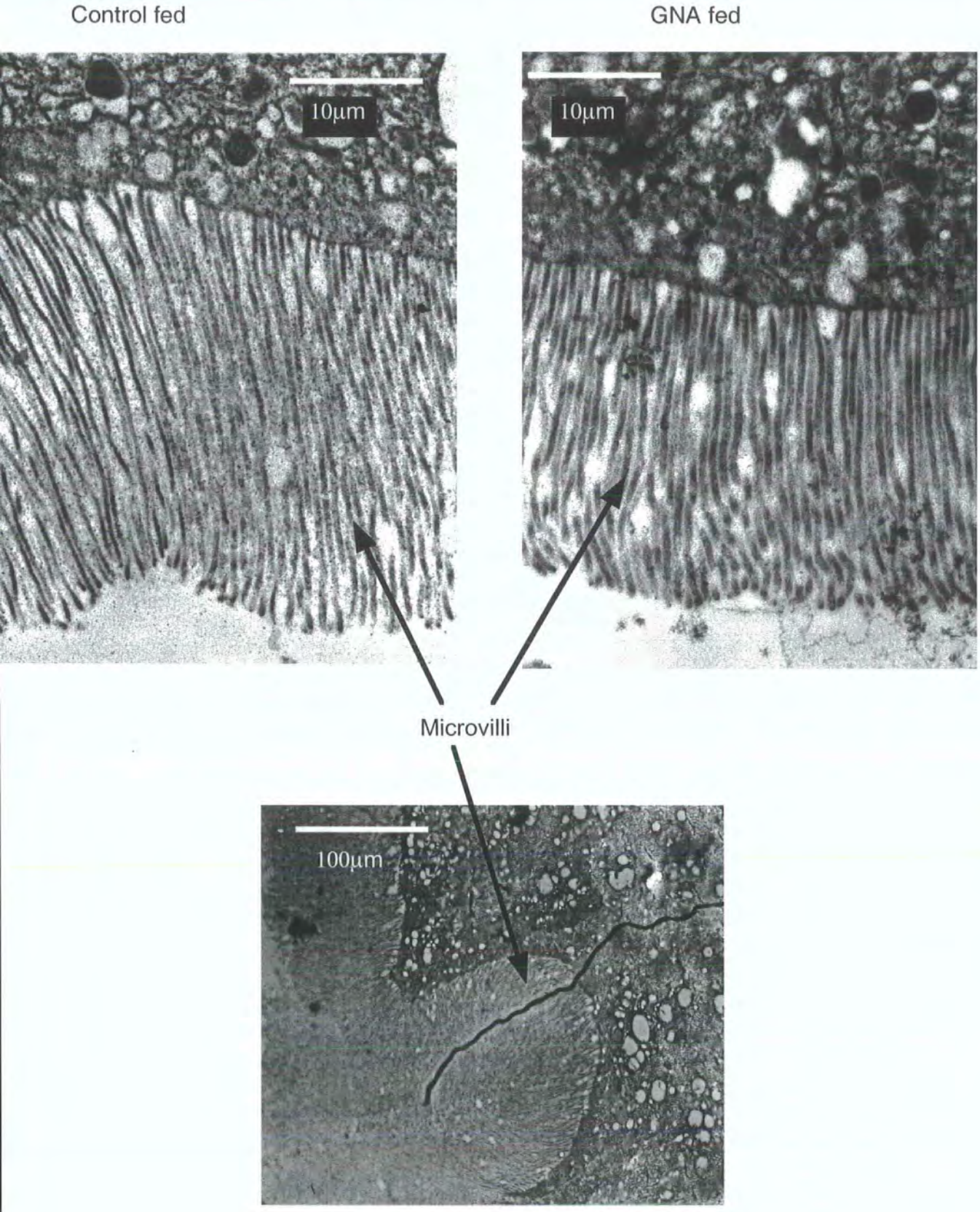
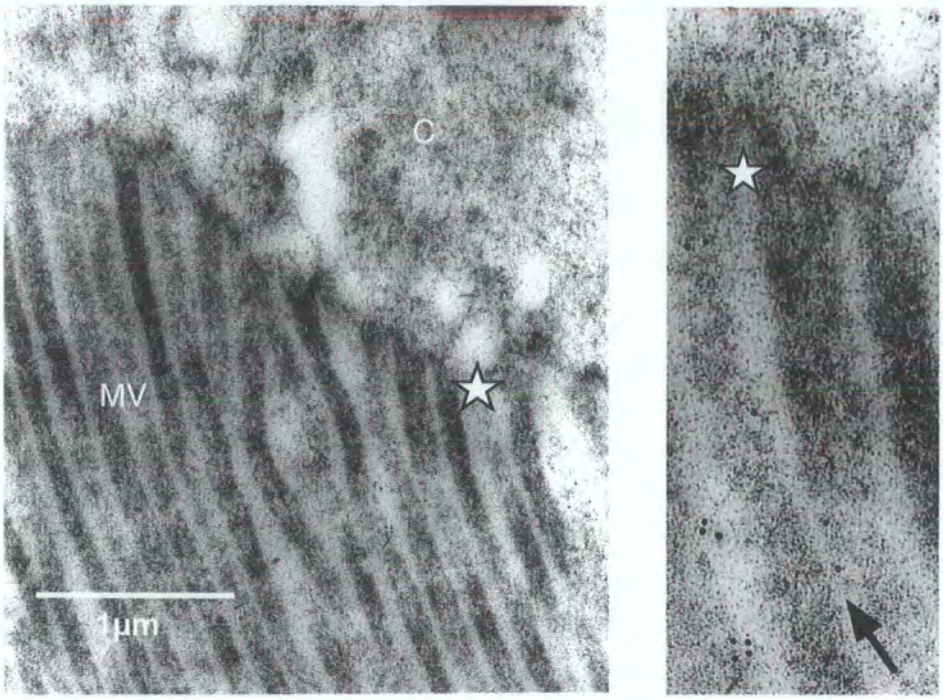


Fig 4.2.2 electron micrographs of the foregut of *L. oleracea* fed on either a control artificial diet or an artificial diet containing GNA at 2% of total protein. Sections were subjected to the immunogold staining procedure to detect the presence of GNA



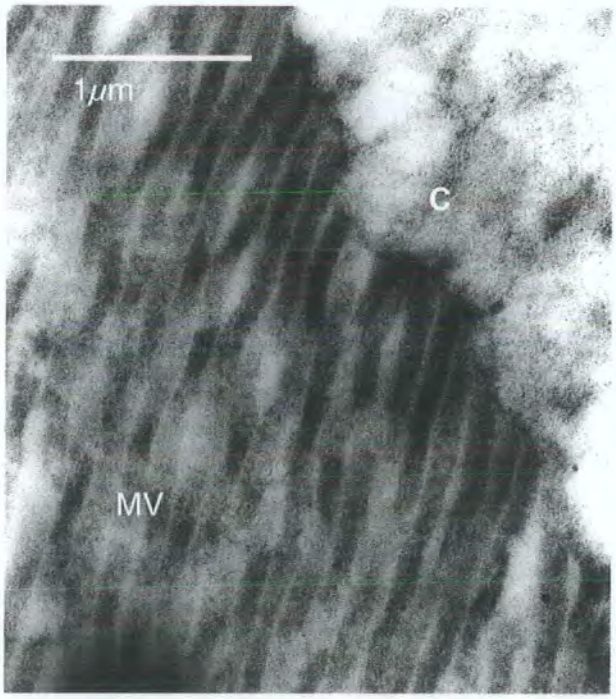
Foregut of GNA fed *L. oleracea*

Legend for all EM plates in chapter 4

MV = Microvilli
C = Cytosol

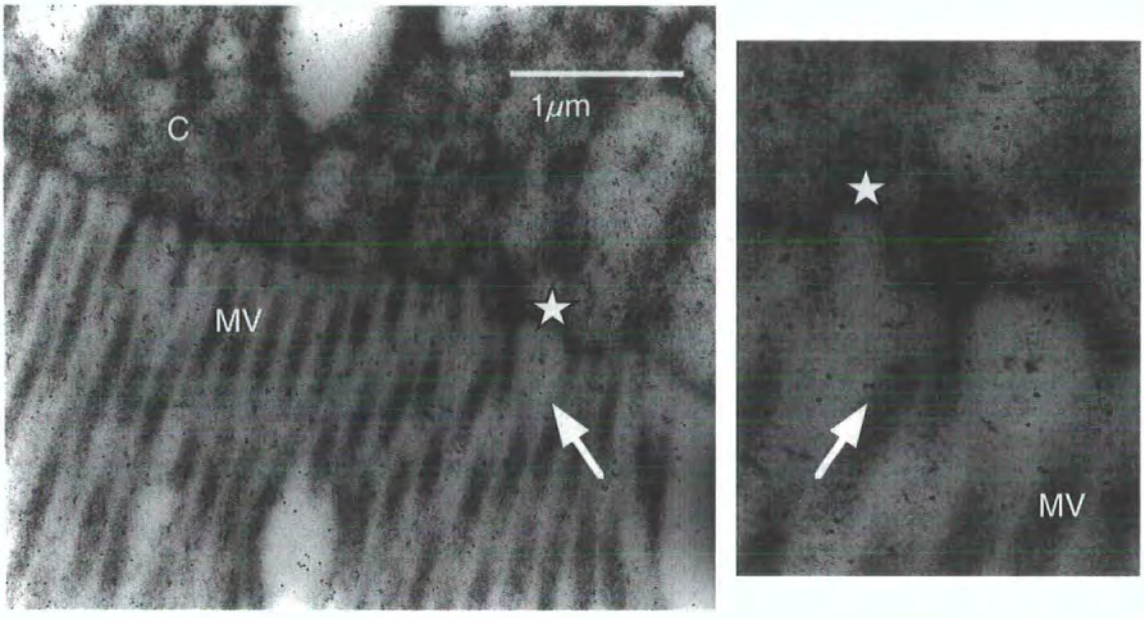
☆ = Area enlarged in right hand size panel

➘ = Black or white arrow points to gold particle, indicating GNA binding.

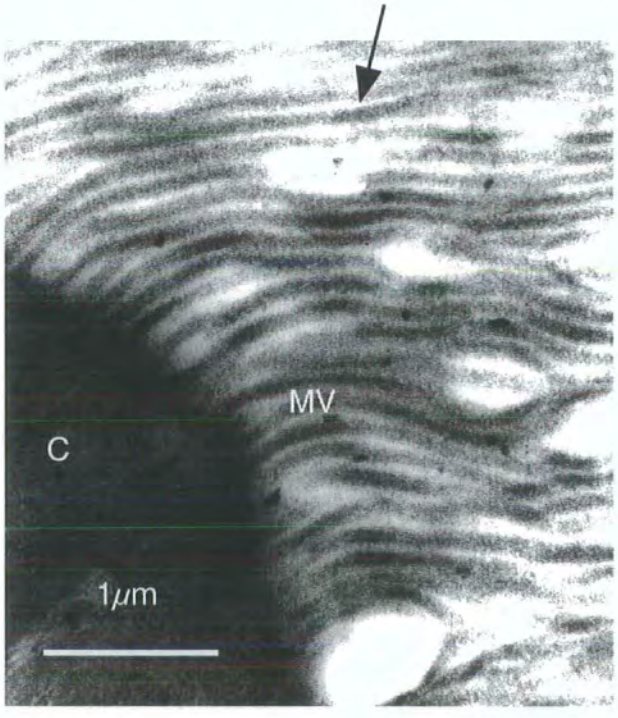


Foregut of control fed *L. oleracea*

Fig 4.2.3 electron micrographs of the midgut of *L. oleracea* fed on either a control artificial diet or an artificial diet containing GNA at 2% of total protein. Sections were subjected to the immunogold staining procedure to detect the presence of GNA

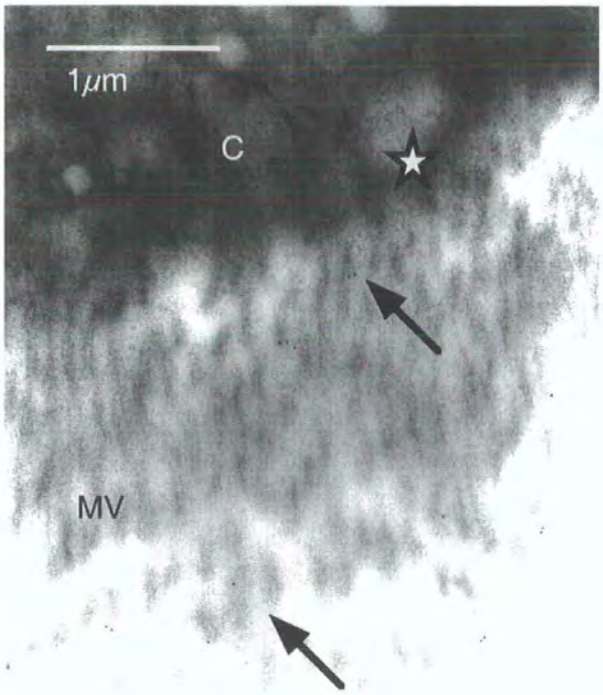


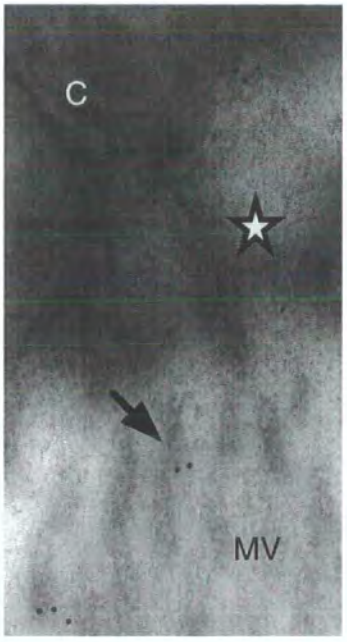
Midgut of GNA fed *L. oleracea*



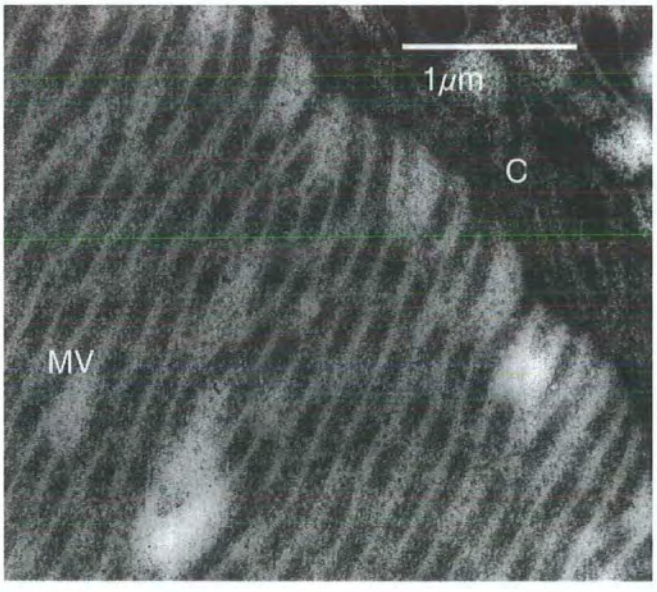
Midgut of control fed *L. oleracea*

Fig 4.2.4 electron micrographs of the hindgut of *L. oleracea* fed on an artificial diet containing GNA at 2% of total protein. One section followed the normal immunogold staining procedure to detect the presence of GNA and the second section was incubated in buffer instead of primary antibody.





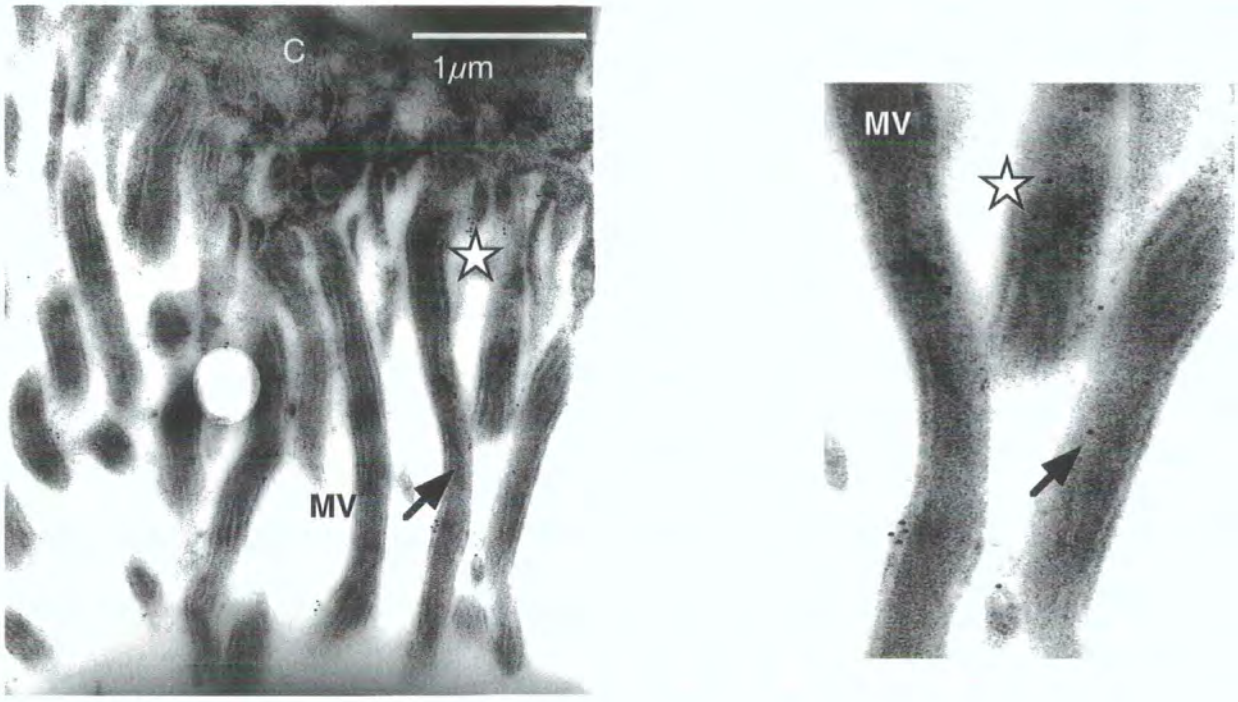
Hindgut section of GNA fed *L. oleracea*.

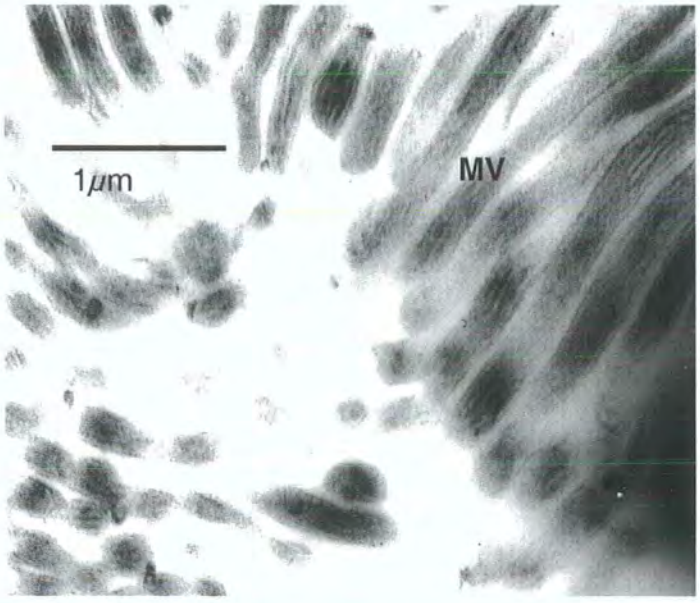


Hindgut section of GNA fed *L. oleracea*.
No primary antibody was used.

Fig 4.2.5 electron micrographs of the malpighian tubules of *L. oleracea* larvae fed on either a control artificial diet or an artificial diet containing GNA at 2% of total protein. Sections were subjected to the immunogold staining procedure to detect the presence of GNA

Electron micrograph of a section from the malpighian tubules from GNA fed *L. oleracea* larvae.





Electron micrograph of malpighian tubules from control fed *L. oleracea* larvae

The same trend of distribution was observed in sections from the hindgut area and malpighian tubules, with the presence of GNA being detected both along the microvilli as well as on the cytosolic side of the epithelial membrane (Plate 4.2.4 and 4.2.5).

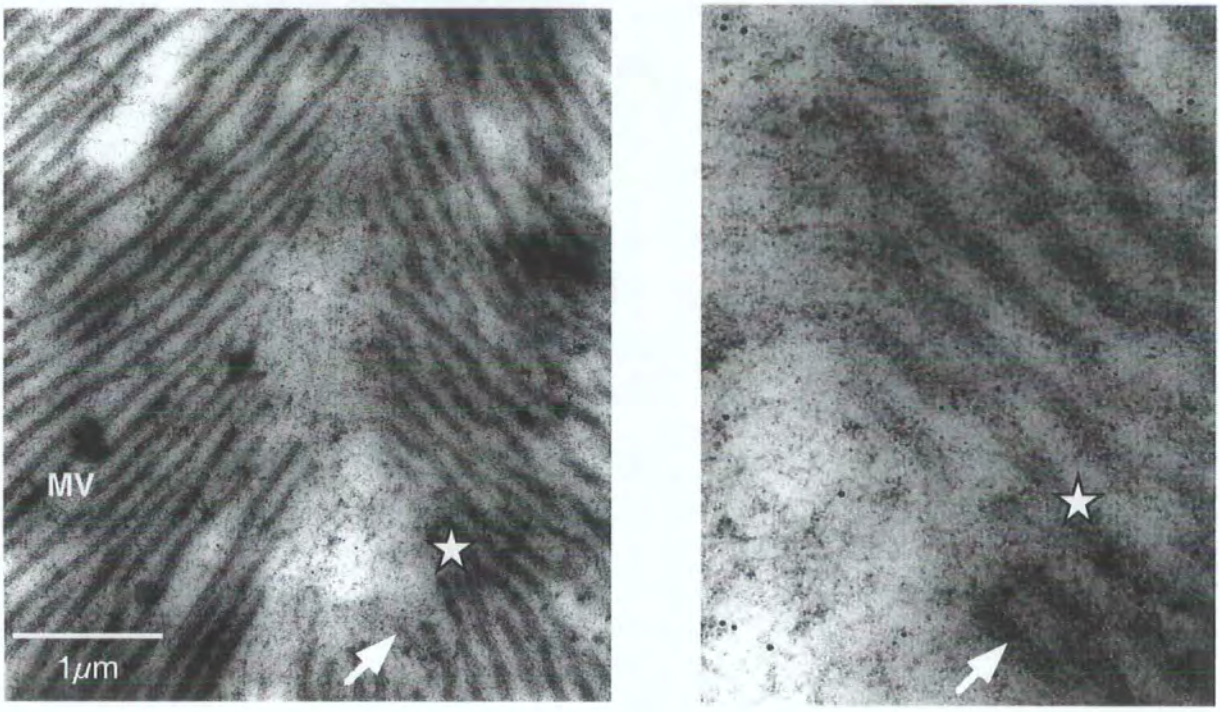
No gold particles (indicative of the presence of GNA) were observed in any of the controls, which composed of control sections taken from either control fed larvae (same protocol as test species) or GNA fed larvae, with the primary antibody step omitted from the immunogold protocol (plate 4.2.4). Thus evidence for the validity of the experimental procedure was demonstrated.

***In vitro* localisation of Con A in *L. oleracea* larvae.**

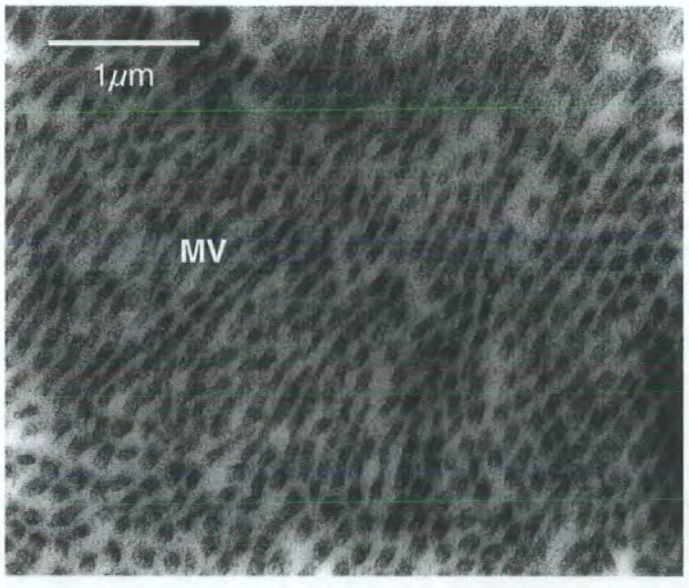
Sections of the alimentary tract of Con A fed *L. oleracea* larvae are presented in plates 4.2.6-4.2.11. The distribution pattern of gold particles (indicating the presence of the lectin Con A) in the foregut, midgut, hindgut and malpighian tubule regions was similar to that observed in GNA fed larvae (see plates 4.2.2-4.2.5). That is to say gold particles were distributed along the microvilli, as well as in the cytosol on the other side of the gut epithelium. As with the GNA fed sections no indication of localised Con A binding was exhibited.

No gold particles (indicating binding) were observed in any of the control sections which comprised of control fed larvae, (same protocol as test species), and Con A fed larvae with the primary antibody step omitted from the immunogold protocol (plate 4.2.7). Providing evidence for the validity of the results.

Fig 4.2.6 electron micrographs of the foregut of *L. oleracea* larvae fed an artificial diet containing Con-A at 2% of total protein. One section was subjected to the immunogold staining procedure to detect the presence of Con-A, the other section followed the same procedure except the incubation in primary antibody step was omitted

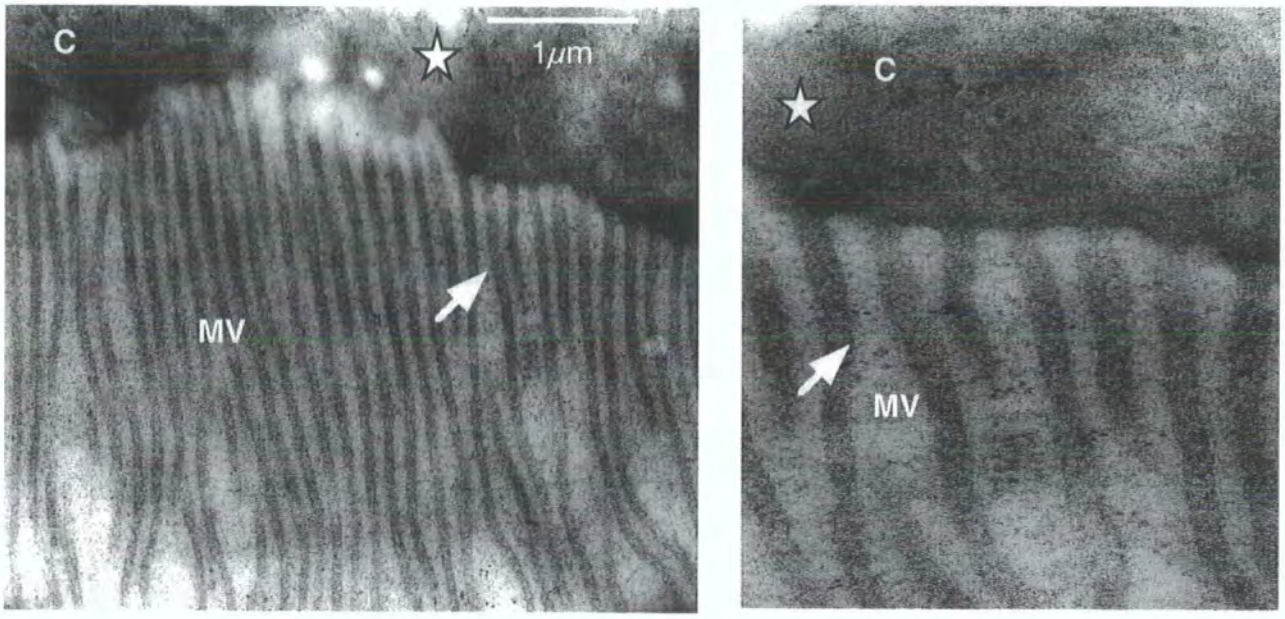


Electron micrograph of foregut section from Con-A fed *L. oleracea* larvae.

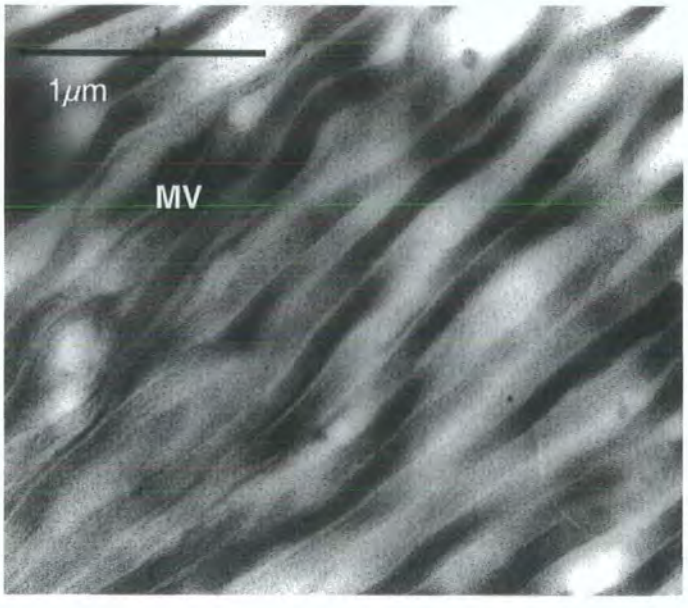


Electron micrograph of foregut section from Con-A fed *L. oleracea* larvae. No primary antibody was used

Fig 4.2.7 electron micrographs of the midgut of *L. oleracea* fed on either a control artificial diet or an artificial diet containing Con-A at 2% of total protein. Sections were subjected to the immunogold staining procedure to detect the presence of Con-A

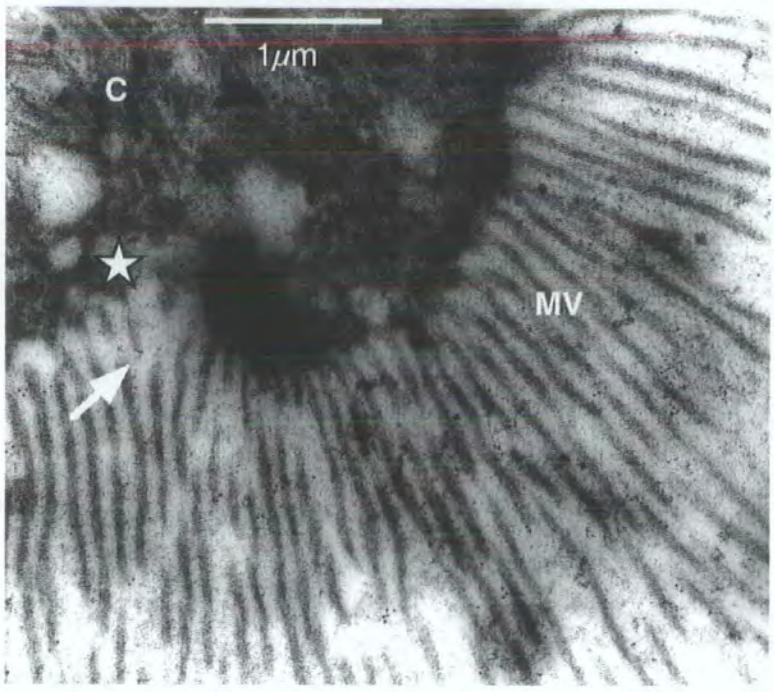


Electron micrograph of midgut section from Con-A fed *L. oleracea* larvae.

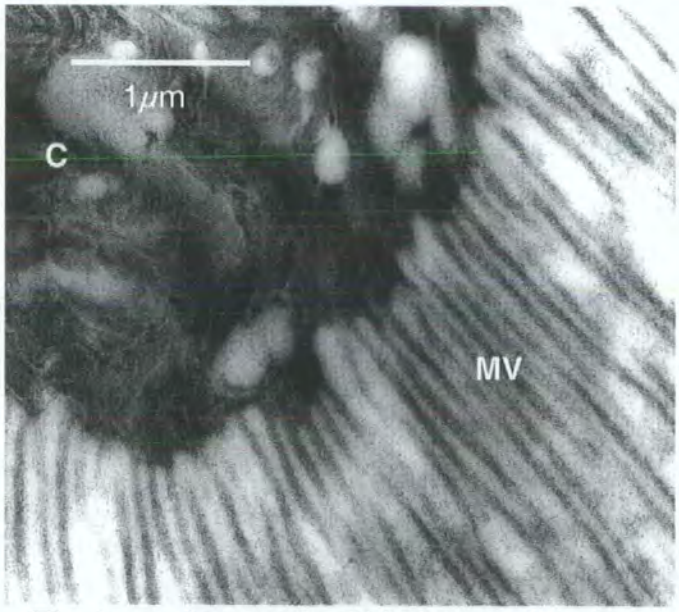


Electron micrograph of midgut section from Control fed *L. oleracea* larvae

Fig 4.2.8 electron micrographs of the hindgut of *L. oleracea* fed on either a control artificial diet or an artificial diet containing Con-A at 2% of total protein. Sections were subjected to the immunogold staining procedure to detect the presence of Con-A

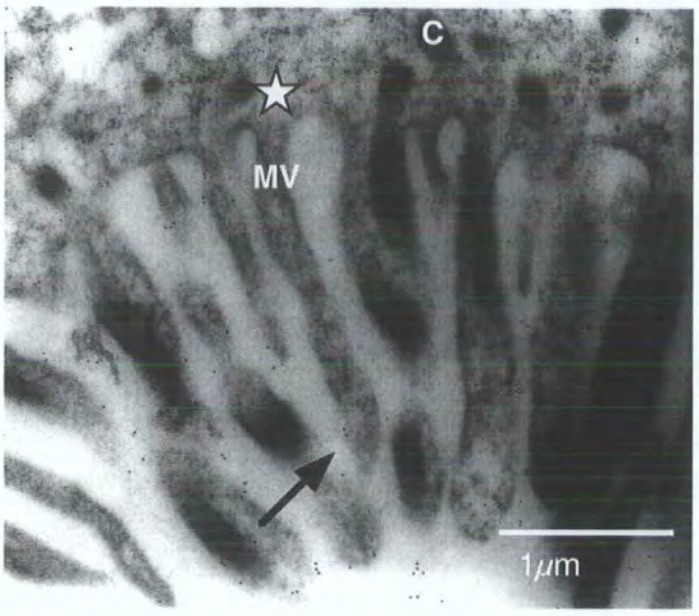


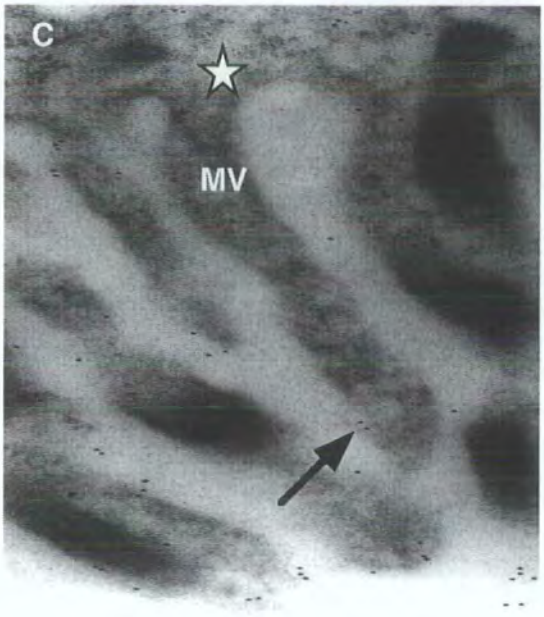
Electron micrograph of hindgut section from Con-A fed *L. oleracea* larvae.



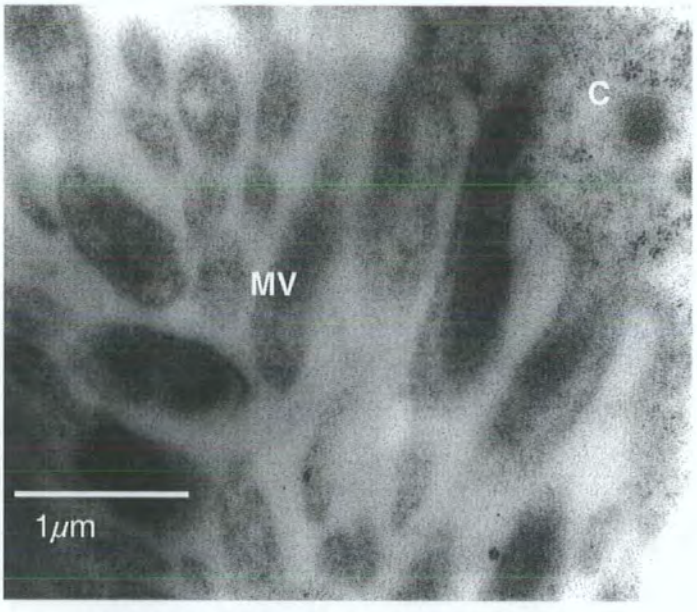
Electron micrograph of hindgut section from control fed *L. oleracea* larvae,

Fig 4.2.9 electron micrographs of the malpighian tubules of *L. oleracea* fed on either a control artificial diet or an artificial diet containing Con-A at 2% of total protein. Sections were subjected to the immunogold staining procedure to detect the presence of Con-A





Electron micrograph of a section from the malpighian tubules from Con-A fed *L. oleracea* larvae.



Electron micrograph of malpighian tubules from control fed *L. oleracea* larvae.

4.3.3 Fluorescent localisation of GNA by tissue blots of the gut of *L. oleracea*

Tissue blots of *L. oleracea* larvae were prepared as described in section 4.2.4. Plate 4.3.1 shows a tissue blot at low magnification (×10 objective lens) incubated with GNA-RITC. The red fluorescence indicative of the presence of GNA-RITC is immediately apparent and is particularly marked when compared to the control tissue incubated in BSA-RITC for which no fluorescence was observed.

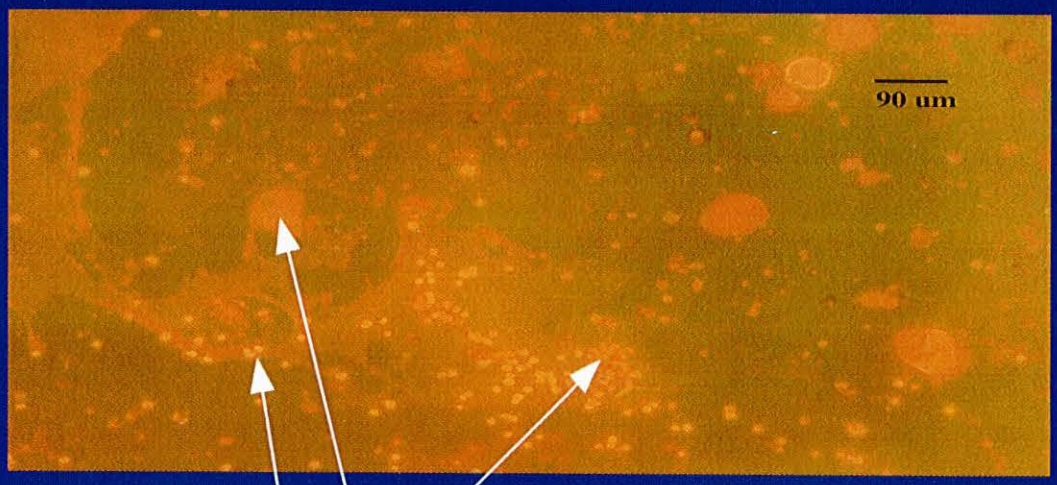
Plate 4.3.3 is a light microscope picture at low magnification ×10 objective lens, giving a clear indication of structure of the tissue on the slide, without any interference due to fluorescence.

4.3.4 Fluorescent localisation of GNA on the peritrophic membrane of *L. oleracea*

Plates 4.3.4-4.3.7 shows sections taken from the peritrophic membrane of *L. oleracea* larvae cut ventrally and splayed open onto a slide. The peritrophic membrane sections were then incubated in GNA-FITC/BSA-FITC as described in section 4.2.5. Plate 4.3.4 shows a section of peritrophic membrane incubated in GNA-FITC, which exhibits speckles of fluorescence indicating the presence of GNA-FITC. The distribution of fluorescence indicates that GNA does not appear to be localised in any one area of the peritrophic membrane.

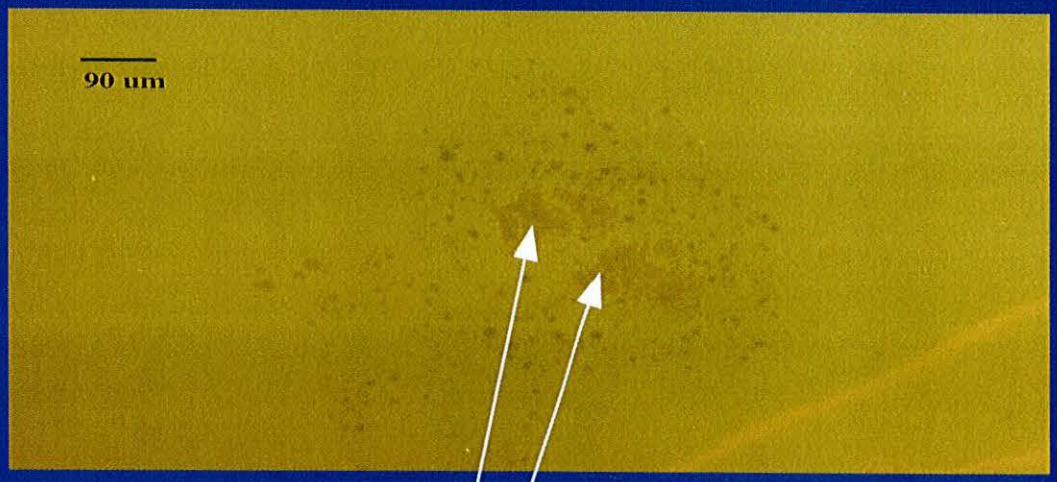
Plate 4.3.5 shows a control fresh tissue section of the peritrophic membrane (incubated in BSA-FITC) of *L. oleracea* at the same magnification as plate 4.3.4

Plate 4.3.1 tissue blot of *L. oleracea* larvae incubated in GNA-RITC and viewed with a Nikon diaphot UV microscope



Bits of tissue from *L. oleracea* midgut with red fluorescence being GNA-RITC

Plate 4.3.2 tissue blot of *L. oleracea* incubated in BSA-RITC and viewed with a Nikon diaphot UV microscope



Bits of tissue from *L. oleracea* midgut

Plate 4.3.3 light microscope image of a tissue blot of the gut of *L. oleracea*

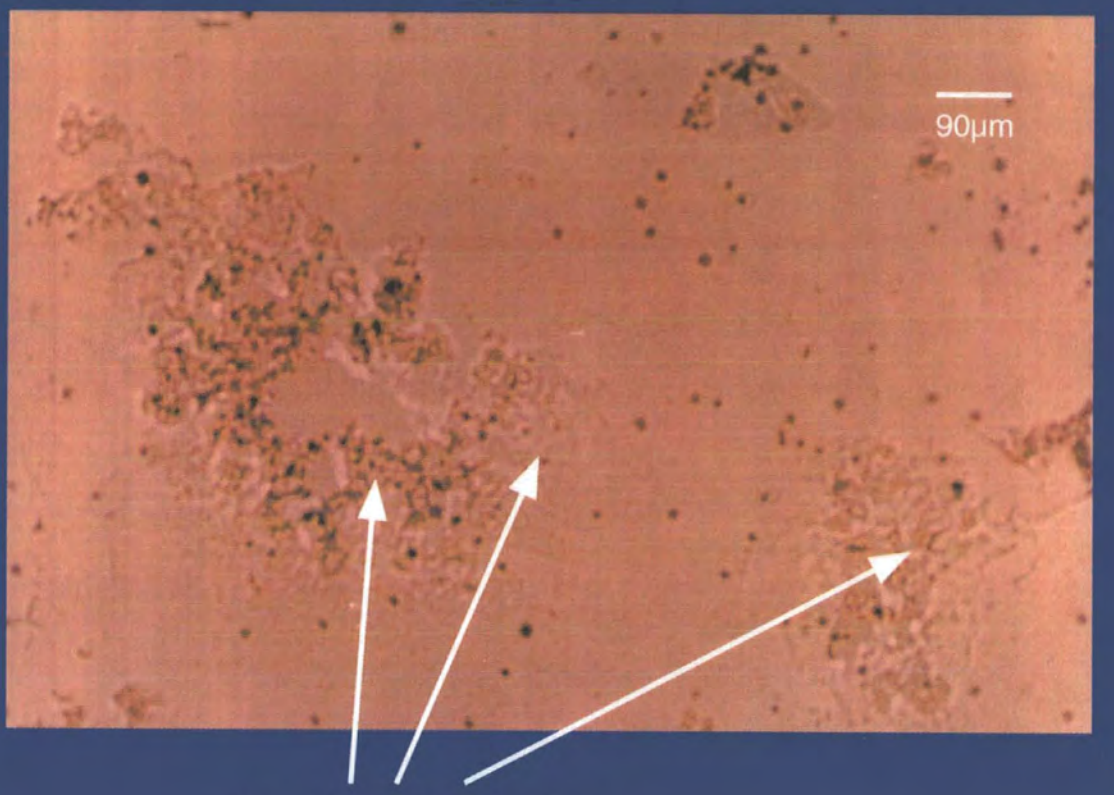
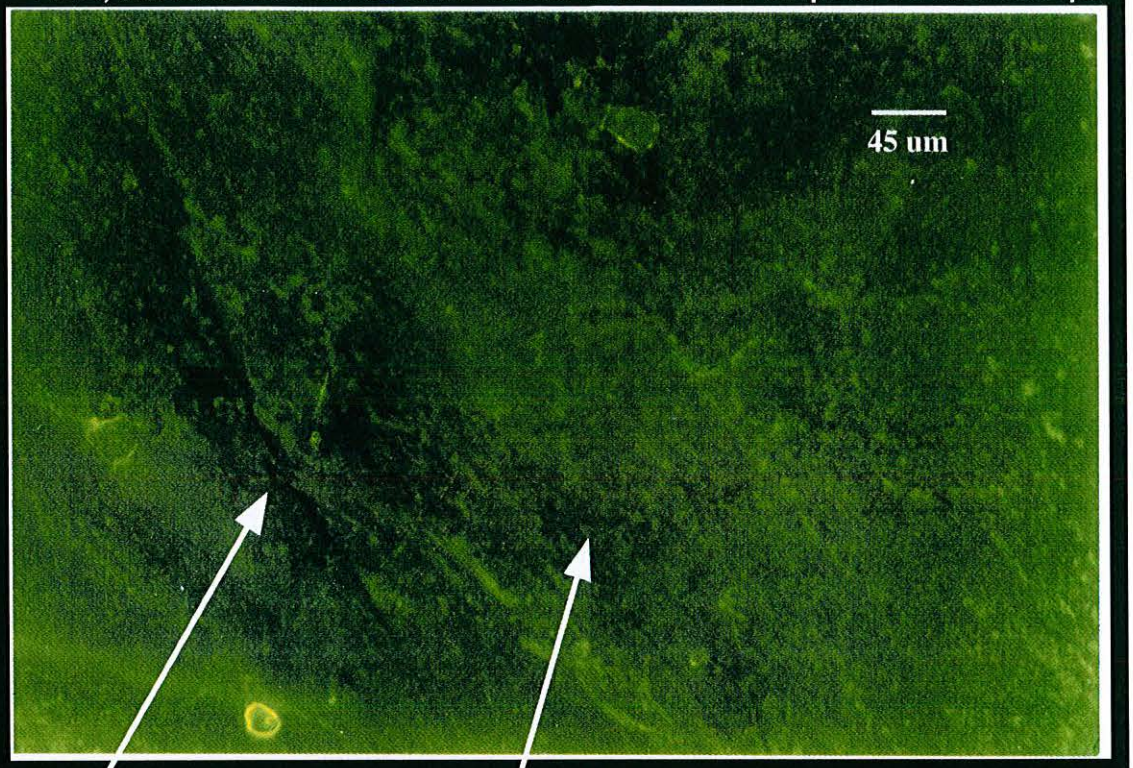


Plate 4.3.4 peritrophic membrane of *L. oleracea* larvae incubated in GNA-FITC, washed with buffer and viewed with a Nikon diaphot UV microscope



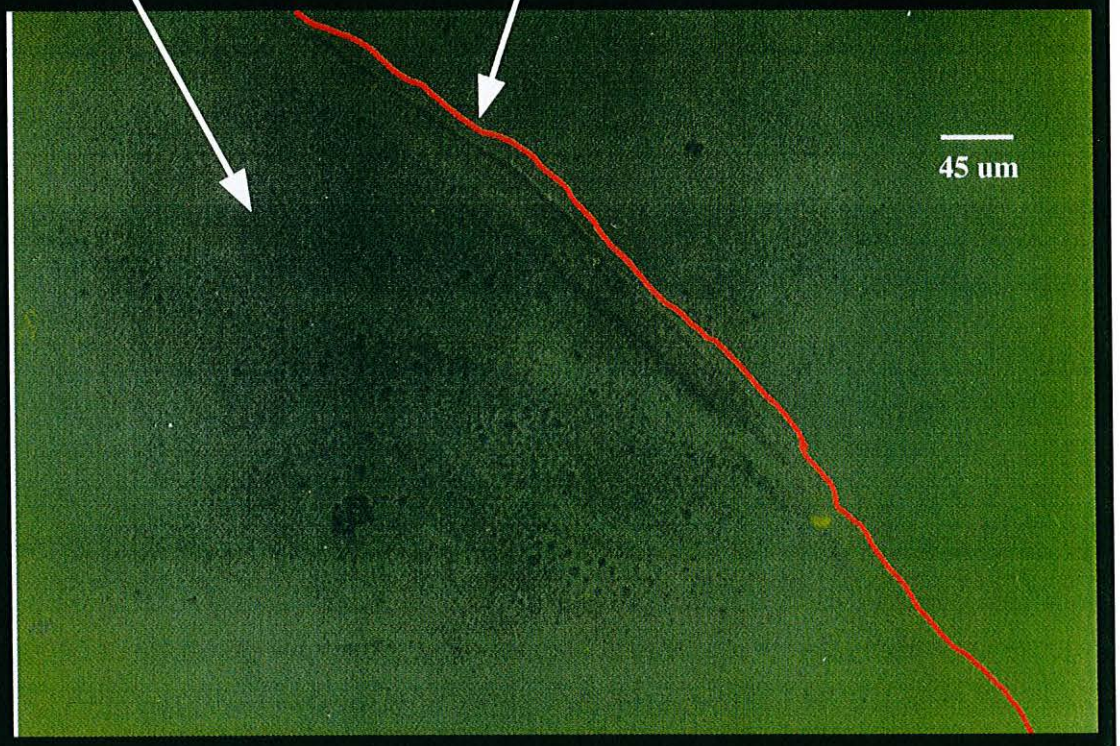


Plate 4.3.5 peritrophic membrane of *L. oleracea* larvae incubated in BSA-FITC, washed with buffer and viewed with a Nikon diaphot UV microscope

Plate 4.3.6 peritrophic membrane of *L. oleracea* incubated in GNA-FITC, phase contrast light microscope image (duplicate of plate 4.3.4).

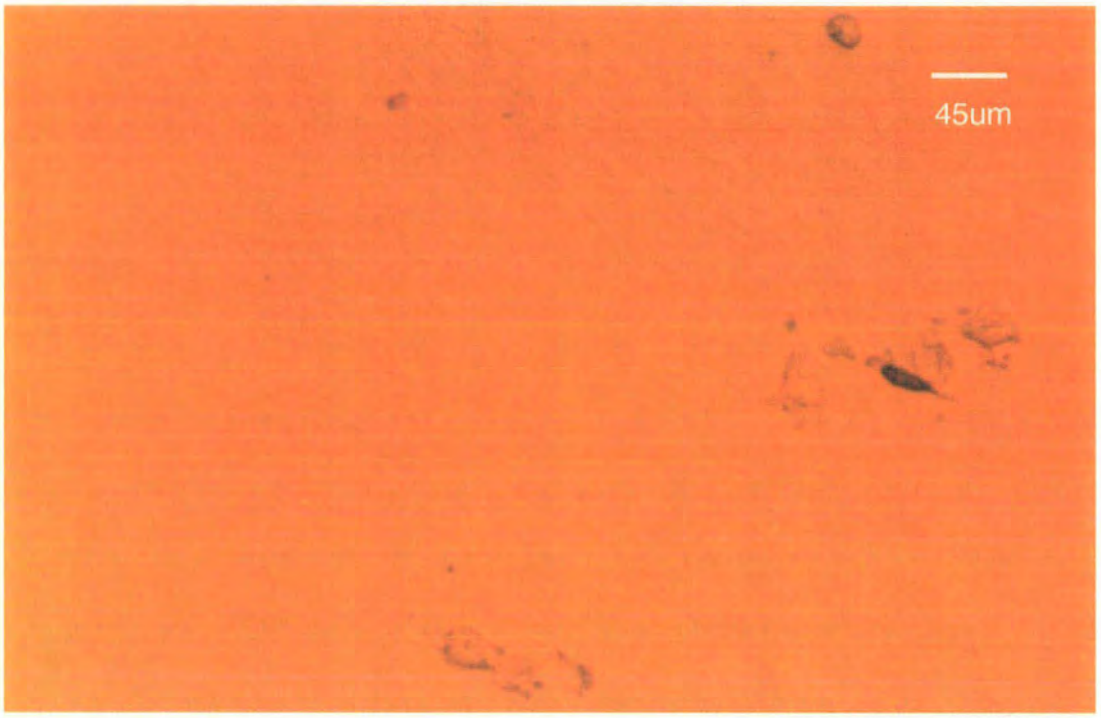


Plate 4.3.7 peritrophic membrane of *L. oleracea* incubated in BSA-FITC, phase contrast light microscope image (duplicate to plate 4.3.5).

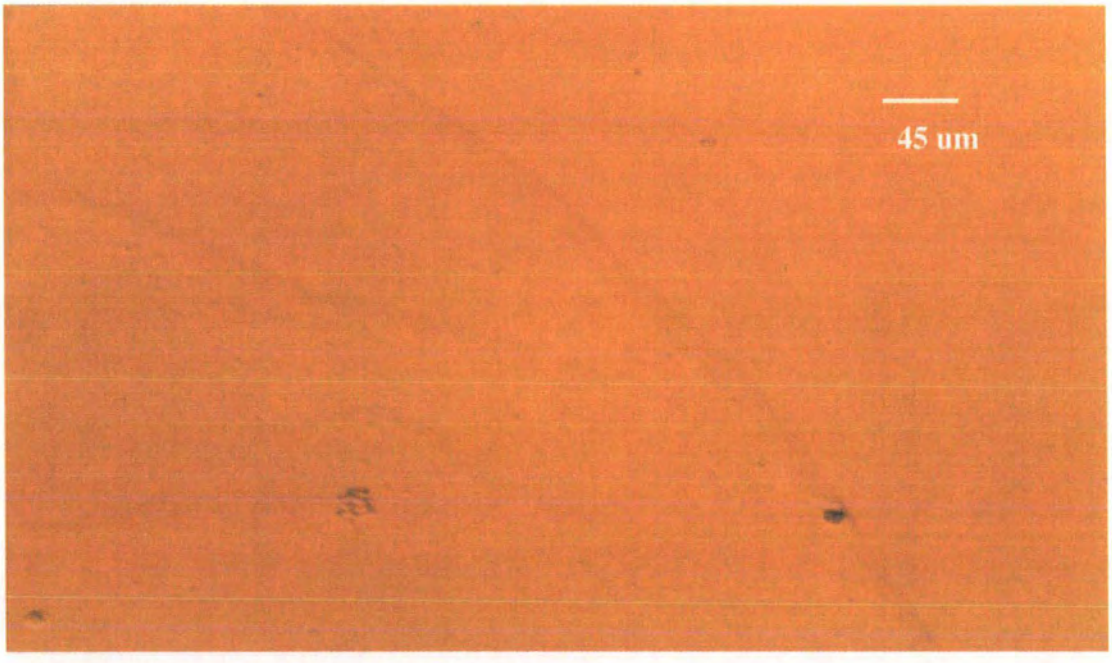
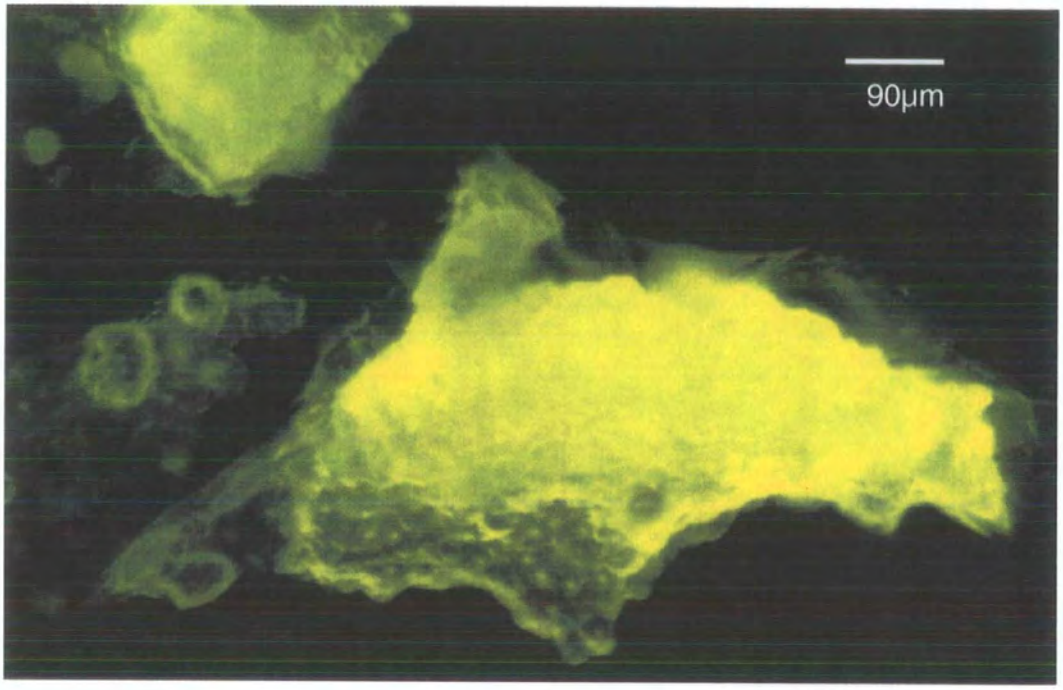


Plate 4.3.8 columnar orientation of or the peritrophic membrane of *L. oleracea* larvae incubated in GNA-FITC, washed in buffer and viewed with a Nikon diaphot UV microscope



(×20 objective lens). The lack of the speckled fluorescence observed in GNA-FITC incubated sections is immediately apparent.

Plates 4.3.6 and 4.3.7 are the (twin) light microscope images of plates 4.3.4 and 4.3.5, respectively showing fresh tissue sections of the peritrophic membrane of *L. oleracea* (magnification ×20). These plates show a clear image of the peritrophic membrane structure.

Plate 4.3.8 shows a section of tissue incubated in GNA-FITC, in which the section is in a columnar orientation displaying more than a simple external view of the peritrophic membrane. The slide allows the inside of the peritrophic membrane to be visualised allowing us an idea of the potential extent to which binding could occur *in vivo,* as such the intensity of fluorescence is amplified.

4.3.5 Fluorescent localisation of GNA on gut tissue

Slides were prepared from the midgut tissue of *L. oleracea* larvae and then incubated in the lipid stain Sudan Black. This was followed by incubating the samples in either GNA-RITC or BSA-RITC as described in section 4.2.4. Plate 4.3.9 (magnification ×20 objective lens) indicates the presence of GNA-RITC, which binds extensively to midgut gut tissue. Some localised binding is indicated by non-uniform fluorescence GNA-RITC stained sections. Plate 4.3.10 shows a control section incubated in GNA-RITC designed to complement plate 4.3.9 (magnification ×20 objective lens). Although overexposed, it illustrates the absence of the pattern of fluorescence caused by GNA-RITC binding, with just the background fluorescence showing through.

Plate 4.3.9 splayed gut section of *L. oleracea* larvae incubated in Sudan black and then GNA-RITC washed with distilled water and viewed with a Nikon UV diaphot microscope

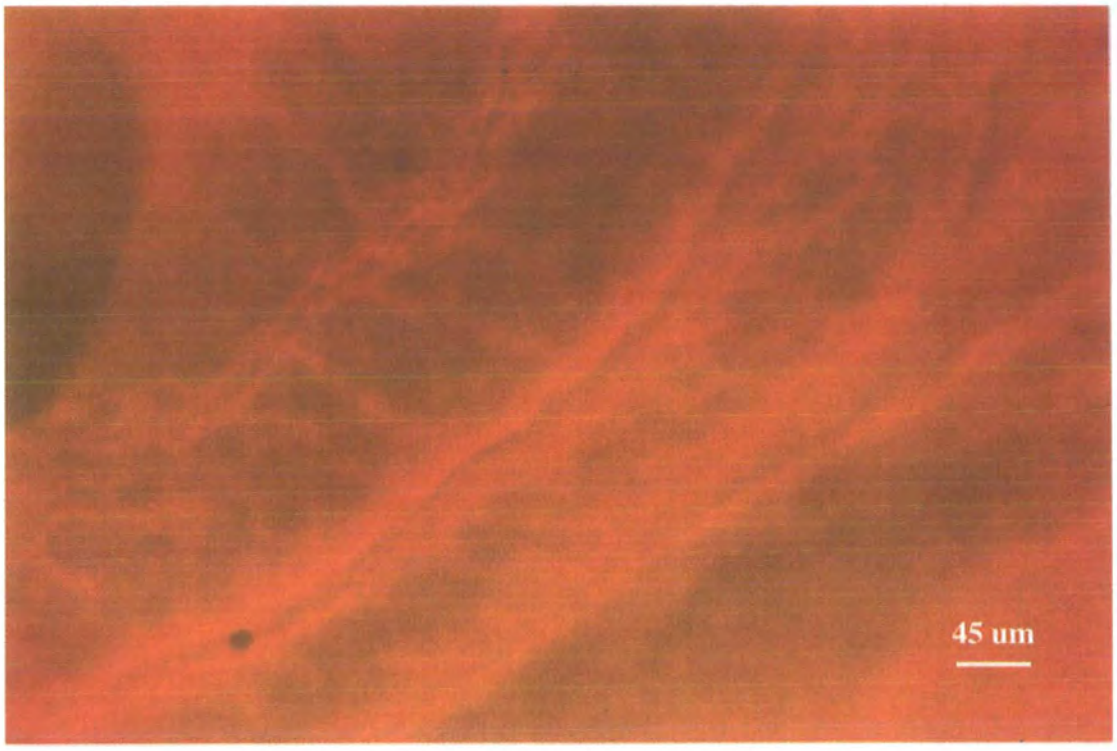


Plate 4.3.10splayed gut section of *L. oleracea* larvae incubated in Sudan black and then BSA-RITC washed with distilled water and viewed with a Nikon UV diaphot microscope

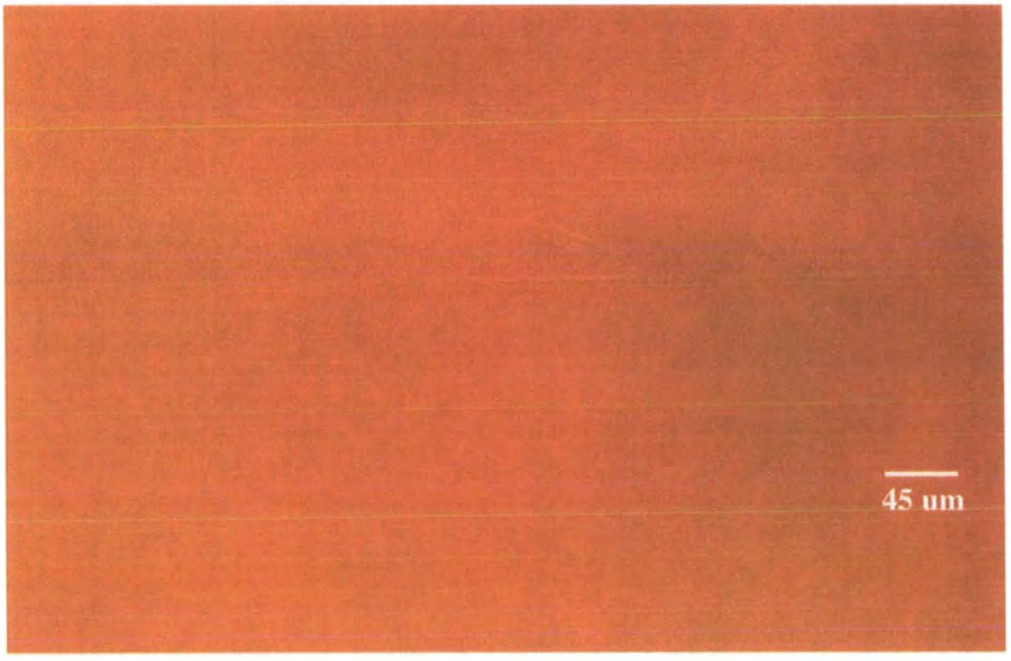


Plate 4.3.11 midgut section of *L. oleracea* incubated in GNA-FITC washed with distilled water and viewed with a Nikon diaphot UV microscope

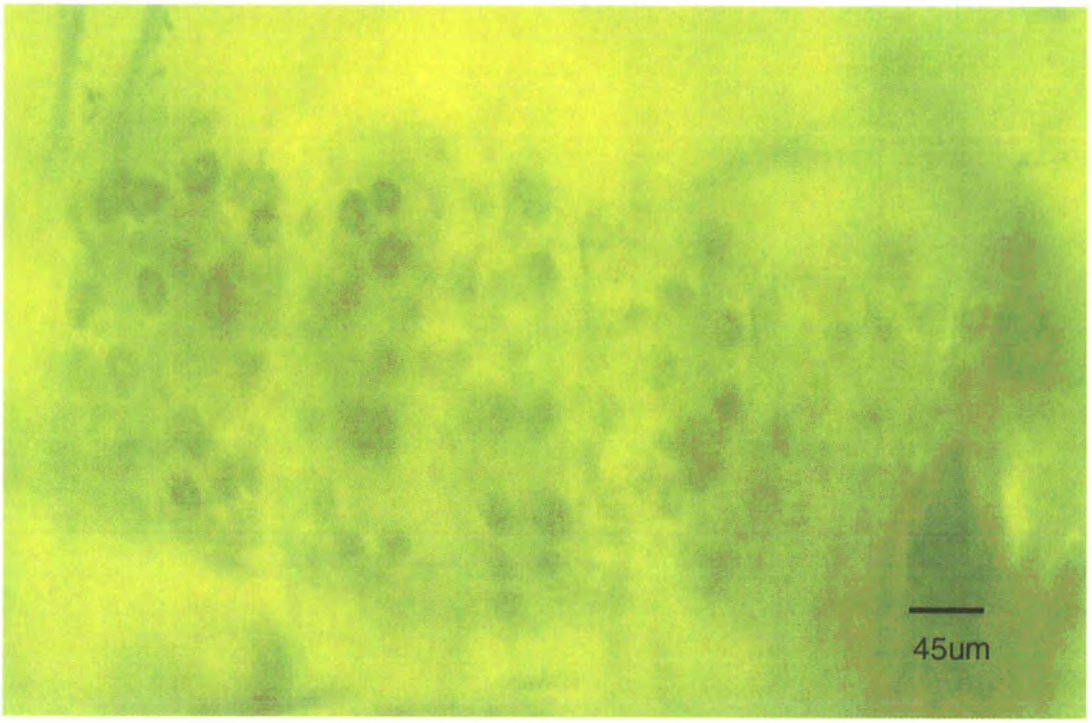


Plate 4.3.12 midgut section of *L. oleracea* incubated in BSA-FITC washed with distilled water and viewed with a Nikon diaphot UV microscope

Plate 4.3.13 light microscope section of Sudan black stained midgut of *L. oleracea*

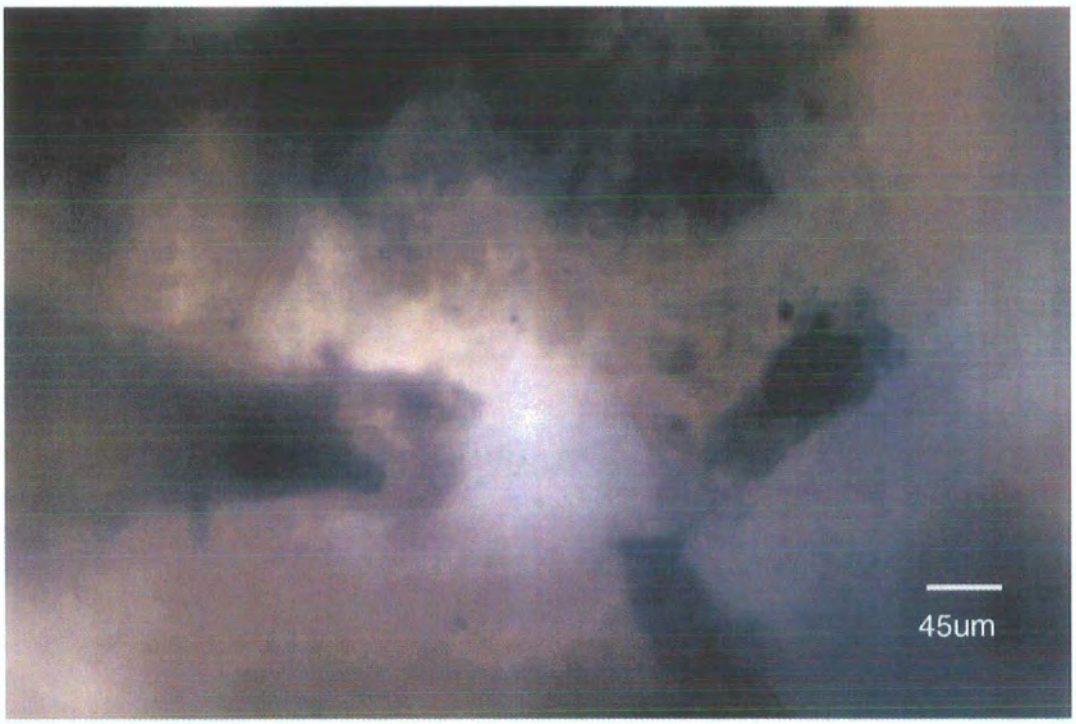


Plate 4.3.11 shows a midgut section incubated in GNA-FITC (magnification ×20 objective lens). It is apparent that the detail and determination of GNA binding is not as obvious as that observed using a rhodamine fluorochrome. However, in comparison with the control section (plate 4.3.12) in which diffuse fluorescence can be observed the section incubated in GNA-FITC (plate 4.3.11) shows more intense and localised fluorescence indicative of GNA binding.

Plate 4.3.13 is a light microscopic image (magnification ×20 objective lens), of the *L. oleracea* midgut to give an idea of the structure under observation. The black staining is lipid binding Sudan black, the extent of which indicates a high lipid content of the sections.

4.3.6 Localisation of GNA binding sites on LR White embedded tissue sections by use of GNA-FITC.

Slides were prepared as described in section 4.2.7 and incubated in either GNA-FITC or BSA-FITC.

Plate 4.3.14 shows a complete section (T.S.) of the *L. oleracea* midgut the green fluorescence produced by GNA-FITC indicative of the extent of GNA binding. A considerable amount of binding occurs at the external surface of the gut (adjacent to haemolymph). Plate 4.3.15 is again a midgut T.S. incubated in GNA-FITC but at a higher magnification. Fluorescence in this section clearly indicates the presence of GNA bound to the section. GNA binding occurs across the section from the luminal area of the microvilli across the cytosol to the external surface of the gut.

Plate 4.3.14 section of midgut from *L. oleracea* incubated in GNA-FITC washed with distilled water and viewed with a Nikon diaphot UV microscope

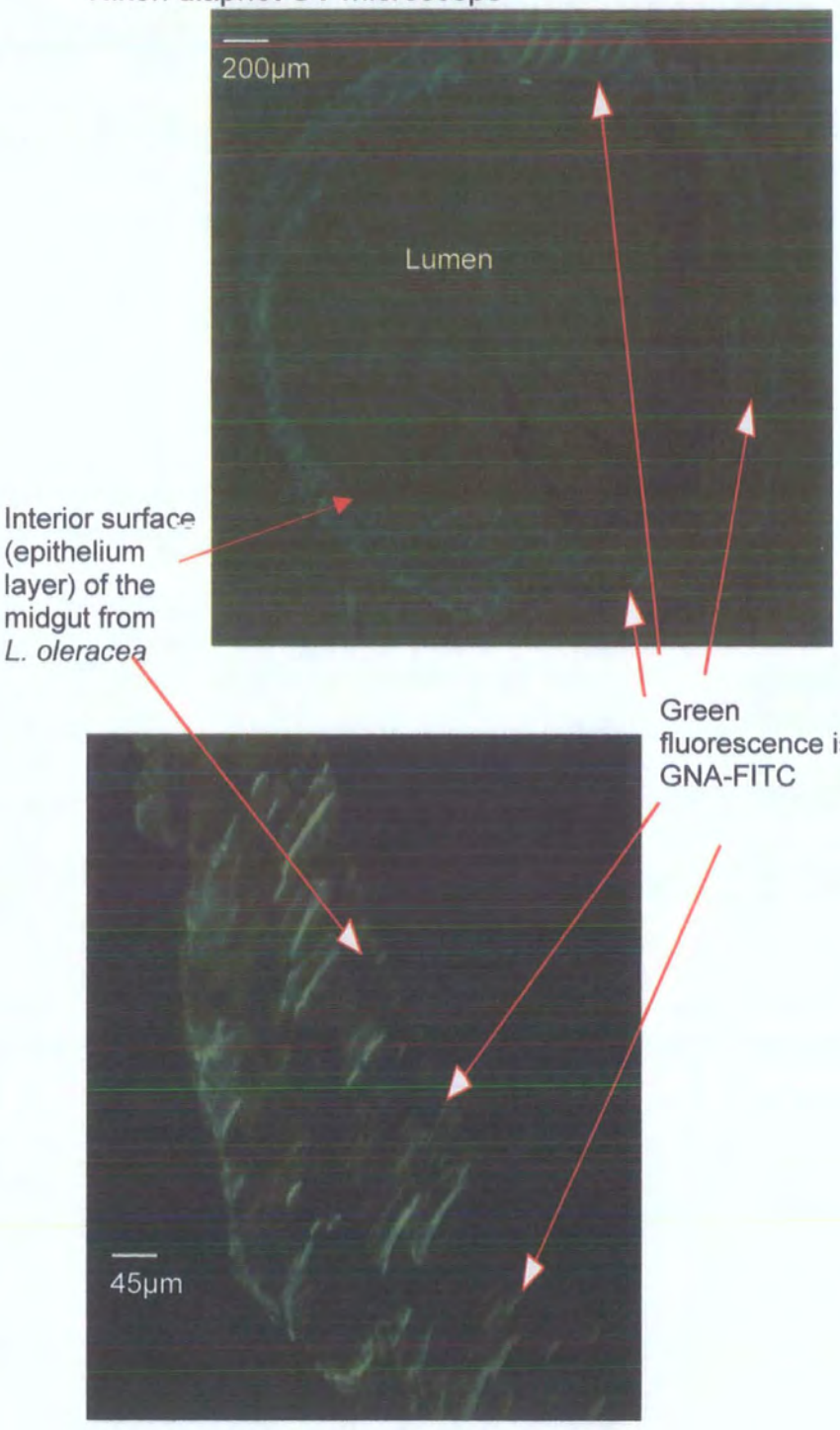
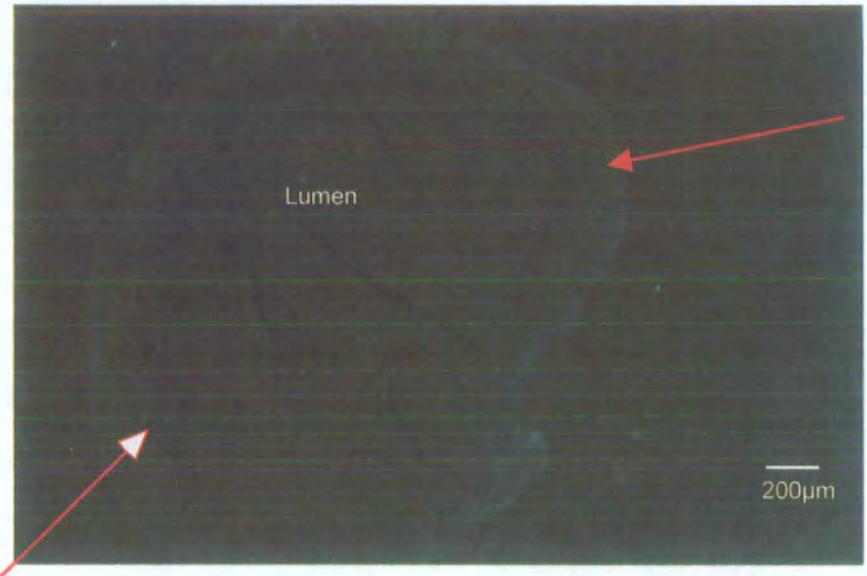


Plate 4.3.15 section of midgut from *L. oleracea* incubated in GNA-FITC, washed with distilled water and viewed with a Nikon diaphot UV microscope.

Plate 4.3.16 section of midgut from *L. oleracea* incubated in methyl mannose pyranoside followed by incubation in GNA-FITC, washed with distilled water and viewed with a Nikon diaphot UV microscope.



Green fluorescence is GNA-FITC

Interior surface (epithelium layer) of the midgut from *L. oleracea*

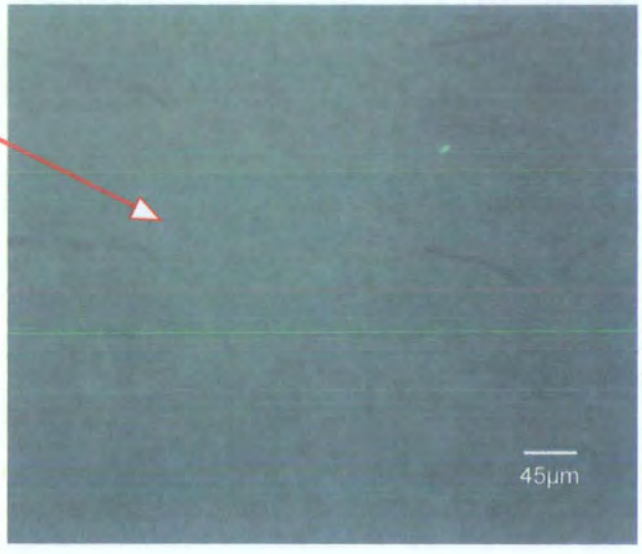


Plate 4.3.17 section of midgut from *L. oleracea* incubated in BSA-FITC, washed with distilled water and viewed with a Nikon diaphot UV microscope.

Plate 4.3.16 is identical to plate 4.3.14 except that it shows a section incubated with α methyl-mannopyranoside to block the mannose residues thought to act as the GNA binding sites. Although binding of GNA-FITC is still apparent, fluorescence is considerably reduced compared to results obtained for GNA-FITC sections represented by plate 4.3.14. Plate 4.3.17 shows a representative control section, which was incubated in BSA-FITC, no fluorescence is exhibited, indicating no binding of BSA-FITC, and the absence of autofluorescence.

4.4 Detection of Lectin Binding to the Tomato Moth Larvae *L. oleracea* by non histological means.

Apart from histological methods lectin binding can also be investigated biochemically using extracted proteins. Lectin binding *in vivo* can be examined be probing western blots of proteins extracted from lectin fed larvae with antibodies to the lectin in question. Alternatively binding can be examined *in vitro* by probing western blots of proteins extracted from larvae with the lectins of interest.

4.4.1 Detection of GNA and Con A binding to crude extracts of gut proteins from *L. oleracea* larvae and Rats.

Crude extracts of protein were taken from the intestine of a rat and from the midgut of *L. oleracea* as described in section 2.8. Proteins were separated by SDS-PAGE (SDS-polyacrylamide gel electrophoresis). Proteins on one gel were visualised by Coomassie blue staining, and proteins on the second gel were transferred to a nitrocellulose filter. The filter was probed with either Con A or

GNA; bound lectin was subsequently detected using antibodies (see section 4.2.12). The gel in fig 4.4.1 shows a large amount of protein present, (in addition to fat causing smearing). The Western blot shown in fig 4.4.1 demonstrates that GNA binds *in vitro* to proteins extracted from both rat and *L. oleracea* larval gut tissue. GNA appears to bind to a greater number of glycopolypeptides in larval gut extracts as compared to rat gut extracts, and is indicative of a greater abundance of mannose residues as constituents of larval gut proteins.

The Coomassie stained gel in fig 4.4.2 shows a large amount of protein loaded onto the gel (again smeared due to fat content). As for GNA the Western blot shown in fig 4.4.2 indicates a greater abundance of receptors for Con A in the *L. oleracea* extract than in the rat gut extract. In comparison with the blot incubated with GNA (fig 4.4.1), the Con A lectin binds to many more gut proteins than the GNA lectin in both the *L. oleracea* larvae and the rat samples.

4.4.2. Detection of Con A binding to different fractions from the *L. oleracea* midgut.

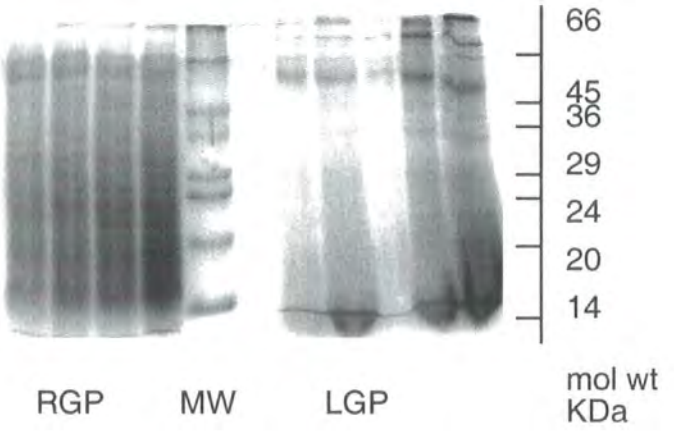
Due to the problems associated with the crude gut extracts, a brush border membrane preparation (BBMV) from the midgut of *L. oleracea* was carried out as described in section 2.11. Protein samples of BBMV proteins (10 µg) were separated by SDS-PAGE. Proteins on one gel were visualised by Coomassie blue staining, and proteins on the second gel were transferred to a nitrocellulose filter. The filter was probed with Con A lectin, and bound Con A was detected by an antibody system (see section 4.2.12).

Fig 4.4.1 GNA binding to gut proteins from *L. oleracea* larvae and rats.

Binding of GNA to gut proteins from rats and *L. oleracea* larvae. 10μg aliquots of LGP (*Lacanobia oleracea* gut proteins) and RGP (rat gut proteins) were visualised by coomassie blue staining in the SDS-PAGE gel. A replicant gel containing 10μg of protein per lane was transfered to nitrocellulose by electrotransfer. The Western blot was incubated in GNA at 1mg/ml. GNA binding was probed for using anti GNA antibodies.

SDS-PAGE GEL

TOTAL PROTEIN



MW = SDS 7 molecular weight marker

WESTERN BLOT

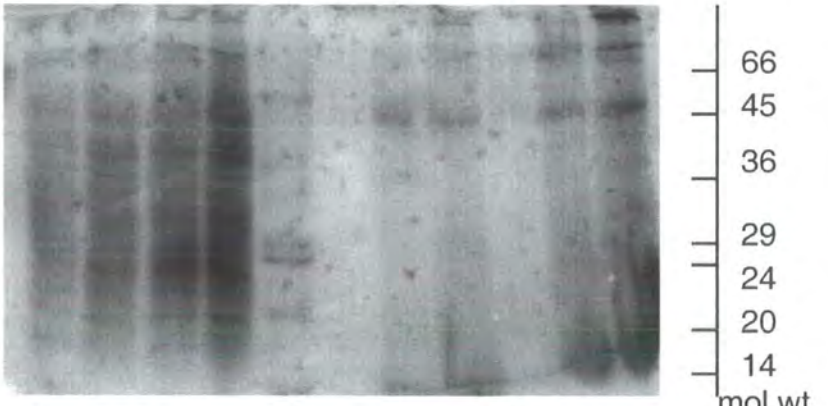
GNA BINDING

Fig 4.4.2 Con-A binding to gut proteins from *L. oleracea* larvae and rats.

Binding of Con-A to gut proteins from rats and *L. oleracea* larvae. 10μg aliquots of LGP (*Lacanobia oleracea* gut proteins) and RGP (rat gut proteins) were visualised by coomassie blue staining in the SDS-PAGE gel. A replicant gel containing 10μg of protein per lane was transfered to nitrocellulose by electrotransfer. The Western blot was incubated in Con-A at 1mg/ml. Con-A binding was probed for using anti Con-A antibodies.

SDS PAGE GEL

TOTAL PROTEIN



LGP MW RGP

MW = SDS 7 molecular weight marker

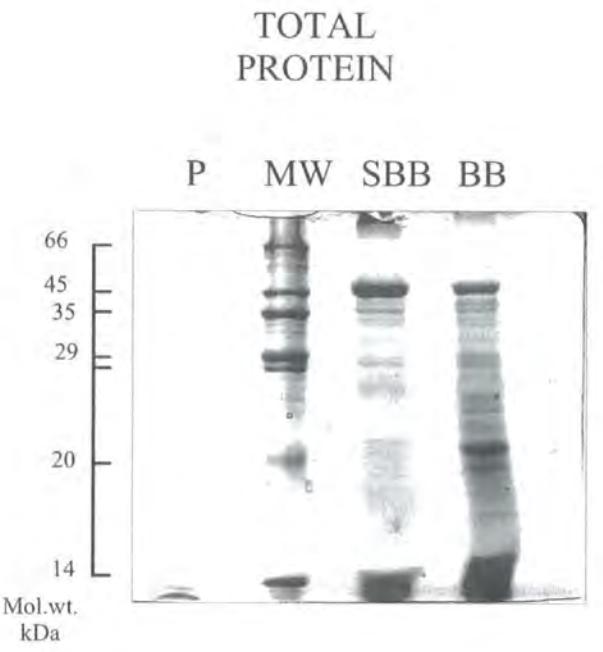
WESTERN BLOT

CON A BINDING

RGP LGP

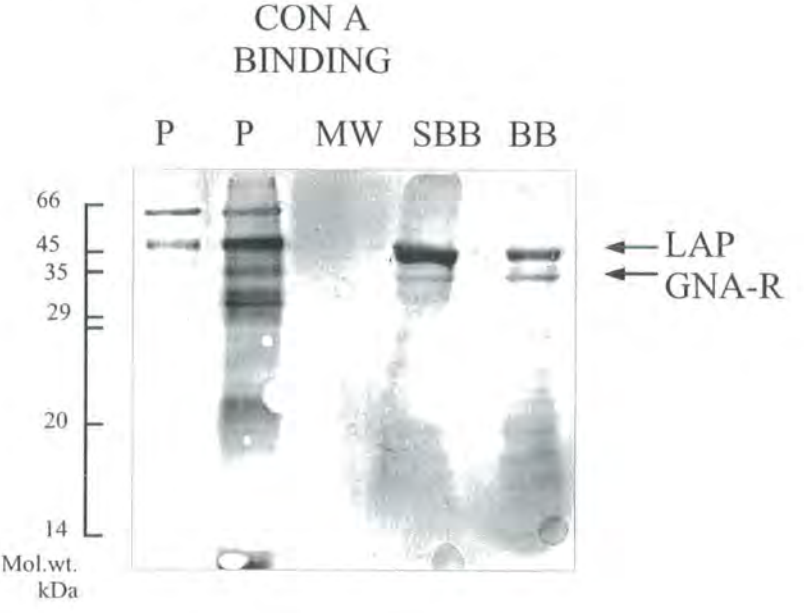
Fig 4.4.3 Con A binding to *L. oleracea* gut proteins

Binding of Con-A to BBMV preparation from *L. oleracea* larvae. 10μg aliquots of each fraction were visualised by coomassie blue staining in the SDS-PAGE gel (top). A replicant gel containing 10μg of protein per lane was transfered to nitrocellulose membrane by Western bloting. The membrane was incubated in Con-A at 1mg/ml. Presence of Con-A was detected utilising anti Con-A antibodies (bottom).



Key

P = peritrophic membrane (2 loadings on blot)
BB = brush border membrane vesicles
SBB = proteins solubilised from brush border membrane vesicles
MW = mol. wt. markers
LAP = leucine amino-peptidase N
GNA-R = GNA receptor



The Coomassie stained gel in fig 4.4.3 shows clearly separated bands in the different fractions of the preparation. The Western blot shown in fig 4.4.3 indicates that Con A binds *in vitro* to glycopolypeptides present in the peritrophic membrane of the *L. oleracea* larvae as well as to the brush border membrane vesicles and soluble brush border membrane vesicles prepared from *L. oleracea* larvae.

4.4.3 Detection of Con A and GNA binding to midgut, hindgut, malpighian tubules, fat body and haemolymph tissue from the *L. oleracea* larvae.

(Carried out by, and reproduced with the permission of Dr E Fitches, University of Durham).

Proteins were extracted from tissue dissected from *L. oleracea* larvae fed for 5 days on control diet or diet containing either GNA or Con A (2% of total protein) The samples were boiled prior to separation in the presence of 2xSDS sample buffer and 10% ß-mercaptoethanol. 20µg aliquots of protein in the gel were detected by Coomassie blue staining (fig 4.4.4a). Replicate gels were blotted onto nitrocellulose (Schleicher and Schuell BA85) and probed with either anti GNA or anti Con A antibodies. Bound antibodies were detected using peroxidase labelled secondary antibodies, followed by treatment with ECL reagents and exposure to X-ray film. The resultant films in fig 4.4.4b and 4.4.4c show that both GNA and Con A bind to all areas tested. When compared to the lectin standards of 10ng and 20ng it can be said that less than 10ng has become bound to any one of the protein fractions.

Fig 4.4.4 Detection of Con-A and GNA binding to midgut, hindgut, malpighian tubules, fat body and haemolymph from *L. oleracea* larvae

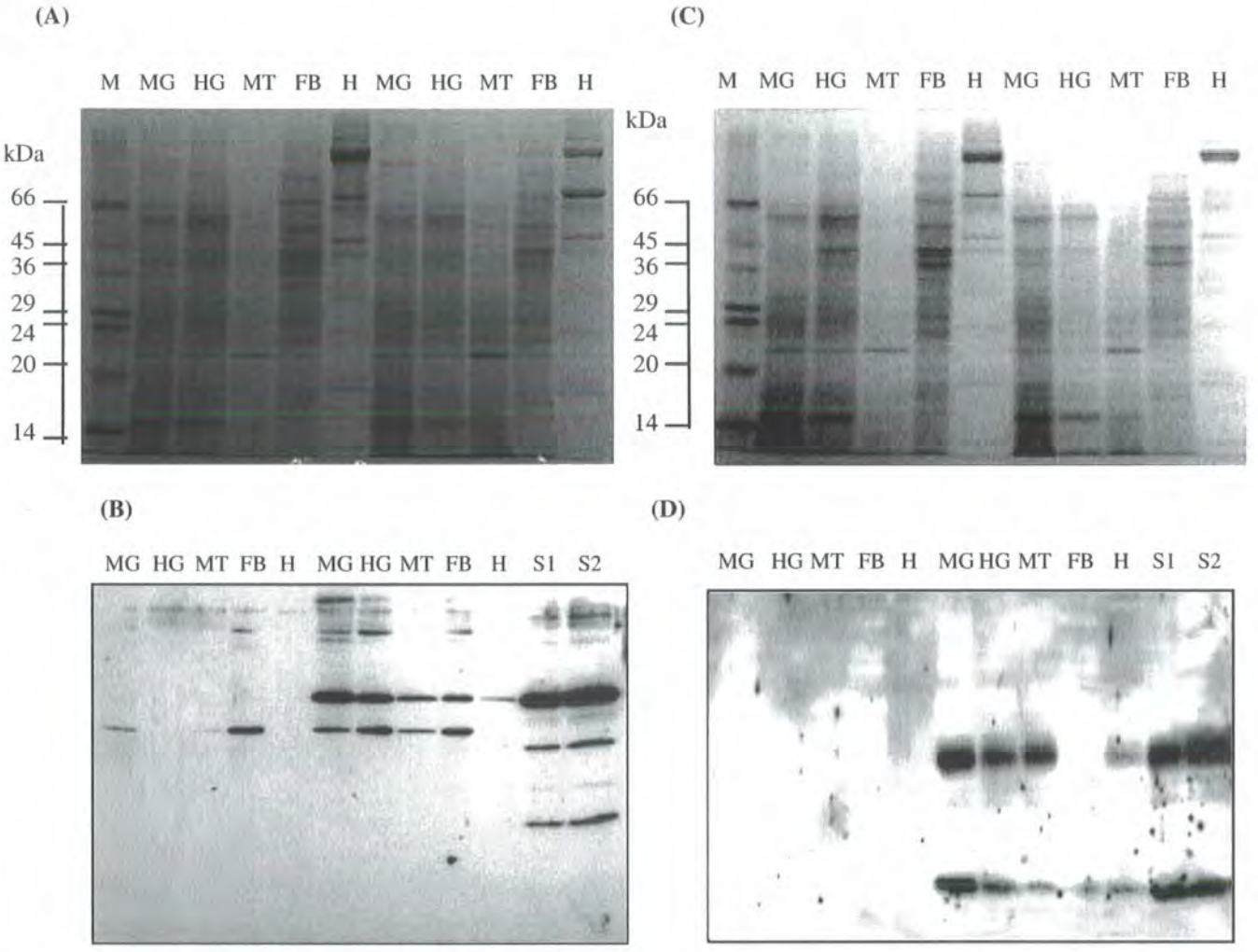


Fig 4.4.4A & 4.4.4B Binding of Con A to *L. oleracea* midgut, hindgut, malpighian tubule, fat body and haemolymph glycopolypeptides in vivo. Proteins (20 mg aliquots) in (A) were detected by Coomassie blue staining. Replicate gel (B) (10 mg aliquots) was blotted onto nitrocellulose and probed with anti-GNA antibodies. Loading is as follows: (A) lane 1: (M) molecular weight marker; lanes 2-6 are extracts from control-fed larvae, lane 2: (MG) midgut; lane 3: (HG) hindgut; lane 4: (MT) malpighian tubule; lane 5: (FB) fat body; lane 6: (H) haemolymph; lanes 7-11 are extracts from Con A-fed larvae, loaded as described for lanes 2-6. (B) Lanes 1-5: extracts from control-fed larvae, loaded as described for lanes 2-6 in (A); lanes 6-10: extracts from Con A-fed larvae, loaded as described for lanes 2-6 in (A); lanes 11 and 12 are Con A standards (10 & 20 ng, respectively).

Fig 4.4.4C & 4.4.4D Binding of GNA to *L. oleracea* midgut, hindgut, malpighian tubule, fat body and haemolymph glycopolypeptides in vivo. Proteins (20 mg aliquots) in (A) were detected by Coomassie blue staining. Replicate gel (B) (10 mg aliquots) was blotted onto nitrocellulose and probed with anti-GNA antibodies. Bound antibodies were detected using peroxidase-labelled secondary antibodies followed by treatment with ECL reagents and exposure to X-ray film. Loading is as follows: (A) lane 1: (M) molecular weight marker; lanes 2-6 are extracts from control-fed larvae, lane 2: (MG) midgut; lane 3: (HG) hindgut; lane 4: (MT) malpighian tubule; lane 5: (FB) fat body; lane 6: (H) haemolymph; lanes 7-11 are extracts from GNA-fed larvae loaded as described for lanes 2-6. (B) Lanes 1-5: extracts from control-fed larvae, loaded as described for lanes 2-6 in (A); lanes 6-10: extracts from GNA-fed larvae, loaded as described for lanes 2-6 in (A); lanes 11 and 12 are GNA standards (10 & 20 ng, respectively).

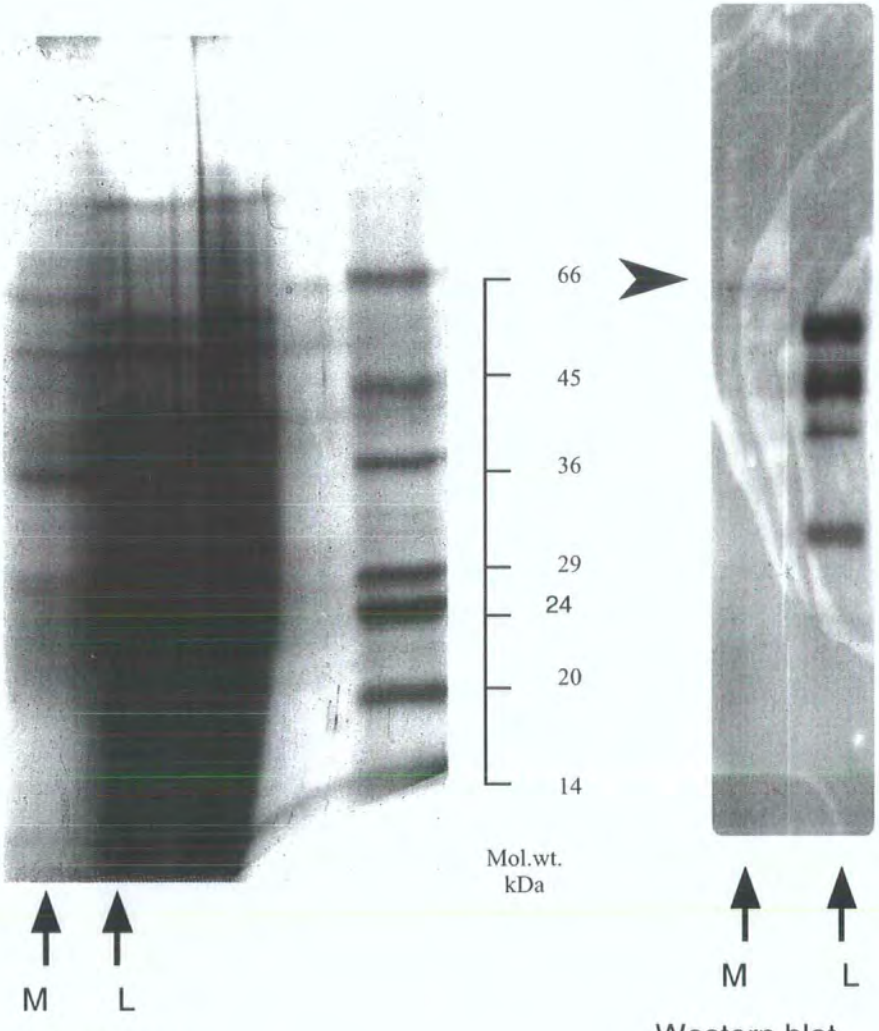
4.4.4 Detection of PHA binding to the midgut of *L. oleracea* larvae *and M. persicae.*

Fourth instar *L. oleracea* larvae were fed on an artificial diet containing DIG labelled PHA (see section 2.4.1) at 2% of total protein for 3 days and chased with control artificial diet for 1 day (diet information in section 3.2.1). 20µg aliquots of protein were separated on duplicate SDS-PAGE gels, and visualised by Coomassie blue staining, the protein on the second gel was transferred to a nitrocellulose filter (Schleicher and Schuell BA85) by electroblotting. The filter was probed with anti DIG antibodies to detect DIG-labelled PHA, bound antibodies were detected using the DIG Glycan Differentiation Kit. On the same gel 20µg of gut proteins from mature *M. persicae* fed on an artificial diet containing DIG labelled PHA were separated, visualised and probed for the presence of PHA in the same manner as the larval gut proteins.

As can be seen in fig 4.4.5 PHA is capable of binding to both the midgut fraction from *L. oleracea* larvae as well as the gut of *M. persicae.*

Fig 4.4.5 PHA binding to midgut of *L. oleracea* and *M. persicae*

Binding of PHA to gut proteins from *L. oleracea* and *M. persicae in vivo.* Gut proteins (20μg) aliquots) from PHA fed insects were visualised by coomassie blue staining of an SDS-PAGE gel, a replicate gel was transfered to nitrocellulose by western blotting presence of DIG labelled PHA was detected using anti-DIG antibodies.



SDS-PAGE
coomassie stained
gel

Western blot

Legend:

L = *Lacanobia oleracea* midgut (20µg protein loaded).

M = *Myzus persicae* midgut (20µg protein loaded).

➤ = Indicates PHA binding to a protein band in gut of *Myzus persicae*

4.5 Discussion

The techniques utilised in this chapter give results that are complementary; thus the histological evidence presented for GNA and Con A binding to *L. oleracea* gut tissue is corroborated by binding studies utilising SDS-PAGE and Western blotting techniques, confirming that lectins bind to *L. oleracea* gut tissue *in vitro* and *in vivo*.

The use of light microscopy and the VECTOR ABC kit in lectin immunolocalisation studies facilitated relatively quick observations of the binding characteristics of the snowdrop lectin GNA to gut sections of *L. oleracea* larvae. Plates 4.1.1 to 4.1.5 demonstrate that GNA binds to receptors across the whole section, from the lumen to the outside of the section, where it would come into contact with the haemolymph. This method is limited as the magnification restrictions of light microscopy make it impossible to determine exactly where the lectin is binding. However, light microscopy does allow us to determine if a lectin has the ability to bind to the structure in question or not. Additionally the biotin-streptavidin system can detect small quantities of a lectin if required, as massive replication of the reporter molecule is possible (Adams 1992), and further end product amplification can be achieved if required (Chen *et al.*, 1996). However, to clarify the situation and to look at structural changes the use of electron microscopy must be employed.

Plate 4.2.1 demonstrates that in the short term neither the Con A or GNA lectins showed any detrimental effect on the morphology of the brush border membrane,

when compared to control sections, although it is difficult to determine if either lectin had a mitogenic effect on the epithelial membrane (which is a possibility). Indeed GNA acts as a poor growth factor on the intestine of rats, although no toxic effects were observed following ten days exposure to GNA (Puzstai *et al.*, 1990).

Electron micrographs of sections from GNA fed *L. oleracea* larvae provide further evidence for GNA binding to the larval alimentary tract. GNA is present throughout the alimentary tract and is found in the cytosol from where it can presumably be transported to other areas within the larvae including the haemolymph (Fitches *et al.*, 2001). GNA has also been seen to be transported from the lumen into the haemolymph of the rice brown planthopper *Nilaparvata lugens.* (Powell *et al.*, 1998)

The pattern of lectin binding within the different areas of the *L. oleracea* gut (fore, mid and hindgut and malpighian tubules), is similar. However, the greatest extent of binding occurs in the midgut region. In all areas, binding is exhibited from the area of the microvilli all the way across the epithelial membrane and into the cytosol and on to the outside of the section, where lectins would come into contact with the contents of the haemolymph. As such it is likely that GNA can find its way into the haemolymph through this route.

Of particular interest is that both GNA and Con A bind to the malpighian tubules of *L. oleracea* larvae (although to a lesser extent than to gut regions), because the malpighian tubules act to concentrate and conserve nutrients and essential

ions while eliminating unwanted water (Dow 1986). Both lectins have the potential via binding to disrupt these processes within the exposed insect. Additionally the discovery of GNA bound to the malpighian tubules and its epithelial membrane indicates a further opportunity for lectin retention, as well as providing an additional route for Con A and GNA to migrate into the haemolymph.

The results from the lectin immunolocalisation studies in which GNA and Con A are found to be present throughout the alimentary tract has been backed up by the biochemical studies presented in section 4.4, in which the binding of the GNA and Con A lectins to tissues (such as the midgut) of the *L. oleracea* larvae has been demonstrated.

As none of the sections showed any form of localised binding it would appear logical that there is no particular area of the epithelial membrane at which GNA crosses the into the cytosol. Con A fed larvae exhibited a similar pattern of lectin distribution, to GNA fed larvae, with Con A present in the cytosol and along the epithelial membrane. Again as for GNA, Con A does not accumulate in specific regions of the gut, indicating no apparent specialised mechanism for lectin movement across the gut epithelium. The lack of lectin localisation allows the formation of a hypothesis that movement of GNA and Con A lectins is probably due to a relatively simple method, such as endocytosis. Endocytic uptake of lectins has been demonstrated to occur for PHA and GNA in rats. Puzstai and co-workers previously demonstrated that lectins which bind avidly to epithelial cells are also readily endocytosed and transcytosed (Puzstai *et al.*, 1990).

Whilst lectin binding to *L. oleracea* tissue has been demonstrated, to elucidate the mechanisms of insecticidal action of both GNA and Con A upon *L. oleracea* larvae further investigations are required. By comparison the toxicity of PHA to mammals has been well documented. PHA is known to exert an effect on a number of areas of the rat (Van Damme *et al.*, 1998) it can effect the systemic immune system of rats (Pusztai *et al.*, 1993), and is known to have a mitogenic effect on the gut epithelium (Palmer *et al.*, 1987). This results in disruption of normal ion and carbohydrate absorptive mechanisms and bacterial overgrowth of the small intestine (Donatucci *et al.*, 1987), (Dobbins *et al.*, 1986). PHA has a similar lethal effect on the western tarnished plant bug, *Lygus hesperus* (severe disruption of the epithelial layer) (Habibi *et al.*, 2000).

The mannose binding lectin from the peanut *Arachis hypogaea* (PNA), binds to the midgut epithelium of the lepidoptera *Chilo partellus* and it is hypothesised that the lectin depresses development via a binding induced antinutrititive mechanism (Law & Kfir 1996). Although unlikely due to no observed drop in feeding it is possible that Con A and GNA exert their toxic effects (at least to some extent) in the same manner.

The most common methods for detecting proteins and lectins in particular is to use antibodies. However, the production of antibodies is both expensive and lengthy. Therefore, by employing a technique to determine lectin binding that

does not require antibodies (such as the use of fluorescence), the costs and the time involved to complete such a study will be substantially reduced.

There are a number of drawbacks involved in the use of fluorescent labels, firstly fluorescence is ephemeral, secondly it can be difficult to make out structure of the tissue (twinkling stars in the dark effect), and thirdly problems attributable to tissue autofluorescence (Brooks *et al.*, 1997). However, in many cases these problems can be overcome.

The use of tissue blots proved quite effective in demonstrating the potential for lectin binding, although no further information could be gained, due to the lack of structural integrity of the tissue examined. In addition it is possible that lectin binding may not occur *in vivo* because this method exposes all the larval tissue to the lectin, not simply the tissue found along the length of its digestive tract. The fresh tissue experiments involving the use of "splayed out" midgut tissue of course overcomes the above problem. Unfortunately autofluorescence due to the high lipid content of the larval midgut necessitated the use of the lipid stain Sudan black, this together with the judicial use of filters reduced but did not eliminate tissue autofluorescence. As such only subjective comparisons relating to the intensity of fluorescence could be made. The RITC fluorochrome allowed a better definition of fluorescence than the FITC fluorochrome with GNA appearing to collect in channels in the gut. Although some idea of the nature of lectin binding could be determined from the results, binding localisation could not be determined due to the inability to distinguish any structures using this technique.

The technique of incubating fresh tissue in fluorescently labelled lectin worked best when employed to investigate lectin binding to the peritrophic membranes of *L. oleracea* larvae. As demonstrated in plate 4.3.4, the speckled effect of the fluorescence indicates the position of lectin binding sites. It is known that the peritrophic membrane is porous (Tellam 1996) and therefore possible that GNA is blocking the pores of the peritrophic membrane due to localisation of mannose receptors at these sites, and by so doing exerts a toxic effect similar to that suggested by Eisemann *et al.*, 1994, who determined that wheat germ agglutinin (WGA) lectin binds to the peritrophic membrane of the blowfly larvae *Lucilla cuprina* and reduced its permeability. Eisemann *et al* suggest that this reduced permeablility of the peritrophic membrane combined with WGA binding to apical membranes of the epithelial membrane and reduced ingestion of diet could lead to starvation of the insect.

The use of embedded tissue sections part 4.3.4 clearly demonstrated lectin binding and gave the best result from incubating specimens in fluorochrome conjugated lectins. It allows easy visualisation of structures and once the slides are prepared they can be stored indefinitely for future use with a lectin conjugate. Of course a drawback is the initial preparation time, the fact that it is not fresh tissue and that fluorescence is ephemeral. Indeed it proved difficult to find a good fixative for the *L. oleracea* larvae, due to a high chitin content. As such it must be remembered that the amount of binding observed may have been decreased by the fixation protocol which can be a critical factor (Hoyer & Kirkeby 1996).

As mentioned earlier, the detection of GNA and Con A lectin binding to the *L. oleracea* larvae by non-histological means in section 4.4, confirms the results obtained from histological studies, and vice versa. The larvae in section 4.4.4 were fed on an artificial diet containing either Con A or GNA, and the resultant Western blot clearly indicates that both Con A and GNA bind *in vivo* to the different dissected sections of the gut of the *L. oleracea* larvae. In addition to which it also provides evidence that both GNA and Con A have been transported from the lumen to the haemolymph. The non-histological evidence in section 4.4.1 and 4.4.2 demonstrated that both Con A and GNA lectins bind *in vitro* to the brush border membrane proteins of the small intestine of rats, although to a lesser extent than observed binding to brush border membrane proteins from *L. oleracea* larvae.

Con A bound more avidly than GNA to both rat and *L. oleracea* extracts and this corresponds with the relative toxicity of the two lectins. In mammals Con A adversely effects the brush border membrane (Nakata & Kimura 1985) (Lorenzsonn & Olsen 1982), as well as causing cytotoxic effects (Lorenz-Meyer *et al.*, 1985), particularly in young animals (Weaver & Bailey 1987). Although GNA does have some effect, it is not known to have a long term effect on mammals (Pusztai & Bardocz 1996). Both Con A and GNA have insecticidal properties. For example Con A exerts a detrimental effect on both *L. oleracea* (Gatehouse *et al.*, 1999) and *Lucilia cuprina* (Eisemann *et al.*, 1994). GNA (which has been tested for insecticidal properties to a greater extent than Con A, as it has no long term effect on mammals) has a detrimental effect on sugarcane

whitegrubs (Allsopp & McGhie 1996), as well as on *L. oleracea* larvae (Fitches *et al.*, 1997).

In vitro tests carried out by incubating Western blots of a brush border membrane preparation (BBMV) are a further way to analyse lectin binding. The blot of a BBMV preparation from *L. oleracea* incubated in Con A (fig 4.4.3) not only indicates that Con A binds to the various fractions of the midgut, but also indicates the major protein to which Con A binds. This is thought to be leucine-amino-peptidase N, because it has been demonstrated that Con A causes an increase in aminopeptidase activity (Fitches *et al.*, 1998).

PHA has been shown to have an adverse effect on *L. oleracea* (chapter 3), and the *in vitro* binding study here provides evidence for lectin binding. Whilst the reason for toxicological effects cannot be determined the mechanisms of toxicity may be similar to those seen in mammals (Puzstai *et al.*, 1990). As PHA binds to the gut of *M. persicae*, it may also have a toxic effect on the aphid but this remains to elucidated. However, due to the toxicity of PHA to animals there is little interest in producing crops expressing the PHA lectin and it has little use in the field of crop protection.

It has been hypothesised that the amount of lectin binding is proportional to its toxic effect and that toxicity does not appear to occur without lectin binding (Harper *et al.*, 1995). Although it must be remembered that not all lectins which bind to various fractions/areas of species are toxic, as such it obvious that a lot more research is required to answer the questions regarding toxicity.

Chapter 5

Binding of the snowdrop lectin Galanthus nivalis agglutinin (GNA) in the peach/green potato aphid *M. persicae*

5.1 Introduction

M. persicae is an important pest species capable of colonising a number of economically important crops. Studies have shown this species to be susceptible to the effects of the snowdrop lectin GNA (Sauvion *et al.*, 1996).

This chapter aims to demonstrate a protocol for rapid determination of lectin binding to aphid guts (in this case utilising GNA), and to investigate the reason for the toxicity of lectins to aphids using GNA as a model. Confocal and fluorescence microscopy utilising fluorescently labelled GNA was used to investigate if toxicity of GNA to *M. persicae* could be attributable to binding of the lectin *in vivo*.

5.2 Materials and methods

5.2.1 Detection of lectin presence *in vivo* in *M. persicae*

Adult aphids of the same stage of development were removed from plants and put onto artificial diet. After 24 hours the aphids were divided into 3 treatment groups of 20 and fed for 2 days on three different diets; control artificial diet, artificial diet containing FITC conjugated GNA at 0.25mg/ml and an artificial diet containing FITC conjugated BSA at a concentration of 0.25mg/ml. Proteins were conjugated with FITC as described in chapter 2. After 2 days the aphids were viewed using a Nikon diaphot UV microscope. All three treatments were then put onto control artificial diet and the aphids were viewed over the next 4 days.

5.2.2 Detection of lectin binding in the gut of *M. persicae*.

Adult aphids removed from plants were placed into aphid feeding chambers containing artificial diet overnight to produce nymphs. Nymphs were reared on artificial diet for 5 days.

On day 5 the aphids were split into three groups and exposed to control, GNA-FITC and BSA-FITC artificial diets as described in section 5.2.1 Aphids were kept on this diet for a period of 48 hours after which the three treatment groups were "chased" with control artificial diet for time periods between 4 hours and 1 week. At the end of each specified time period the aphids were sacrificed and the guts dissected out into 1xPBS and viewed immediately with a Bio rad μ radiance scanning system confocal microscope.

5.3 Results

All plates presented show a representative sample from the experiments carried out.

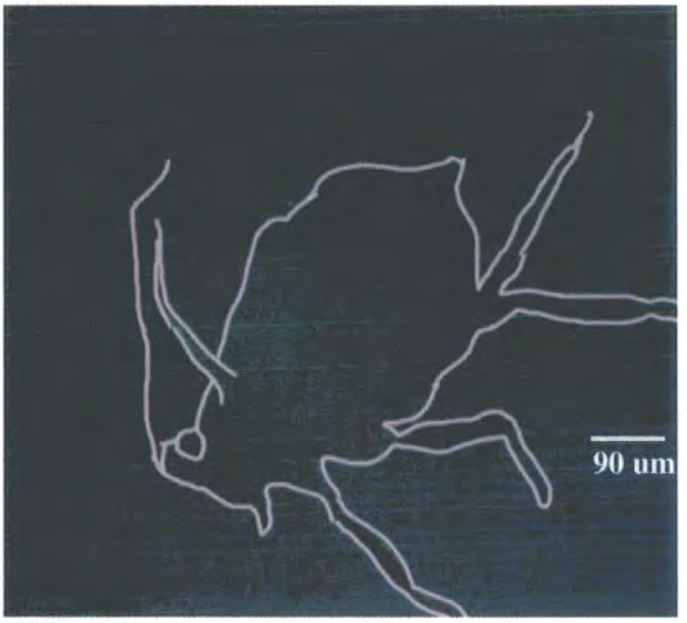
5.3.1 Detection of lectin presence *in vivo* in *M. persicae*

Plate 5.1 shows representative aphids that were fed on the three types of test artificial diets (control, GNA-FITC or BSA-FITC containing diets) described in 5.2.1, and then chased with artificial diet only for 48 hours and viewed with a UV diaphot microscope. Plate 5.1A shows an aphid from the control treatment group. As no fluorescence was observed the aphid has been outlined for ease of viewing. Plate 5.1B shows an aphid fed on the artificial diet containing GNA-FITC. Fluorescence from GNA-FITC is clearly visible and was concentrated in the central area of the aphid. Plate 5.1C shows an aphid fed on the artificial diet containing BSA-FITC. Fluorescence from the BSA-FITC was not as high as that exhibited in the GNA-FITC fed aphid and was less localised.

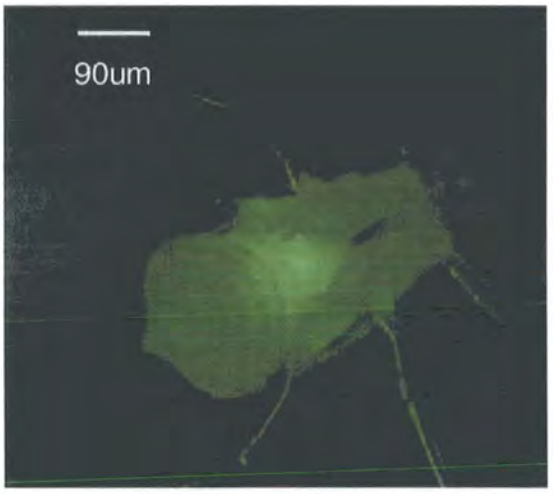
Plate 5.2 shows aphids from the three different feeding regimes as previously described in section 5.2.1, which were subsequently chased with artificial di*et al*one for 96 hours.

Plate 5.2A is an image of *M. persicae* fed on artificial diet containing BSA-FITC, all traces of fluorescence have disappeared and the aphid had to be outlined for ease of viewing. Plate 5.2B shows a *M. persicae* fed on an artificial diet containing GNA-FITC, unlike aphids exposed to BSA-FITC those exposed to

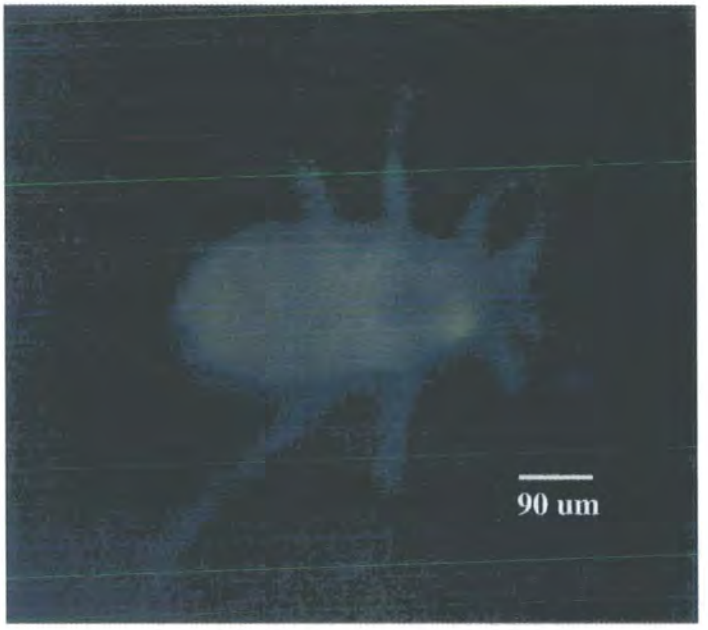
Plate 5.1 *M. persicae* fed for 48 hours on three types of artificial diet, control diet or diets containing GNA-FITC or BSA-FITC. Insects were then chased for 48 hours on artificial di before viewing with a Nikon diphot UV microscope



5.1 A



5.1 B



5.1 C

Key

5.1 A = *M. persicae* fed on artificial diet for 96 hours.

5.1 B = *M. persicae* fed on GNA-FITC for 48 hours and chased with artificial diet for 48 hours.

5.1 C = *M. persicae* fed on BSA-FITC for 48 hours and chased with artificial diet for 48 hours.

Plate 5.2 *M. persicae* fed for 48 hours on artificial diet, containing GNA-FITC or BSA-FITC. Insects were then chased for 96 hours on artificial diet before viewing with a Nikon diphot UV microscope

Plate 5.2 A

KEY

Plate 5.2 A = *M. persicae* fed on BSA-FITC for 48 hours and chased with artificial diet for 96 hours.

Plate 5.2 B = *M. persicae* fed on GNA-FITC for 48 hours and chased with artificial diet for 96 hours.

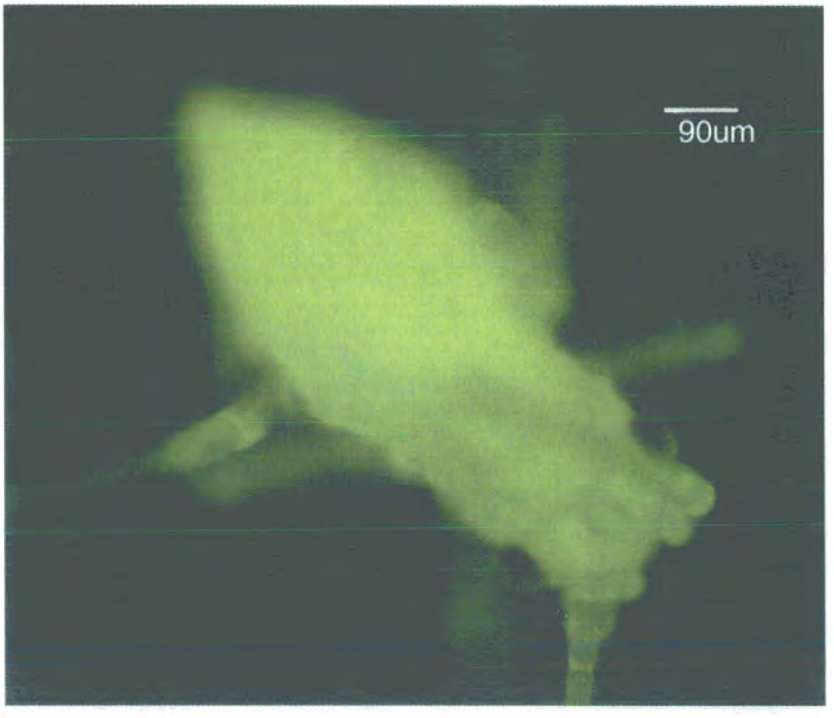


Plate 5.2 B

GNA-FITC exhibited fluorescence throughout their bodies after being chased with artificial diet for 96 hours.

5.3.2 *In vivo* detection of GNA binding in the gut of *M. persicae* nymphs.

As described in section 5.2.2, *M. persicae* nymphs were fed on an artificial diet containing either GNA-FITC, Albumin-FITC, or a control diet with no fluorochrome for a period of 48 hours, followed by a chase period of up to 96 hours with control artificial diet for up to 96 hours. Sacrificed larvae had their guts dissected out into PBS and viewed with a Bio-Rad confocal microscope immediately after dissection.

No fluorescence was observed in *M. persicae* fed on the artificial diet containing BSA-FITC and chased with artificial diet containing no fluorochrome after 24 hours, or at subsequent time points see Plate 5.3. The same result was observed in guts dissected from control fed aphids (i.e. no autofluorescence).

Plate 5.4 is a confocal image of the foregut of *M. persicae* fed on an artificial diet containing GNA-FITC and subsequently chased on artificial diet for 24 hours, the yellow-green colouration is GNA-FITC. GNA was seen to be present in the foregut area where it was concentrated on the boundary as well as being found in the central luminal area. The GNA also bound to the haemocytes that remained present in the PBS incubation solution.

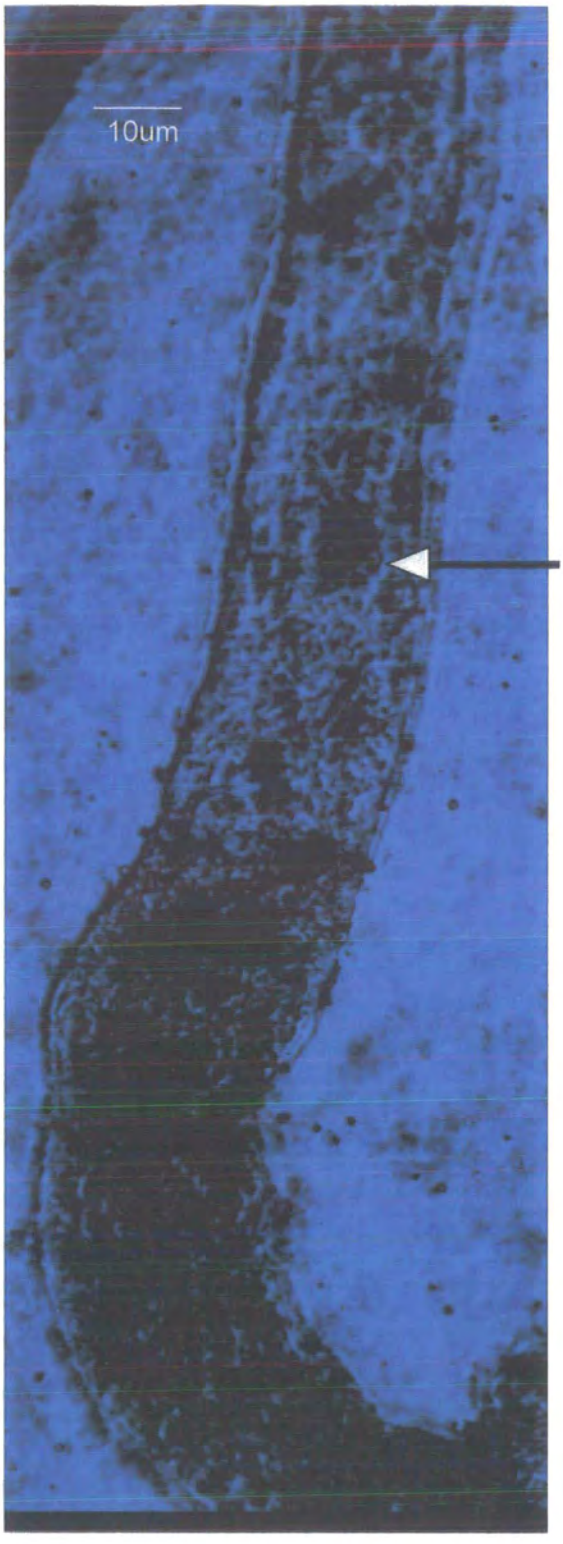
Plate 5.5 shows that after being chased for 48 hours with artificial diet (containing no fluorochrome) the GNA-FITC remained present in the foregut. However, the

power of the laser had to be increased for this image, at this time point no GNA-FITC remained in the lumen of the foregut, with fluorescence being restricted to the gut membrane.

Plates 5.6- 5.8 Show representative and comparable images of the midgut area of *M. persicae*, fed on GNA-FITC and chased with artificial diet for 24, 48 and 96 hours respectively (as described in section 5.2.2). Plate 5.6 demonstrates that GNA-FITC was present throughout the whole of this part of the gut. It was present at the exterior of the stomach, and internally along the cuboidal, and columnar cells, as well as between the rods of the striated border (see fig 1.7 in chapter 1).

Fluorescence in plate 5.7 is less than in plate 5.6 and is more concentrated towards the outside of the section. Plate 5.8 demonstrates that when chased with control diet for 96 hours an even more marked decrease in fluorescence is observed, the section shows the same gut area as that displayed in plates 5.6 and 5.7, but the GNA is almost solely concentrated towards the exterior (tunica propria) of the midgut area of the aphid.

Plate 5.3 Foregut of *M. persicae* fed on BSA-FITC for 48 hours and chased with artificial diet for 24 hours



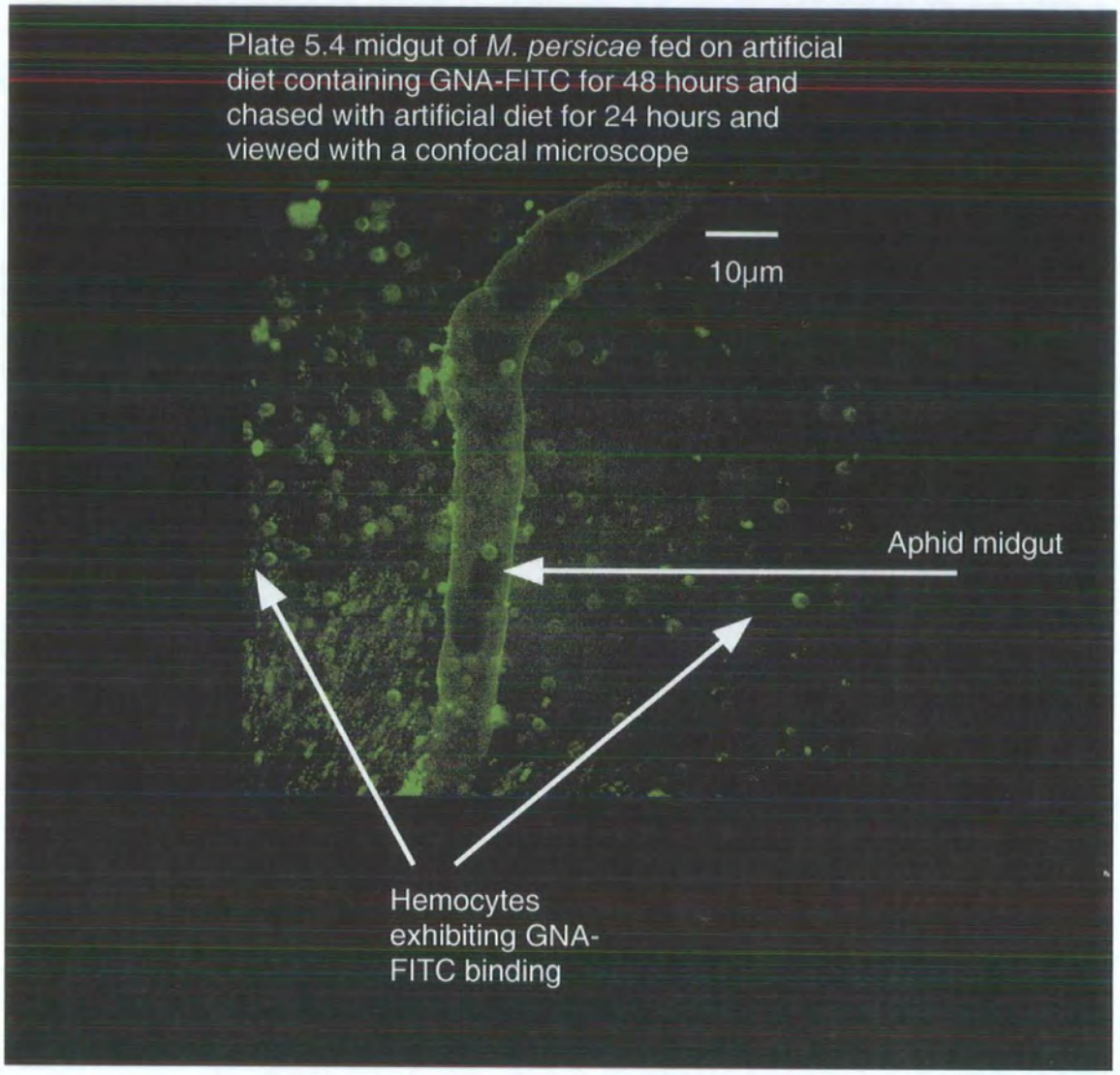


Plate 5.4 midgut of *M. persicae* fed on artificial diet containing GNA-FITC for 48 hours and chased with artificial diet for 24 hours and viewed with a confocal microscope
10µm
Aphid midgut
Hemocytes exhibiting GNA-FITC binding

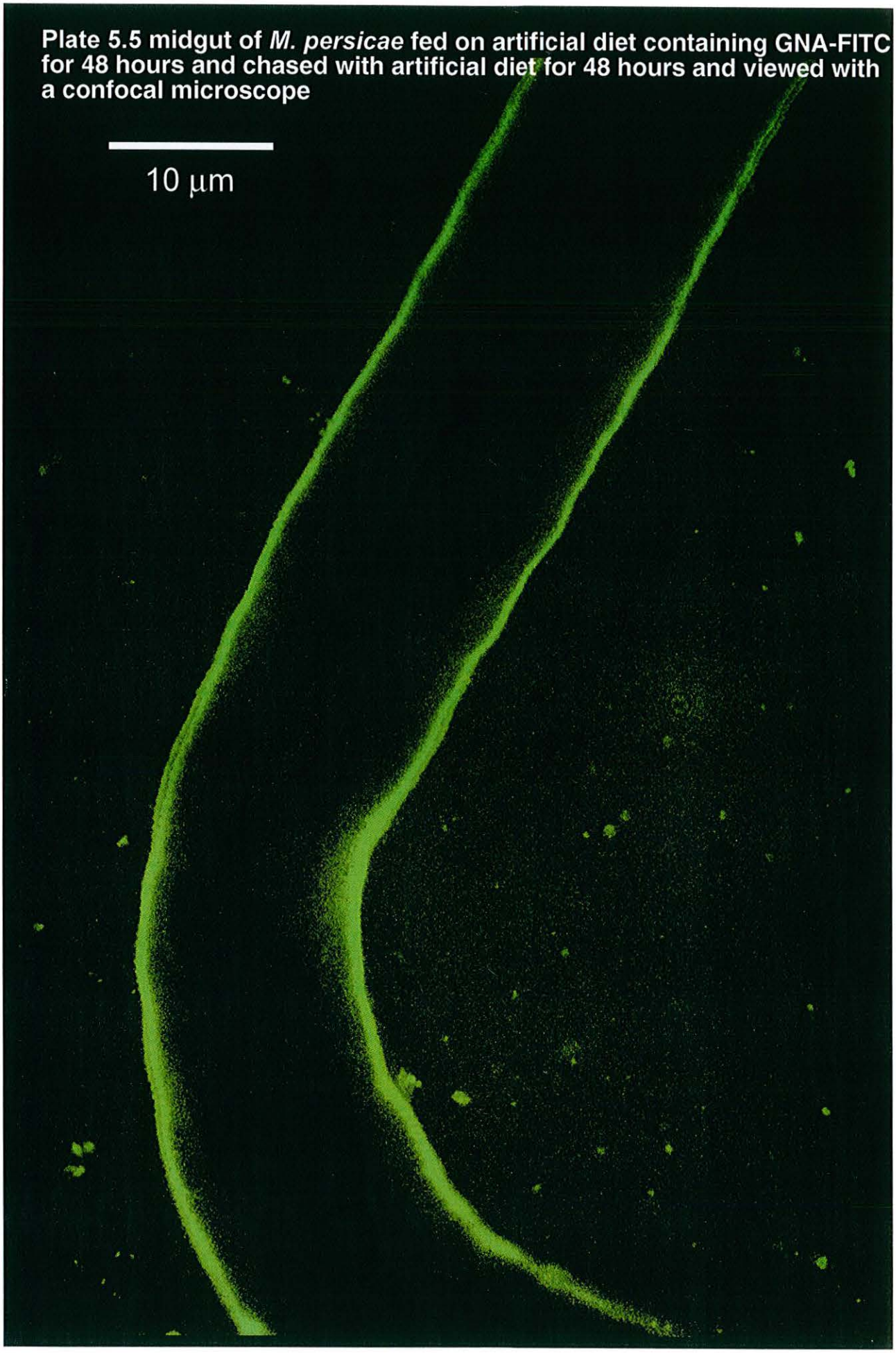


Plate 5.5 midgut of *M. persicae* fed on artificial diet containing GNA-FITC for 48 hours and chased with artificial diet for 48 hours and viewed with a confocal microscope

Plate 5.6 & 5.7 midgut of *M. persicae* fed for 48 hours on GNA-FITC than chased for 24 hours Plate 5.6 (top), and 48 hours Plate 5.7 (bottom) . Sections were visualised using a confocal microscope.

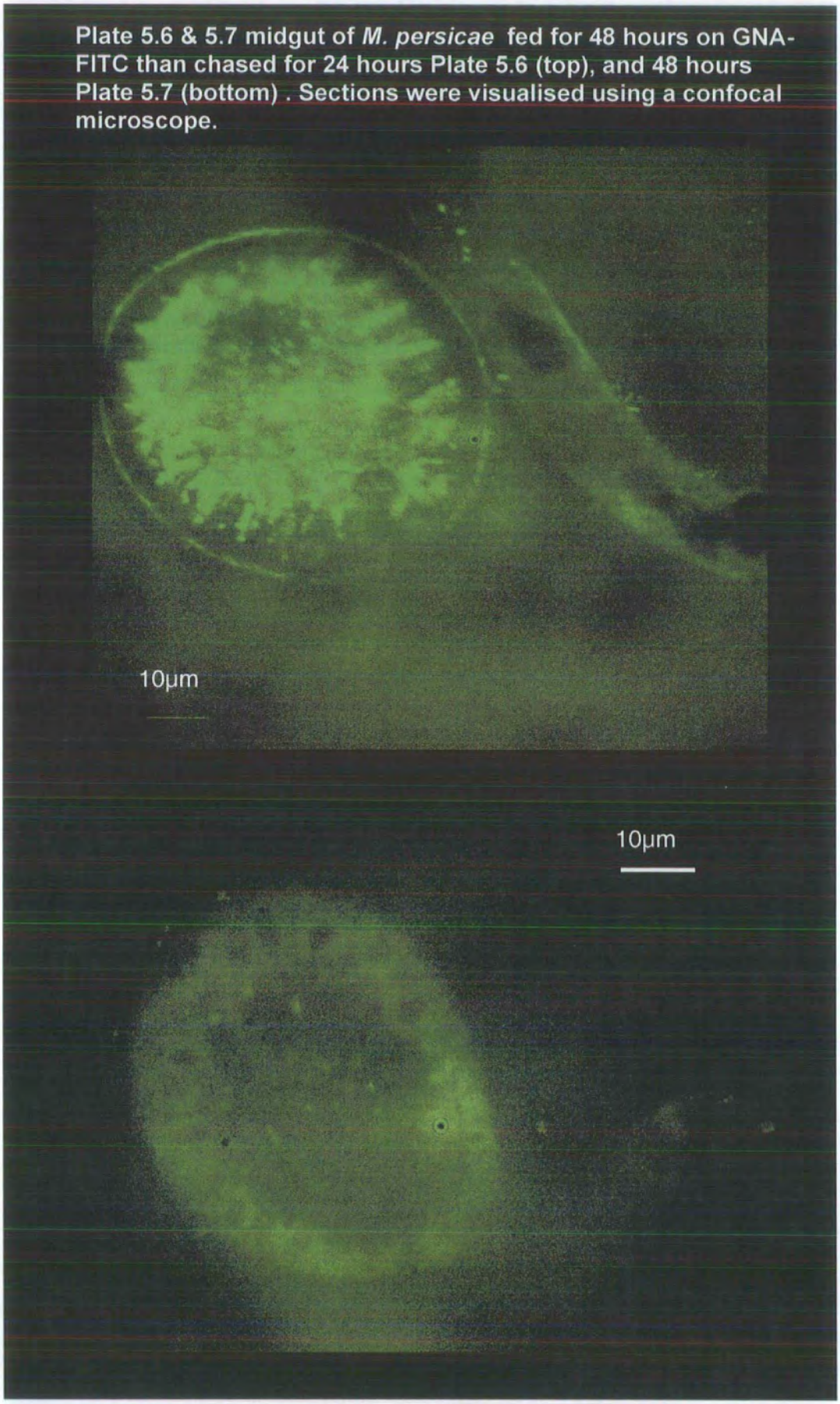
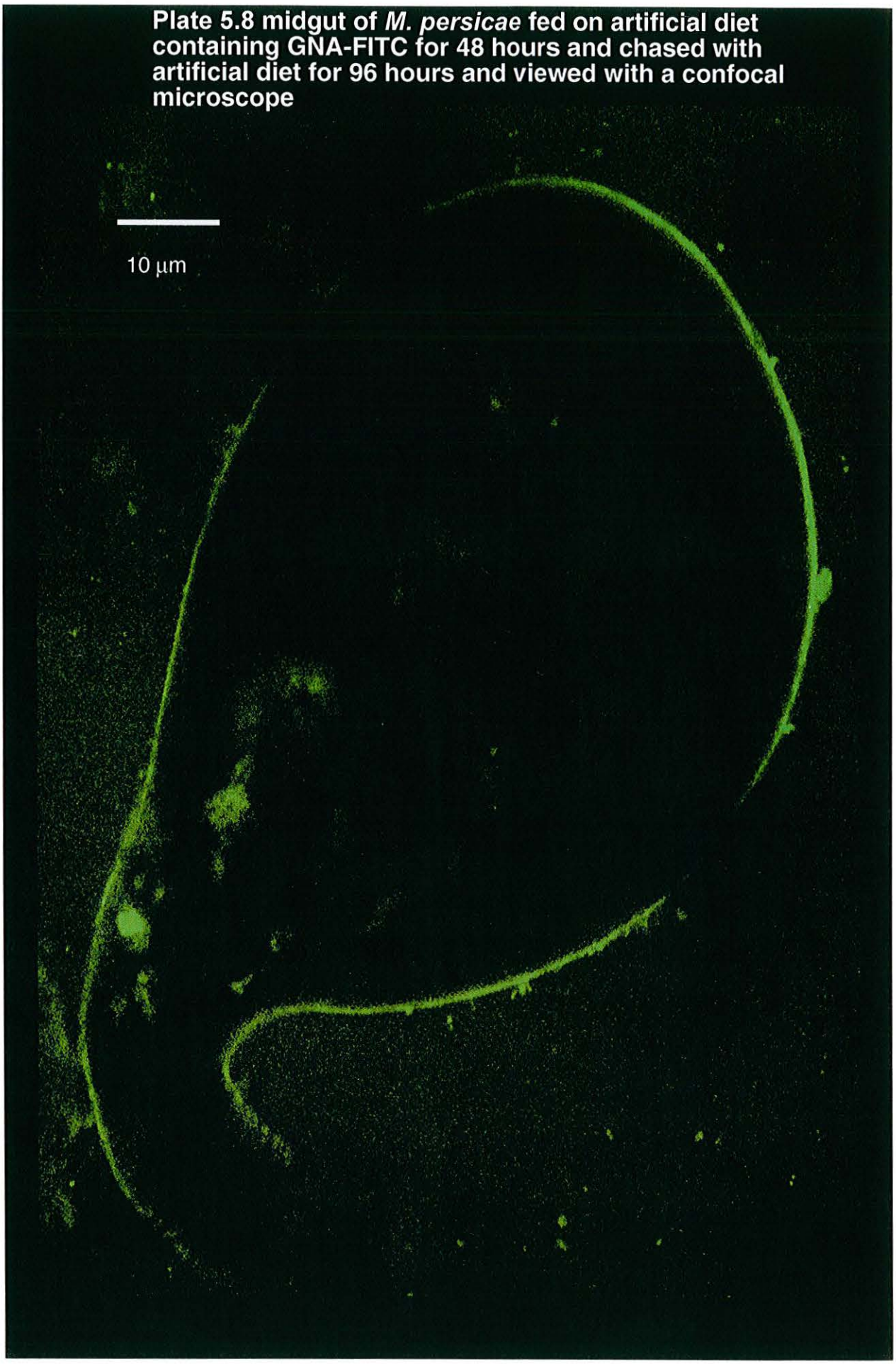


Plate 5.8 midgut of *M. persicae* fed on artificial diet containing GNA-FITC for 48 hours and chased with artificial diet for 96 hours and viewed with a confocal microscope



5.4 Discussion

Results presented for whole aphid in section 5.3.1 indicate that aphids fed on BSA-FITC retain fluorescence within their body after being chased with normal artificial diet for 48 hours, after which fluorescence disappears totally, suggesting that the BSA-FITC has been rapidly processed and excreted. Conversely aphids fed on GNA-FITC retain fluorescence within their body after being chased with normal artificial diet for at least 96 hours. Direct visualisation of localised GNA-FITC was possible at the 48hour chase time point. At the 96hour chase time point with artificial di*et al*one, localisation of fluorescence became less distinct/defined, suggesting that the GNA-FITC conjugate is initially retained within a specific area of the aphid (presumably via binding to exposed mannose residues), and is subsequently dispersed throughout the body of the insect. The advantage of this experiment is that it removes the uncertainty associated with the effects of dissection. However, it does not provide definitive evidence with regard to if and where in the aphid GNA binding occurs.

The results from section 5.3.2 examined GNA binding in more detail and removed some of the speculation regarding the fate of GNA.

Plate 5.3 indicates that when fed a diet containing albumin-FITC the fluorochrome is not detectable within the aphid gut after being chased with artificial diet for 24 hours, indicating that the BSA-FITC is either not taken up by the insect and is simply excreted, or it is transported through the foregut to the

midgut and crosses the epithelial membrane, the albumin–FITC conjugate would the be broken down, the protein digested and the fluorochrome excreted. This result is consistent with, and supports the results for whole aphids in section 5.3.1.

The foregut of the aphids fed on GNA-FITC for 12 hours exhibited fluorescence for at least 7 days after feeding. Initially the GNA was seen to be bound to the sides of the foregut and present in the lumen of the foregut.

It is likely that GNA becomes detached from its position in the foregut due to abrasion, cells sloughing off, or by developing a higher affinity for something in the aphids diet. As the luminal area of the *M. persicae* has a germ free gut (Grenier *et al.*, 1994), it is unlikely that GNA is interacting with gut microfauna as occurs in rats, where GNA reduces pathogen numbers in the lower part of the small bowel and large intestine (Naughton *et al.*, 2000).

As with the whole aphids, fluorescence of GNA-FITC in the guts of dissected aphids decreased with increased length of exposure to control diet. Indeed at the 48 hour time point the power of the laser was increased to allow better visualisation of the fluorescence produced by GNA-FITC. Of particular interest is that the GNA binds to haemocytes. This is in agreement with Fitches *et al.*, 2000 who found GNA to be present in the haemolymph of *L. oleracea* and to be bound to *L. oleracea* haemocytes (Raemaekers 2000 pers.comm). GNA is known to be toxic *to M. persicae* (Sauvion *et al.*, 1996) and the delivery of the lectin to the haemolymph and subsequent binding to haemocytes may be part of the

mechanism by which lectins exert detrimental effects on insects, in analogy to the effect of PHA in rats (Leivestadt *et al.*, 1988) (Greer & Pusztai 1985).

It is known that GNA has the ability to resist degradation in the gut (Van Damme *et al.*, 1998). This was confirmed to be the case in *M. persicae* by the results presented in section 5.3, in which GNA was observed to remain bound to the gut for at least 1 week. Fluorescence dissipates with time (as seen in both the foregut, and the midgut sections) indicating a decrease in the amount of GNA present in the aphids digestive tract. This evidence for GNA binding *in vivo* to the digestive tract of *M. persicae* confirmed previous *in vitro* binding studies in *M. persicae* (Down *et al.*, 2000).

In conclusion the results indicate that a large percentage of the ingested GNA became strongly bound to the gut epithelial membrane of *M. persicae* and that with time a percentage of the lectin is transported across the epithelial membrane to the cytosol and subsequently into the haemolymph.

Further research is required to allow determination of the reason for the significant effect on aphid mortality caused by GNA on *M. persicae* (Sauvion *et al.*, 1996). As such we can only hypothesise that the detrimental effects of the lectin are in part to effect of GNA upon haemocytes, and/or that GNA blocks or disrupts the normal metabolic functions of the insect. Other studies indicate that toxicity of lectins to be attributable to disruption of the gut epithelial membrane (Powell *et al.*, 1998, King *et al.*, 1982). However, at the level of microscopy employed here it is impossible to determine if the GNA lectin causes a disruption of the *M. persicae* epithelial membrane.

Chapter 6

Binding of the Snowdrop Lectin *Galanthus nivalis* in the Two Spot Ladybird Larvae *A. bipunctata*

6.1 Introduction

In any method of pest control one does not wish to exert a detrimental effect on beneficial insects or on non-target organisms. The two spot ladybird (a known beneficial insect) was chosen as a test species to investigate toxicity of plant lectins on a tritrophic level. This chapter forms part of a larger project investigating the toxic effects of GNA on non target species. As lectin binding appears to be a prerequisite for toxicity (see chapter 1), the binding potential of GNA to the larval midgut and any subsequent effects of GNA on the ultrastructure of the gut have been examined. GNA has been shown to be non toxic to *A. bipunctata* larvae (Down *et al.*, 2000), and therefore these studies were aimed at investigating whether this was attributable to an absence of lectin binding within exposed tissue.

6.2 Materials methods

6.2.1 Immunolocalisation of GNA in *A. bipunctata* at the electron microscope level.

The aphid *M. persicae* was used as a delivery system to introduce GNA to the *A. bipunctata* larvae. On a daily basis, 12 chambers containing approximately 50

neonate to mid-instar aphids were prepared as follows: 6 were supplied with artificial diet, containing 0.1% w/v GNA, and 6 were supplied diet with GNA replaced by casein. The aphids were fed on diet for 4 days, before being fed to the ladybird larvae, with the diet sachets being replaced every 2 days to limit fungal contamination of the diet.

The ladybird larvae were individually placed in feeding chambers made from the lid of a 3.5cm petri dish lined with a piece of dry filter paper, and covered with a layer of parafilm over the top. Aphids were supplied to the larvae on a daily basis to appetite. The *A. bipunctata* larvae were fed from egg hatch until reaching 4th instar. Larval midguts were dissected, on ice, from the ladybird larvae and immediately placed in fixative (2.5% paraformaldehyde, 2.5% gluteraldehyde, 2% sucrose in Sorensens buffer at pH 7.4 prepared as described by Glauert, (1975) and cut into smaller sections. After 3 hours of fixation, at room temperature and spinning on a wheel, the pieces of gut were washed for 3x 5-10 minutes in Sorensens buffer pH 7.4, rinsed in distilled water, and dehydrated by incubation in 50% ethanol for 2x 5-10 minutes followed by incubation in 70% ethanol for 3x 10 minutes. Gut pieces were then placed in a 70% ethanol/LR white resin mix for 30 minutes before transferring to pure LR white resin for 3-4 hours. Sections were incubated with fresh resin overnight, then again with fresh resin for 3-4 hours, and embedded in capsules containing LR white resin at 50-55°C for 24hours.

Ultra thin sections (60-80µm thick) were cut on a microtome (Leica Ultracut) and mounted on formvar and carbon coated nickel grids (150 mesh, hexagonal). For

immunohistochemical labelling, sections were incubated for 10 minutes in heat inactivated goat serum before incubating overnight at 4°C in a 1:100 dilution (in Tris-HCl buffer pH 7.5) of antibody raised against recombinant GNA. Sections were washed for 4x 5 minutes in Tris-HCl buffer pH 7.5, and 1x 5 minutes in Tris-HCl buffer pH 8.2, before incubating for 1 hour in anti-rabbit 10nm gold-conjugated IgG (1:20 dilution in Tris-HCl buffer pH 8.2). The above Tris-buffers were prepared according to the guidelines sent with the gold conjugate secondary antibody (Agar Scientific, Stansted), except that the sodium azide was omitted. Sections were washed for 1x 5 minutes in Tris-HCl pH 8.2, and 4x 5 minutes in distilled water, then stained for 10 minutes in 1% aqueous uranyl acetate and 10 minutes in lead citrate (Reynolds 1963). Sections were washed between and after staining for 5x 5 minutes in distilled water and viewed using a transmission electron microscope (Philips EM400T). This procedure was repeated on midguts dissected from larvae, which had been reared on *M. persicae* fed control artificial diet.

6.3 Results

6.3.1 The effect of GNA on the morphology of the ladybird larvae *A. bipunctata* midgut.

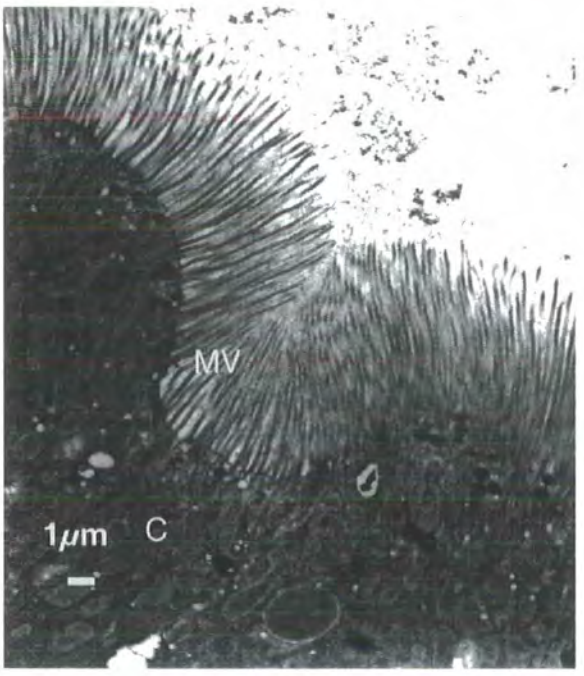
Comparing representative electron micrographs of the midgut of *A. bipunctata* larvae fed on control or GNA dosed aphids (plate 6A), it is apparent that no disruption of the brush border membrane is exhibited. Furthermore no noticeable differences in the ultrastructure of the microvilli were observed.

6.3.2 Detection of GNA in the lumen, epithelial and cytosol areas of the two spot ladybird larvae *A. bipunctata*.

Plate 6.B is a representative electron micrograph of the same structure as Plate 6A, but at a higher magnification. The section has been subjected to the immunogold labelling procedure (section 6.2.1). Gold particles indicating presence of GNA are found along the microvilli as well as on the other side of the brush border epithelial membrane in the cytosol. Plate 6C is an enlargement of a section of plate 6B allowing better visualisation of the gold particles conjugated to GNA and as such lectin binding is clearly exhibited.

Plates 6D & 6E are control sections, plate 6D is a representative section of the midgut from the control fed ladybird larvae which underwent the same staining procedure as plate 6B, and plate 6E is a representative section of midgut from the GNA fed larvae, which followed the same labeling procedure as the other sections, except that the primary antibody step was omitted.
The absence of gold particles in these sections verified the validity of the experiment.

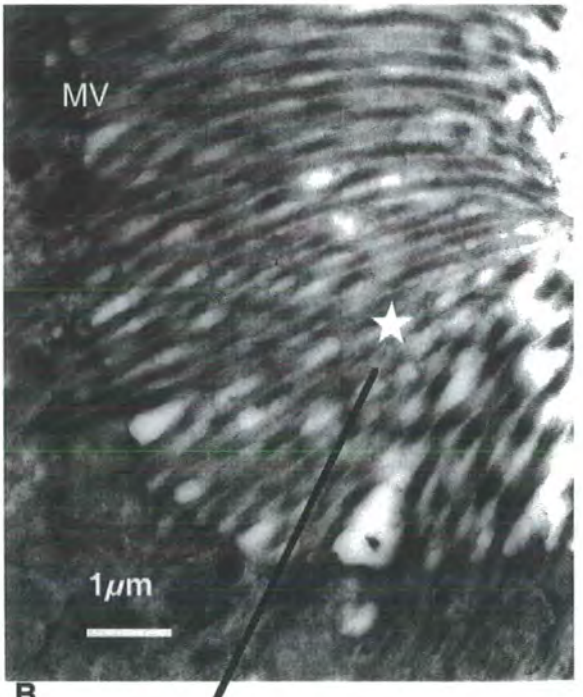
Plate 6A-C electron micrographs of the midgut of GNA fed *A. bipunctata* larvae



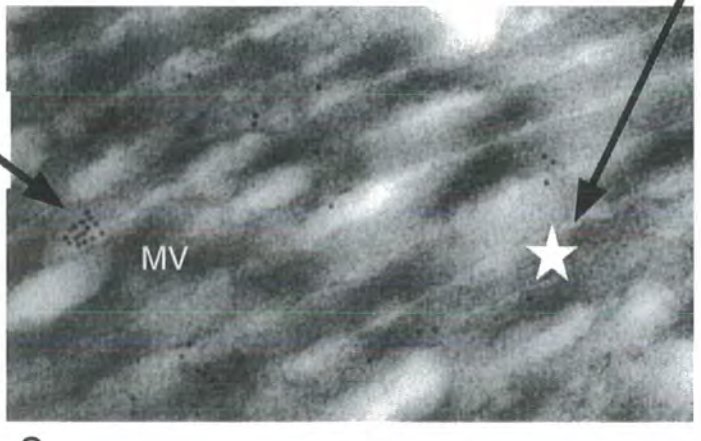
A

Legend To *A. bipunctata* larvae micrographs

A = Low power micrograph of GNA fed *A. bipunctata.*
B = GNA fed *A. bipunctata* with imunogold particles indicating binding of GNA.
C = Close up of B.
MV = Microvilli
C = Cytosol

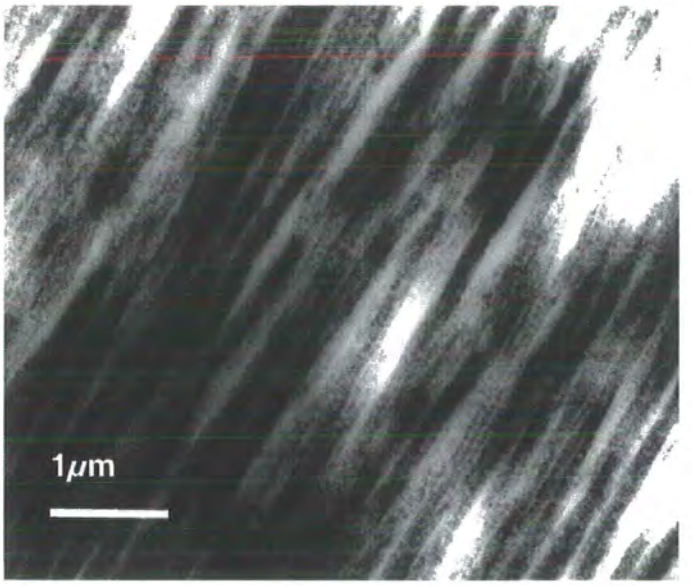


B

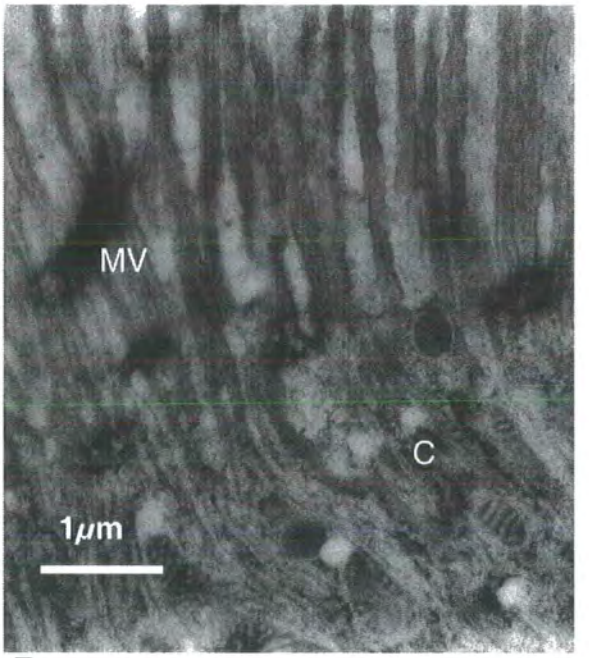


C

Plate 6D-E electron micrographs of the midgut of control and GNA fed *A. bipunctata* larvae



D



E

Legend To *A. bipunctata* larvae micrographs
D = Control fed *A. bipunctata* underwent the same staining procedure as plate B.
E = GNA fed *A. bipunctata* but the primary antibody step was ommited.
MV = Microvilli
C = Cytosol

6.4 Discussion

Plates 6B-6E clearly demonstrate that GNA binds to the epithelial membrane of the ladybird larvae, and that GNA is present in the cytosol. As such it is apparent that GNA has been transported across said membrane into the cytosol. A feature of some lectins such as PHA, is their mitogenic effect on epithelial cells causing the disruption of the microvilli the result of which is often expression of a toxic effect (Pusztai & Bardocz 1996). Indeed PHA induces lesions, which seriously effect the morphology of the gut epithelial membrane of rats (King *et al.*, 1986). In *A. bipunctata* larvae although GNA binds to the epithelial membrane in the midgut of the ladybird larvae, no disruption of the microvilli is apparent. This allows us to hypothesise that if GNA were to exert a toxic effect on the ladybird larvae, it would not be due to a mitogenic effect on the epithelial membrane, or by the emergence of lesions. The snowdrop lectin GNA was found to bind across the midgut sections of the GNA fed ladybird larvae, with no non-specific binding exhibited in the control sections.

An SDS-PAGE gel, and subsequent Western blot of the 2 spot ladybird midgut protein (not shown), has further backed up the immunogold evidence of lectin binding. The Western blot was incubated in recombinant GNA and evidence of binding of GNA to the protein bands of the insects midgut was determined by ECL detection (Down *et al.*, 2000).

Due to the lack of localised binding of GNA, the mechanism of transport across the epithelial lining of the midgut into the cytosol of the ladybird larvae is unknown. This is also the case in the midgut of the tomato moth larvae, *L.*

oleracea with the lectins GNA and Con A (chapter 4), and with GNA in the brown planthopper (BPH) *Nilaparvata lugens* (Powell *et al.*, 1998). In all these examples no localised binding was found and the lectins exhibited a similar binding pattern along the epithelial membrane and in the cytosol. The mechanism of transport is also unclear in the intestine of rats fed on PHA due to lack of localised binding (King 1986). Although, it is known that PHA exerts some of its toxicity by its adverse effect on the epithelial membrane (Bardocz *et al.*, 1997).

The difference between the *A. bipunctata* larvae and *L. oleracea* larvae is that GNA exerts an insecticidal effect upon *L. oleracea* larvae (Fitches *et al.*, 1997), but not to *A. bipunctata* larvae (Down *et al.*, 2000). However, it has been reported that when adult *A. bipunctata* were fed for 12 days on the peach potato aphid *M. persicae* which had been colonising GNA expressing transgenic potatoes, ladybird fecundity, egg production and longevity significantly decreased over the next 2-3 weeks. (Birch *et al.*, 1999). This work is contradicted by a recent study, in which no significant differences between treated and control ladybirds was exhibited (Down *et al.*, 2000). The study by Down *et al*, was more comprehensive and with a larger sample size, which combined with the study investigating the effect of GNA on the *A. bipunctata* larvae indicates that the majority of scientific evidence supports the fact that GNA does not exert a toxic effect on *A. bipunctata*.

Chapter 7

Effect of Lectins on the Polyphenoloxidase Activity in the Tomato Moth (*L. oleracea.*) Larvae

7.1 Introduction

As discussed in chapter 1, while there are many theories with regard to toxicity of lectins to insects, the actual mechanism of lectin toxicity is unknown. This chapter investigates a potential mechanism of lectin toxicity, utilising *L. oleracea* larvae as a test model. GNA and PHA were chosen as test lectins because it is known that both bind to the lepidopteran gut and that GNA is found in the insect haemolymph (Fitches *et al* 2001).

7.2 Materials and methods

7.2.1 Systemic effects of GNA on the polyphenoloxidase system in *L. oleracea*

Forty newly ecdysed fourth instar *L. oleracea* were selected, 20 were placed on a artificial diet containing GNA at 2% of total protein and the remaining 20 were placed on an artificial diet with casein replacing the GNA (diet was made up as described in section 2.4). The *L. oleracea* were fed *ad libitum* for 5 days, at which point haemolymph was extracted from the larvae by piercing the skin on the underside of the larvae with a hypodermic needle. Haemolymph was collected with glass capillary tubing and stored on ice (collection of haemolymph was carried out alternately from test/control fed pools, to minimise differences in

cascade initiation due to injury). Haemolymph from every two individuals was pooled into one eppendorf and incubated at room temperature for 30 minutes. After 30 minutes 10µl of each sample of haemolymph was added to 3 replicates of 100µl of 2mM DOPA in 50mM sodium phosphate buffer (pH 6.5) in a flat bottomed microtitre plate. A negative control of 110µl of 2mM DOPA in buffer was used. The microtitre plate was read over time using a Spectra Max plus plate reader at 470nm.

7.2.2 Effect of GNA, PHA, and SKTI on the polyphenoloxidase cascade

150 newly eclosed fourth instar *L. oleracea* larvae were removed from the insect culture and 30 of each were placed on an artificial diet (made up as described in section 2.3), containing either PHA, GNA, SKTI, at 2% of total protein, and 30 were placed on an artificial diet with GNA at 2% of total protein plus 3.02mg of SKTI, and the final 30 were fed on a control diet with casein replacing the test protein. The groups of *L. oleracea* where then fed to excess for up to 6 days. Haemolymph was extracted on days 2, 4 and 6 (as described in section 7.2.1) and stored on ice. After collection was complete 5µl of haemolymph from each individual (2 replicates per individual) was placed into 200µl of 2mM DOPA in 50mM Sodium phosphate buffer pH 6.5 and read at 1 minute intervals at 470nm on a Spectra Max plus plate reader for 3.5 hours.

7.2.3 *In vitro* confirmation of GNA in haemolymph

Remaining haemolymph from insects used in section 7.2.2 was collated, and samples of haemolymph from the larvae fed on GNA and sample of haemolymph from larvae fed on casein was mixed with 10 µl of 4X SDS sample buffer and 1µl of ß-mercaptoethanol and boiled for 5 minutes. 20µg of protein from each sample was separated by SDS-PAGE and transferred to a nitrocellulose membrane by western blotting (as described in section 2. 5). The nitrocellulose filter was incubated overnight at 4°C in block (supplied by Roche).

Following washing for 2x10 minutes in TBS pH 7.5 and 1x10 minutes in antisera buffer, the filter was incubated for 2 hours in anti-GNA primary antibody at concentration 1:10,000 in antisera buffer. The filter was washed for 3x5 minutes in antisera buffer and incubated for 2 hours in goat anti rabbit horseradish peroxidase (secondary antibody), at a concentration of 1:10,000 in antisera. After incubation in secondary antibody the filter was washed for 2x5minutes and 1x15 minutes in TBS/Tween-20 (100µl Tween-20/100ml TBS), and 2x5 minutes in water. The filter was then immersed in ECL detection fluid and developed according to manufacturers instructions.

7.2.4 Effects of GNA on haemolymph extracted from the *L. oleracea* larvae

Haemolymph was extracted from 20 fourth instar *L. oleracea* (as described in section 7.2.1). The haemolymph from every 4 larvae was pooled and stored on ice. Half of the haemolymph from each set was centrifuged for 2 minutes at 14000rpm, and the supernatant of cell free haemolymph was removed.

3 replicates per pooled treatment were set up with 10µl of normal haemolymph or 10µl of cell free haemolymph placed into the wells of a microtitre plate which contained 100µl of a 1mg/ml solution of either GNA or casein, dissolved in 2mM DOPA in 50mM Sodium phosphate buffer pH 6.5. The microtitre plate was read at 1 minute intervals at 470nm on a Spectra Max plus plate reader over a 3 hour period. The observed increase in absorbance was not linear; an initial lag period was followed by an increase, which then "tailed off" so that absorbance tended towards a maximum limit. The maximum rate of reaction was estimated from the progress curve and used as a measure of polyphenoloxidase activity.

7.2.5 Statistical analysis

Statistical analysis was carried out using the Microsoft Excel package, using the t-test statistical method (it should be noted that excel only generates P values which are in the results). A p value of 0.05 or less is the considered standard to indicate a significant difference between samples (Dytham 2001).

7.3 Results

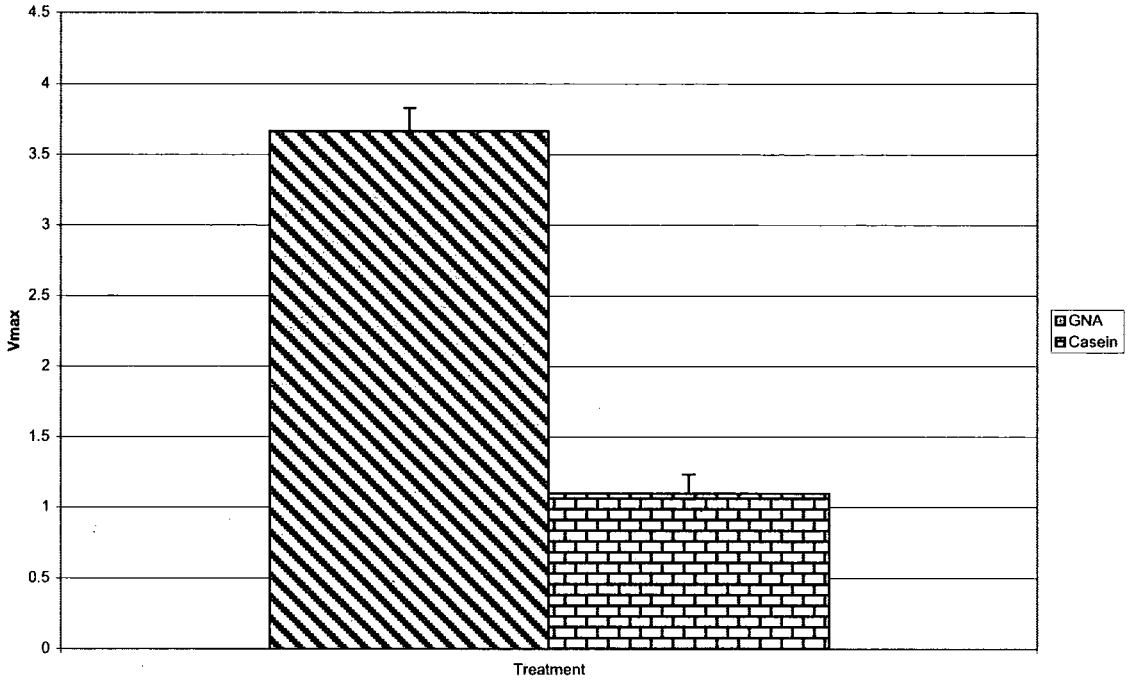
7.3.1 Systemic effects of GNA on the polyphenoloxidase system in *L. oleracea.*

As set out in section 7.2.1, 20 fourth instar *L. oleracea* larvae were fed on a control or GNA artificial diet for 72 hours. Haemolymph was extracted from the larvae and placed into a well of a microtitre plate containing L-DOPA. Phenoloxidase activity was assessed (colour change) over a 3 hour period. The

reaction rates for the phenoloxidase activity of individual larvae are set out below in fig 7.1.

The level of polyphenoloxidase activity is four times higher in the haemolymph extracted from the GNA fed larvae (mean Rate = 3.5) compared with haemolymph from control fed larvae (mean Rate = 0.76) which is statistically significant ($P < 0.001$ with 19 d.f).

Fig 7.1 Effect of GNA on polyphenoloxidase activity in *L. oleracea* larval haemolymph

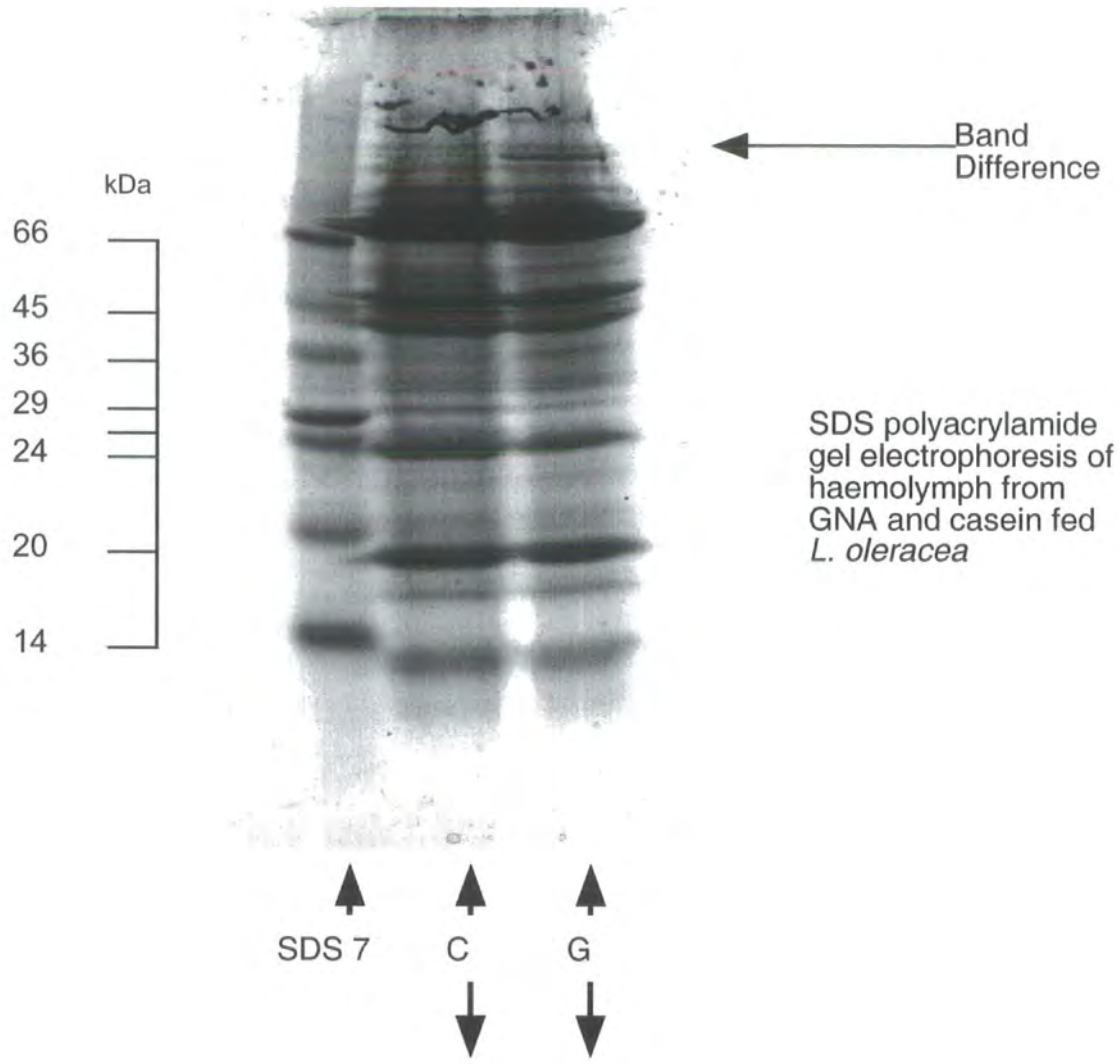


7.3.2 *In vitro* analysis of haemolymph from GNA fed *L. oleracea* larvae

Samples of haemolymph (excess from above experiment) from both the GNA fed larvae and control fed larvae were boiled in 4 x SDS sample buffer in the presence of ß-mecaptoethanol and the proteins separated on a SDS-PAGE gel as described in section 7.2.2. The resultant gel was stained with Coomassie blue (fig 7.2). The sample of haemolymph from the GNA fed larvae exhibits a protein band of approximately 80 kDa that is not present in the sample of haemolymph from the control fed larvae.

As described in section 7.2.2 a further sample of the haemolymph was run on a duplicate SDS-PAGE gel and transferred to nitrocellulose by Western blotting and probed for the presence of GNA (fig 7.2), and provided *in vivo* evidence for the presence of GNA in the haemolymph of the samples.

Fig 7.2 SDS-PAGE gel (top) and Western blot (bottom) of haemolymph from GNA and casein fed *L. oleracea* 20µg of protein was loaded into each lane.



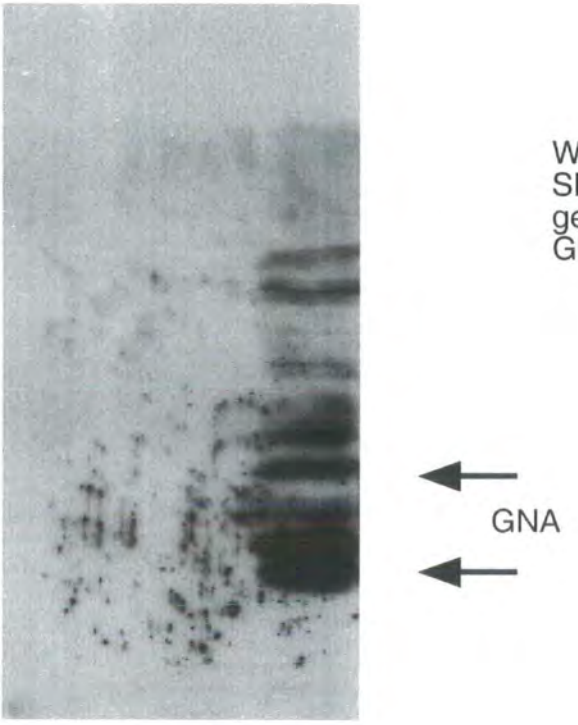
SDS polyacrylamide gel electrophoresis of haemolymph from GNA and casein fed *L. oleracea*

Key

SDS 7 = Size markers

C = Haemolymph from control fed *L. oleracea* larvae.

G = Haemolymph from GNA fed *L. oleracea* larvae



Western blot of above SDS polyacrylamide gel, probed with anti GNA antibody.

7.3.3 Effect of GNA, SKTI, PHA and Casein on the polyphenoloxidase system of *L. oleracea.*

As set out in section 7.2.3, fourth instar *L. oleracea* larvae were fed on an artificial diet containing either GNA, GNA + SKTI, SKTI, PHA or a control diet (30 larvae per treatment). Haemolymph was extracted from the larvae after they had been fed on the different dietary regimes for 2, 4 and 6 days and 5µl of haemolymph was placed into wells of a microtitre plate containing L-DOPA to assay of phenoloxidase activity was obtained. The Rate reaction rate data is presented in fig 7.4 below. Statistical analysis of the rates using the t-test statistical method to compare the control sample with test samples at the different time points was carried out and can be seen in table 7.1 below.

The haemolymph extracted from larvae fed on the PHA lectin showed the highest rate of enzyme activity, followed by that extracted from larvae fed on GNA. Both of these feeding regimes exhibited a significant difference in phenoloxidase activity within larval haemolymph when compared with levels of activity in haemolymph from control fed larvae. The difference is most marked after being fed on the diet regimes for 2 days, and phenoloxidase activity decreases with increased feeding time. Larvae fed on GNA exhibited a significant difference in polyphenoloxidase activity between haemolymph samples taken after 2 and 6 day feeding regimes, (approximately 25%, t-test p value of 0.01 with 19df).

Fed	2 day feed	4 day feed	6 day feed
GNA	< 0.001	< 0.001	< 0.001
GNA-SKTI	> 0.05	> 0.05	> 0.05
SKTI	> 0.05	> 0.05	> 0.05
PHA	< 0.01	< 0.001	< 0.001

Table 7.1 p values of t-test statistical analysis (all with 19 degrees of freedom), of Rate values of phenoloxidase activity within *L. oleracea* haemolymph, comparing control fed samples and test fed samples at each time point.

The SKTI feeding regime exhibited no significant difference in phenoloxidase levels when compared with levels in control fed larvae. Larvae fed on GNA-SKTI initially exhibited no significant difference in phenoloxidase levels in extracted haemolymph when compared to controls (two day feed sample), but after being fed on the test diet for 4 and 6 days a significant difference was observed (see table 7.1 above).

Levels of phenoloxidase activity found in the haemolymph of larvae fed on GNA alone were higher than that found in the haemolymph from larvae fed on GNA-SKTI, These levels were analysed using a t-test, and were found to be significant when comparing enzyme activity in haemolymph from larvae fed on the two diets for 2 days, but not in haemolymph from larvae fed on the two diets for 4 and 6 (see table 7.2 below).

2 day feed	< 0.05
4 day feed	> 0.05
6 day feed	> 0.05

Table 7.2 p values of t-test statistical analysis (all with 19 degrees of freedom) comparing Rate values of phenoloxidase activity within *L. oleracea* haemolymph from larvae fed on an artificial diet containing GNA or GNA-SKTI fed samples at each time point.

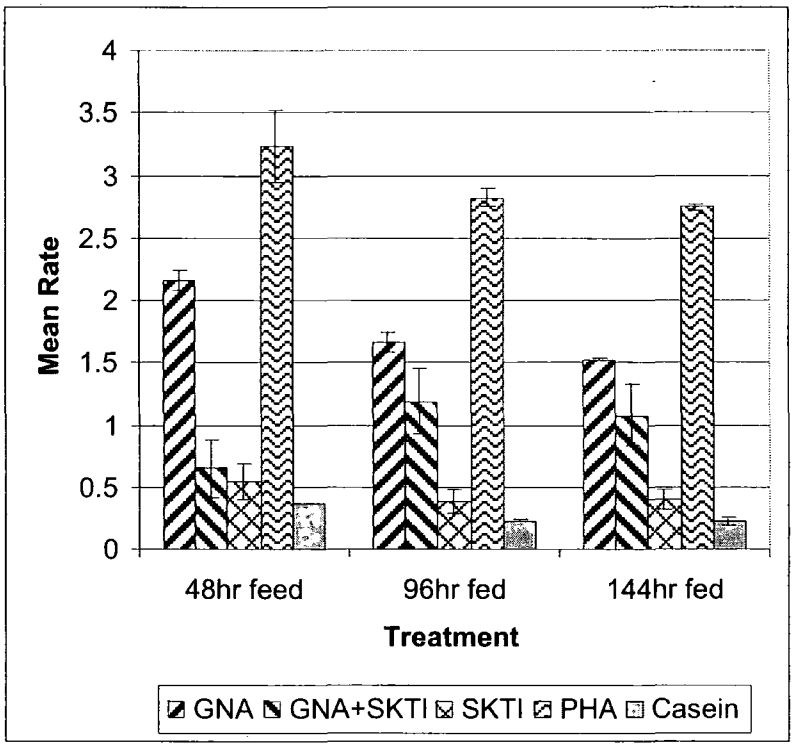


Fig 7.3 Showing the Rate rates of phenoloxidase activity in the haemolymph of larvae of *L. oleracea* after being fed on different artificial diets (bars indicate the standard error, 30 replicates per treatment).

7.3.4 Effects of GNA on Haemolymph extracted from the *L. oleracea* larvae.

As described in earlier in section 7.2.4, haemolymph was extracted from individual larvae, which had been fed on artificial diet for 48 hours and placed in a microtitre plate well containing a 1mg/ml solution of either GNA or casein and assayed for phenoloxidase activity over a 3 hour period. Cell free haemolymph samples were treated in the same manner, except for being centrifuged for 2 minutes at 14000rpm and decanted off.

The mean reaction rates for phenoloxidase activity are set out below in fig 7.4. Statistical analysis using a t-test indicates no significant differences in phenoloxidase activity in either normal haemolymph samples or in cell free haemolymph, when comparing those incubated with GNA or casein (see table 7.3 below).

	Haemolymph	Cell free haemolymph
GNA v Casein	> 0.05	> 0.05

Table 7.3 p values of t-test statistical analysis both with 9 degrees of freedom comparing phenoloxidase activity in samples incubated with either GNA or casein (control) fed samples.

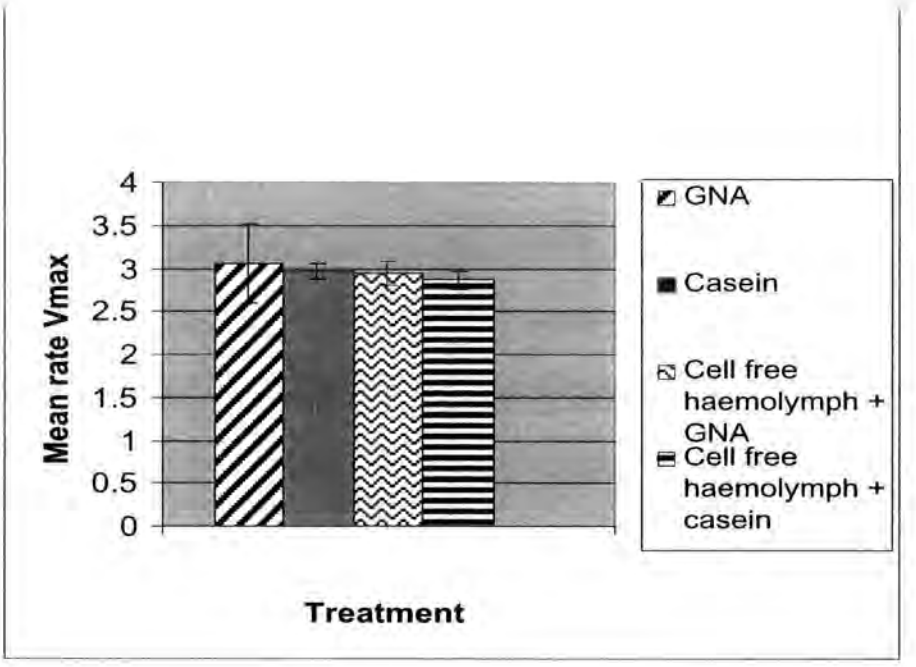


Fig 7.4 Reaction rates of *in vitro* phenoloxidase activity in haemolymph samples extracted from the larvae of *L. oleracea* fed an artificial diet containing GNA or casein, bars on graph indicate standard error (20 replicates per treatment).

7.4 Discussion.

The results in section 7.1 (investigating the *in vivo* effect of GNA on the levels of phenoloxidase in the haemolymph of *L. oleracea* larvae) show a significant difference in the level of phenoloxidase activity between treatments, with the GNA sample showing 78% more phenoloxidase activity than the control sample, indicating that GNA has a systemic effect on the phenoloxidase system.

It is hypothesised that the 80 kDa protein band present in the haemolymph sample from the GNA fed larvae (fig 7.1) was induced, and expressed, due to the presence of GNA within the larvae as a whole, or specifically within the haemolymph (the presence of GNA within the haemolymph of GNA fed larvae was confirmed by western blot [fig 7.2]). Furthermore it is hypothesised that the

80KDa protein is a part of the *L. oleracea* larvae's immune system and in particular part of the phenoloxidase system. A protein of similar size (72 kDa) has been characterised as an activated phenoloxidase, after induction of the immune system of the coleopteran insect *Tenebrio molitor*, (Lee 1999).

The results from experiment 7.2, show an increase in the rate of phenoloxidase activity within the haemolymph of the tomato moth larvae *L. oleracea* fed on the PHA (12 times higher than control after feeding for 144hours on lectin) and GNA (6.7 times higher than control after feeding for 144hours on lectin) lectins when compared with the rate of activity in haemolymph from control larvae fed larvae. The phenoloxidase system can be initiated by a number of factors (as discussed in Chapter one), and the results in this chapter provide evidence that the lectins GNA and PHA induce the polyphenoloxidase system, indicated by the fact that both PHA and GNA cause a statistically significant rise in the rate of phenoloxidase activity. It has been demonstrated that under normal conditions, the prophenoloxidase enzyme is in its inactive zymogen form (Brey 1995) (which would be the case for the haemolymph samples obtained from the *L. oleracea* larvae fed on the control diet), and that serine proteinases are involved in the conversion of the inactive zymogen PPO to phenoloxidase (Soderhall 1994). The addition of a serine proteinase inhibitor should decrease the effects of PHA and GNA on the conversion of the zymogen prophenoloxidase to phenoloxidase. In section 7.3.3 SKTI was employed as a serine proteinase inhibitor preventing activation of phenoloxidase by inhibition of serine proteinases. The results indicate that the larval group fed for 2 days on the diet regime containing GNA

and SKTI, exhibited a significantly lower level of phenoloxidase activity than the group fed for 2 days on GNA alone (see table 7.2). At subsequent feeding times, although not significant, phenoloxidase activity is higher in samples of haemolymph taken from GNA fed larvae than in haemolymph samples taken from GNA-SKTI fed samples (fig 7.4). This supports the hypothesis that PHA and GNA induce the phenoloxidase system, indicated by the fact that SKTI partially negates the effect of GNA on the level of phenoloxidase activity within larval haemolymph. It is assumed that SKTI inhibits a percentage of the serine proteinase activity (caused by GNA), by reducing the conversion of the inert zymogen prophenoloxidase to phenoloxidase. It is likely that a higher dose of SKTI would cause further drops in the level of phenoloxidase activity completely negating the effect of GNA within the haemolymph of the *L. oleracea* larvae. From the three time feeding regimes the larvae fed on the lectins for 2 days exhibited the highest amount of phenoloxidase activity and enzyme activity dropped in the samples taken from larvae fed for 4 and 6 days. This drop in activity suggests that the insect is unable to maintain high levels of phenoloxidase activity.

The effect of PHA and GNA on the constituents of the haemolymph, which are involved in the polyphenoloxidase system, has not been well researched. The insect equivalent to mammalian blood cells are haemocytes of which the granular cells and plasmatocytes play the most active role, particularly in relation to the polyphenoloxidase system (Gupta 1991). It has been demonstrated that

phenoloxidase activity is only seen in the granular cells of *Anopheles albimanus* (Hernandez 1999), and it is stated that, granulocytes and plasmatocytes provide cellular immunity against foreign antigens (Gupta 1986). Fig 7.3 shows that GNA binds to haemolymph samples from *L. oleracea*, in addition it has been shown GNA-GFP (green fluorescent protein) binds predominately to granulocytes and plasmatocytes (Raemaekers, R. J. M. 2000 Pers. Comm).

It is logical that haemocytes could act as elicitors of the polyphenoloxidase system in the haemolymph of *L. oleracea* larvae, and that interaction of haemocytes with lectins (GNA and PHA specifically), will have an effect on the insect immune system. This hypothesis is backed up by the fact that GNA binds to haemolymph samples, and to certain haemocyte types. It is hypothesised that PHA would also bind to haemolymph samples due to its wide binding specificity (Van Damme 1998), which has been shown to be the case for the PHA-E and PHA-L forms of the PHA lectin (Fitches 2001)

The results from the *in vitro* experiment in section 7.3, in which the haemocyte sample, was extracted and then exposed to GNA, show no differences in the level of polyphenoloxidase. The reason for this negative response is unknown, but two general hypotheses may be suggested. First the effect of GNA on the phenoloxidase system is systemic, and secondly that the induction and development of higher levels of phenoloxidase requires a longer lag period than was tested. It is thought that responses immune responses can take a considerable period of time to develop. (Chadwick 1991).

As GNA in particular and PHA to a lesser extent have the ability to avoid degradation in digestive systems (see chapter 1) and to remain attached to receptors, it is hypothesised that *in vivo,* PHA and GNA have time to cause a response leading to the induction of the phenoloxidase system.

Chapter 8

Characterisation of leucine aminopeptidase (Con A receptor) in the gut of *L. oleracea* by isolation of a cDNA clone encoding this enzyme

8.1 Introduction

Leucine aminopeptidase an abundant protein found in the guts of many lepidopteran species, and is known to be the Bt receptor in some lepidoptera. Additionally leucine aminopeptidase is thought to be the major receptor for Con A lectin within the tomato moth larvae *L. oleracea*. Thus it was of interest to characterise this receptor to increase knowledge of the lectin-receptor interaction

8.2 Materials and Methods

A cDNA library from the gut of *L. oleracea* was supplied by Dr Elaine Fitches, and was prepared using the Stratagene ZAP-cDNA synthesis kit.

8.2.1 Polymerase chain reaction (PCR) to produce probe to screen cDNA library.

PCR primers for aminopeptidase N in *L. oleracea* were constructed on the basis of database sequences for aminopeptidase N in 3 lepidopteran species, *Manduca sexta*, *Heliothis virescens* and *Plutella* spp. Primers were chosen on the basis of conservation of amino acid sequence, and low redundancy of codon

usage, using Primer Select software. The primers manufactured by Oswel had the following sequences:

Reverse primer GCRAA5CCYTCRTT5AGCC

Forward primer GC5TTYCC5TGYTAYGAYGARCC

These primers were utilised in a PCR reaction (as follows) to amplify a product estimated to be 600 bases from the cDNA library.

A 100μl aliquot of the cDNA library was removed and centrifuged for 1 minute at 14000rpm to remove impurities, the supernatant was boiled for 10 minutes and placed immediately on ice prior to use. A 50μl PCR reaction mix was made up containing: 5μl of 25mM $MgCl_2$, 5μl 10xPCR buffer, 1μl DNTP, 4.61μl (100pmols/μl) forward primer 5.63μl (100pmols/μl) reverse primer, 0.5μl *Taq* polymerase, 10μl template cDNA, 13.62μl sterile distilled water,

The PCR amplification reaction was carried out on a Perkin Elmer Gene Amp PCR system 2400 thermal cycler to the temperature and time conditions set out in fig 8.2.1.

8.2.2 Agarose gel electrophoresis of DNA

The PCR reaction mix was run out on a 1% agarose gel made in 1xTAE with 5μl of ethidium bromide for visualisation (Sambrook *et al* 1989).

PCR products were visualised with a UV transilluminator and the bands of approximatelyimately 600bp cut out. The Quiagen quick kit was used to separate

DNA from the agarose by gel purification according to the manufacturers instructions.

8.2.3 Cloning of PCR products.

The PCR product from section 8.2.2 was inserted into TOPO TA cells using the Invitrogen cloning kit according to manufacturers instructions, with the following modifications; 50μl and 100μl of the TOPO TA (bacterial transformation) were grown up overnight at 37°C, on 2 LB plates respectively containing 50μg/ml of kanomycin and X-Gal. 20 white bacterial colonies were picked off each plate and each colony was deposited into 5ml of LB broth and grown up overnight at 37°C with shaking.

Plasmid DNA was isolated from the LB broth using the Promega miniprep kit according to the manufacturers instruction (centrifugation protocol), and digested using the EcoR1 digestive enzyme for 3 hours at 37°C, the digestion mix containined the following components: 20μl plasmid DNA prep, 1μl EcoR1, 3μl Promega buffer R and 6μl of sterile water. 15 μl of the digest was run on a 1% agarose gel as described in section 8.2.2 and as a band of 600bp was visualised the sample was sent for sequencing.

8.2.4 DNA sequencing

DNA sequencing was carried out by the University Of Durham on an automated DNA sequencer (ABI model 377), using the dideoxy terminator chemistry method (Sanger *et al* 1977) and fluorescent dye linked M13 forward and reverse primers.

Sequences obtained were analysed for homology to known sequences in the EMBL nucleotide sequence database via blast searches (http://www.ncbi.nlm.nih.gov/BLAST/ 18/02/2000).

8.2.5 Preparation and radio-labelling of probe with ^{32}P-dCTP

Following restriction with EcoR1 and subsequent gel purification (see sections 8.2.3 and 8.2.2 respectively), 100ng of the DNA probe (31μl) was boiled for 5 min and placed directly on ice for 10 min. The probe was labelled with ^{32}P-dCTP by adding the following components in strict order 31μl DNA, 10μl OLB, 2μl BSA (10mg/ml), 5μl ^{32}P-dCTP (50 μCi) and 2μl Klenow enzyme (10mg/ml) and then mixed and incubated overnight at room temperature. The radio-labelled probe was separated from unincorporated nucleotides by chromatography on a Sephadex G50 column.

An estimate of total counts per minute was made by liquid scintillation in a Packard Tricarb liquid scintillation analyser. The probe was estimated to be labelled to $1.6x10^{-8}$.cpm/μg DNA.

8.2.6 Titration and screening of the *L. oleracea* cDNA library with radiolabelled probe.

Titration of the phage library was carried out as below, to determine the volume of the cDNA library required to produce an appropriate number of plaques for primary screening of the cDNA library.

MRF host cells were grown up overnight at 30°C in 50ml of LB broth with 0.2% w/v maltose, 10mM $MgSO_4$ and tetracycline (12.5μg/ml), after which the MRF cells were spun down by centrifugation and resuspended to an OD600 of 1 in 10mM $MgSO_4$.

A serial dilution of the cDNA library (100μl final volume) in SM buffer was made and 200μl of MRF cells were added to the 10^{-3}-10^{-7} serial dilutions. All dilutions were incubated for 15min at 37°C, mixed with 3ml of pre-warmed NZY top agar (48°C) and poured onto pre-warmed NZY plates. Plaques were counted after 6 hours incubation at 37°C.

Library plating

MRF cells were grown up and prepared as above. The estimated amount of cDNA for each plate was added to 6ml of the MRF host bacterial cells and incubated for 15 min at 37°C. The MRF mix was then added to prewarmed NZY top agar (48°C) and poured onto prewarmed, large (22x22cm) NZY agar plates. Plates were incubated for 4-6 hours until the plaques reached pinhead size. Prior to lifting plates were placed at 4°C to stop growth and to aid transfer method.

Plaque lifts

A pair of large sheets of nitrocellulose paper were immersed in distilled water to become damp and placed over the plaques in a marked position for 2 and 5 minutes exposure to the bacterial plaques respectively. The filters were then immersed for 5min per solution in 10% SDS, denaturation solution and neutralisation solution, and finally 2xSSC, as per Sambrook *et al* 1989. Filters were subsequently cross linked using a UVXlinker at 150mv

Preybridisation and hybridisation of the filters.

Filters were placed into Techne hybridisation tubes, immersed in prehybridisation solution and incubated at 65°C overnight in a hybridisation oven, after which the prehybridisation solution was poured off and the filters immersed in hybridisation solution, containing the labelled probe (prior to addition probe is boiled for 5min and placed on ice for 10 min). Filters were incubated overnight in hybridisation solution at 65°C and then washed for 2x20 min in 2xSSC/0.1%SDS at 65°C followed by 2x5min washes in 1xSSC/0.1% SSC at room temperature.
Filters were blotted dry and exposed to autoradiograph film for 4 days at -80°C. Films were developed to identify positive colonies.

8.2.7 Preparation and labelling of probe with DIG-dUPT.

The probe was prepared and gel purified as in section 8.2.3 and 8.2.2 respectively. The probe was then labelled with DIG-dUPT by a PCR reaction according to manufacturer's protocol (Roche), with the following modifications:
To determine the optimum amount of the cDNA template to use in the labelling reaction, a serial dilution of template was used. 10μl of each dilution from stock to dilution 10^{-6} was added to the PCR reaction mix (as per 8.2.1), and the PCR was run using the conditions set out in 8.2.1. The resultant PCR products were run out on a 1% agarose gel. Taking the concentration of cDNA at which no band appears as point 1 the optimum concentration of cDNA for the labelling is point

three, i.e. two above the point at which no band appears which in this case was at dilution factor 10^{-2}.

The DIG fragment was incorporated into the probe using the touchdown PCR method in 8.2.1 with the addition of 1 μl of DIG-dUPT per reaction.

The resultant PCR product was run out on a 1 % agarose gel and the slightly heavier less mobile bands with the incorporated DIG were cut out and gel purified using the Qiagen quick kit.

8.2.8 Primary screening of the cDNA library using a DIG labelled probe.

Plaques were grown up on large plates as in section 2.7.6, and plaque lifts were carried out according to the Roche protocol with the following modifications/protocol choices. Duplicate nitrocellulose filters were left on the plates for 1 and 5 minutes, and were cross linked using a Bio-rad GS Gene Linker UV Chamber at 150mv. Filters were placed in Techne hybridisation tubes for 1 hour in a prehybridisation solution consisting of 5x SSC, 0.1% N-lauroylsarcosine, 0.02 % SDS and 1% Roche blocking reagent in (maleic acid buffer). The prehybridisation solution was replaced with hybridisation solution containing the DIG probe [pre-boiled for 5 min and placed on ice for 10 min prior to addition]) and filters were incubated in the hybridisation oven overnight at 68°C, followed by washing for 2x5min in 2xSSC 0.1% SDS at room temperature and 2x15 min in 0.5% SSC 0.1% SDS at 68°C.

Filters were developed using CSPD according to the manufacturers instructions (Roche).

8.2.9 Secondary screening of the cDNA library using a DIG labelled probe.

Positive plaques from the primary screening were cored out from the large plates and incubated overnight at 4°C in 500µl of SM buffer + 10µl of chloroform.

The positive plaque plugs were tested by PCR using the reaction conditions set out in section 8.2.1 using 10µl from the SM buffer, chloroform and plug mix as the PCR template. The PCR product was separated and visualised using a 1% agarose gel, and the positive plug samples indicated by the PCR reaction were taken for use in secondary screening.

For secondary screening, plates were set up in the same manner as the library titration in section 8.2.6, using a serial dilution from original to 10^{-3} serial dilution. Secondary screening was completed as for primary screening in section 8.2.6, except small plates were used.

8.2.10 Characterisation of the Leucine aminopeptidase (Con A receptor) in the gut of *L. oleracea* by PCR

Primers designed around the partial aminopeptidase sequence in fig 8.1 were used in conjunction with commercially available T7 and T3 primers, which were designed around the end sequences produced in the cDNA library by the Stratagene ZAP-cDNA synthesis kit. The forward primer had the sequence dGAA GTT ATG GAA CGG TGT ACT GC designed for use with the T7 primer to generate a fragment of 2-2.5KB.

The Reverse primer had the sequence dCAT GTT CAT ATT ATG AAC CAT TG, designed for use with the T3 primer to generate a fragment of 800BP.

A PCR reaction was set up for both fragments using aliquots of the cDNA library as template following the protocol set out above in 8.2.1 for 30 cycles under the following conditions.

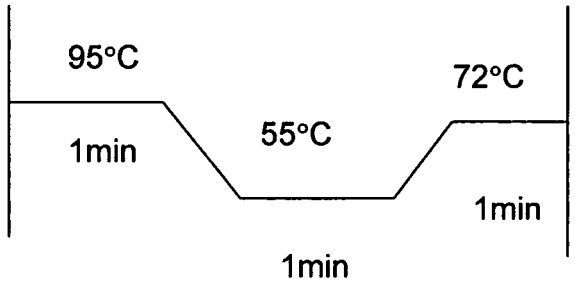


Fig 8.2.2 The PCR conditions designed to amplify a 800bp and 2-2.5KB fragment from the *L. oleracea* cDNA library

The protocol was repeated with differing concentrations of $MgCl_2$ and template in the reaction mix, and with extension times of up to 3 minutes.

The protocol was then altered and a touchdown PCR protocol (as in 8.2.1) except with a 4 min extension time was utilised.

All DNA bands were visualised by agarose gel electrophoresis as in section 8.2.2, correctly sized PCR products were excised, cloned and sent for sequencing.

8.3 Results

8.3.1 Characterisation of the leucine aminopeptidase (Con A receptor) in the gut of *L. oleracea.* by isolation of a cDNA clone encoding this enzyme

In order to characterise the major Con A binding protein, thought to be an aminopeptidase; a cDNA library from the gut of the *L. oleracea* larvae was utilised.

As described in section 8.2.1, a PCR product of approximatelyimately 600 base pairs was obtained from the previously prepared *L. oleracea* gut cDNA library. Sequencing of the resulting clones showed that the insert encoded a protein fragment of approximately. 200 amino acids (seen fig 8.1 below), with a high homology to aminopeptidase N enzymes from several lepidopteran species (http://www.ncbi.nlm.nih.gov/BLAST/ 18/02/2000). The cloned PCR product was used as a probe in screening the gut cDNA library.

```
          GCGTTCCCGTGCTATGACGAACCTGGATTCAAGGCAACTTTCGATATATCTATAACCAGGGACATCGAAGGCTT
          A  F  P  C  Y  D  E  P  G  F  K  A  T  F  D  I  S  I  T  R  D  I  E  G  F
          A  F  P  C  Y  D  E  P  G  F  K  A  T  F  D  I  T  M  N  R  E  -  E  S  F

81        CAACCCGACCTTATCAAACATGCCGACAAGAGCATTCGAAGAGGTCGGTAACGGAAAAATAAAGGAGACTTTCTACACTA
            N  P  T  L  S  N  M  P  T  R  A  F  E  E  V  G  N  G  K  I  K  E  T  F  Y  T
            S  P  T  I  S  N  M  P  I  R  T  T  N  T  L  A  N  G  R  V  S  E  T  F  W  T

161       CTCCCCGTACTTCCACATACTTGTTGGCCTTCATTGTGTCAGGCTACAAAGAAGTAGCTACCAACCACCTATCGACAAGA
            T  P  R  T  S  T  Y  L  L  A  F  I  V  S  G  Y  K  E  V  A  T  N  H  L  S  T  R
            T  P  V  T  S  T  Y  L  L  A  F  I  V  S  H  Y  T  V  V  S  T  N  N  N  A  L  R

241       CCATTCCACATTTACGCTCGCGGTAACATTCCCGCTGGTAGTGGAGACTATTCCTTAAGAGTTGGGTCCCCGCTTCTTGA
            P  F  H  I  Y  A  R  G  N  I  P  A  G  S  G  D  Y  S  L  R  V  G  S  P  L  L  E
            P  F  D  I  Y  A  R  N  N  V  -  G  R  T  G  D  W  S  L  E  I  G  E  K  L  L  E

321       AGTTATGGAACGGTGTACTGCTATTCCTTACTATACAATGGGTACCAATATGAACATGAAGCAGGCTGCCATCCCTGATT
            V  M  E  R  C  T  A  I  P  Y  Y  T  M  G  T  N  M  N  M  K  Q  A  A  I  P  D
            A  M  E  A  Y  T  Q  I  P  Y  Y  T  M  A  E  N  I  N  M  K  Q  A  A  I  P  D

401       TCTCAGCGGGTGCTATGGAAAACTGGGGACTTTTGACTTACAGGGAAGCTCTCATTATCTACGACCCTGAAAATACCAAC
            F  S  A  G  A  M  E  N  W  G  L  L  T  Y  R  E  A  L  I  I  Y  D  P  E  N  T  N
            F  S  A  G  A  M  E  N  W  G  L  L  T  Y  R  E  A  L  I  L  Y  D  P  L  N  S  N

481       AATTTCTACAAACAGCGTATAGCCAATATAATTTCTCACGAAATTGCCCACATGTGGTTCGGCAACCTCGTCACTTGCGC
            N  F  Y  K  Q  R  I  A  N  I  I  S  H  E  I  A  H  M  W  F  G  N  L  V  T  C  A
            H  F  Y  K  Q  R  V  A  N  I  V  A  H  E  I  A  H  M  W  F  G  N  L  V  T  C  A

561       CTGGTGGGACAATCTCTGGCTCAACGAAGGCTTCGC
            W  W  D  N  L  W  L  N  E  G  F  A
            W  W  D  N  L  W  L  N  E  G  F  A
```

Fig. 8.1. Nucleotide and predicted amino acid sequence of *L. oleracea* PCR fragment encoding aminopeptidase N. Amino acid sequence from *Manduca sexta* aminopeptidase N is shown in italics for comparison. Sequences corresponding to the PCR primers used are underlined.

The probe was labelled with ^{32}P-dCTP in order to screen the library as described in section 8.2.6 However, all films produced from the screening of the *L. oleracea* cDNA library with the radiolabeled probe, failed to generate duplicated evidence of positive plaques.

Primary screening using the DIG labeled probe succeeded in generating 25 positive plaques. The PCR (utilising the primers from section 8.2.1) identified 3 positive plaques, which were selected for use in secondary screening as

described in section 8.2.9. However, the secondary screening failed to produce any positive results.

8.3.2 Characterisation of the Leucine aminopeptidase (Con A receptor) in the gut of *L. oleracea* by PCR

As set out in section 8.2.10, attempts were made to generate two PCR fragments. One small fragment of approximately of 700bp was obtained, although it was smaller than the 800bp product anticipated. Sequencing of the resulting clones showed that the insert encoded a protein fragment with a high homology to aminopeptidase N enzymes from several lepidopteran species (http://www.ncbi.nlm.nih.gov/BLAST/ 20/06/2000). See fig 8.2.
Generation of the larger PCR fragment of 2-2.5KB was unsuccessful.

8.4 Discussion

Numerous variations of conditions were attempted in the screening of the cDNA library using both the ^{32}P-dCTP and the DIG-dUPT labeled probes, such as stringency of washing, hybridisation temperatures, film exposure time, all of which was very time consuming and failed to allow characterisation of leucine aminopeptidase from the cDNA library.
The most likely reason for the failure to isolate the entire sequence of the main Con A receptor (leucine aminopeptidase) by PCR amplification is that ideal conditions were not met. Due to time constraints it was not possible to continue work to optimise these conditions. The production of the probe by PCR, and the discovery of a further fragment from the cDNA library also by

PCR, indicates that the probability of a complete sequence for leucine aminopeptidase (Con A receptor) being present in the cDNA library is high.

Chapter 9

Concluding Discussion

After initial handling and technical problems, the of insect bioassays allowed a relatively quick assessment of the toxicity of the lectins tested. For practical reasons, in this work only the tomato moth larvae *L. oleracea* and the peach potato aphid *M. persicae* were used as models. Since lectins can be selective in their toxicity, toxic effects exhibited or not exhibited in these two species may not be duplicated in others. It would have been beneficial to test lectin toxicity on a wider range of species, but time and financial constraints prohibited this. The selectivity of lectin toxicity is demonstrated in chapter 3, where the black mulberry lectin *M. nigra* exerts no apparent toxicity to *L. oleracea* larvae, but exerts a detrimental effect on *M. persicae* by inhibiting normal growth.

The bioassays testing the effect of jacalin and *M. nigra* lectins on *M. persicae* indicated that both lectins exerted a similar effect. The main link between these two lectins is that they both exhibit a binding specificity for galactose (Gal) and N-acetylgalactosamine (GalNAc). The bioassays indicate a probable relationship between the binding specificity of two different lectins and adverse effect on an insect population. However, *M. nigra* lectin did not have an adverse effect *on L. oleracea*, perhaps because *L. oleracea* has less or no receptors for the *M. nigra* lectin (due to insufficient quantities of this lectin being available no binding studies using these two lectins could be carried out). The hypothesis is that by determining receptor specificity and lectin binding specificity, it could be possible to predict toxicity in a given species or family to a predetermined lectin. However,

as yet no link has been determined. WGA has no toxicity to the pea aphid *Acrythosiphon pisum* where as RSII does. Both lectins bind N-acetyl glucosamine but only the RSII lectin exhibits a toxic effect (Rahbe *et al.*, 1995).

The experimental design (described in chapter 3) for the testing of substances within an artificial diet is such that other substances such as protease inhibitors can be tested in the same manner as lectins. Indeed the effect of protease inhibitors on *L. oleracea* has been studied in this way (Gatehouse *et al.*, 1997), and (Gatehouse *et al.*, 1999).

In all instances where lectin toxicity occurs, both in animals and insects, there are no recorded cases where a toxic effect has not been shown to be accompanied by lectin binding (reviewed by, Van Damme *et al.*, 1998; Pusztai *et al.*, 1995, Pusztai 1991).

As seen in chapters 4-6 the lectin GNA binds to gut components in *L. oleracea* and *A. bipunctata* larvae as well as in *M. persicae.* The evidence of lectin binding is clear with the histological and non-histological evidence backing each other up. This is significant as the *in vitro* (non-histological) evidence alone is not proof of lectin binding *in vivo*, because the proteins the lectin binds to on Western blots may not actually be in contact with the gut lumen, and so binding to the gut surface *in vivo* may never occur. Therefore, histological work is vital in confirming that lectins do bind to the surface of the gut.

The histological evidence in chapters 4-6 was unable to point directly to a toxic effect caused by the GNA or Con A lectins tested, unlike the effect of PHA on rats in which histological evidence shows disruption of the epithelial membrane (King 1982).

As mentioned earlier GNA and Con A are known to have toxic effects on larvae of the Tomato moth *L. oleracea* (Gatehouse *et al.*, 1999; Fitches *et al.*, 1997) and on *M. persicae* (Gatehouse *et al.*, 1999; Sauvion *et al.*, 1996). However GNA does not affect *A. bipunctata* larvae (Down *et al.*, 2000) or the parasitic wasp *Eulophus pennicornis* (Bell *et al.*, 1999) indicating that lectin binding is not indicative of toxic effect.

Although lectin binding has not been determined in the adult form of *A. bipunctata*; GNA has been claimed to cause a toxic effect on adults (Birch *et al.*, 1999). However, a similar, but longer term bioassay by Down *et al* (2001) in which *A. bipunctata* were fed *M. persicae* raised on GNA expressing potato plants utilising a larger sample size than that used by Birch *et al.*, (1999), failed to indicate any toxic effects to either the adult or larval form of the two spot ladybird *A. bipunctata* (Down *et al.*, 2001). A recent paper studying tritrophic relationships between GNA expressing transgenic plants, *L. oleracea* and the parasitoid wasp *Eulophus pennicornis*, also determined that expression of GNA although effecting *L. oleracea* did not adversely effect the ectoparasitoid *E. pennicornis* (Bell *et al.*, 1999).

Currently there are a number of hypotheses regarding the mechanisms of toxicity caused by plant lectins, none of which are conclusive.

As discussed in chapter 1, it is known that GNA has a negative effect on a number of insect species. Obviously the size of the dose is important but the nature of negative effects caused by lectins is not fully known, although there are a number of theories, such as that lectins act as anti-feedants, or block areas of the gut from absorbing nutrients. As such all the current theories for toxicity must be considered when determining reasons for the toxicity of lectins.

One known toxic effect of lectins is to inhibit normal growth. Eisemann *et al* 1994 demonstrated that 3 different lectins, Con A, WGA and lentil lectin all inhibited the larval growth of *Lucilia cuprina,* and concluded that the reason normal growth was inhibited by lectins could be due to 3 or more mechanisms: 1) reduced intake of diet, 2) partial blockage of the pores of the peritrophic membrane, 3) direct binding of specific lectins to the midgut epithelial cells, which could affect various functions of the cell membrane. Within *Lucilia cuprina* mechanisms 1 and 2 would cause a restriction of nutrient availability, leading to a subsequent starvation effect, whereas mechanism 3 may seriously effect normal gut processes.

It has been determined that GNA acts as an antifeedant to BPH (seen by a drop in honeydew production), but the mechanism by which the BPH rejects GNA containing food is not known (Powell *et al.*, 1995). The controls, which included aphids fed on the lectin *Pisum sativum* agglutinin (PSA) (non toxic to aphids),

exhibited no antifeedant effect (Powell *et al.*, 1995). In addition to the antifeedant effect, lectins have also been seen to effect the amino acid composition of honeydew of BPH (Powell *et al.*, 1993). However, GNA does not always act as an antifeedant in all species, for example GNA does not cause an antifeedant effect on *L. oleracea* (Fitches *et al.*, 1998).

In chapter 4 it is demonstrated that both Con A and GNA bind to the peritrophic membrane of *L. oleracea*. As such one explanation for toxicity to the lepidopteran could be impaired functionality of the peritrophic membrane, perhaps by blockage of the pores as mentioned above with *Lucilia cuprina*.

The most studied effect of lectin toxicity is its effect on the epithelial membrane of the gut (Van Damme *et al.*, 1998; Pusztai *et al.*, 1995; Pusztai 1991). EM studies in chapter 4 determined that no structural differences were observed between the epithelial membranes from control and GNA or Con A lectins fed specimens. As no disruption of the brush border membrane was observed (as occurs in mammals when fed the lectin PHA [Pusztai *et al.*, 1990]), this is one mechanism of toxicity that can be discounted within *L. oleracea*. However GNA does cause disruption of the midgut epithelial layer in at least one insect, the rice pest BPH, probably resulting in expression of a toxic effect (Powell *et al.*, 1998).
Harper *et al.*, 1995 point out the possibility that the amount of binding of a lectin could be directly proportional to its toxic effect. However Foissac *et al.*, 2000 showed that transgenic rice plants expressing GNA had a similar level of

resistance to the green leafhopper *Nephotettix virescens* (GLH) and the brown planthopper *Nilaparvata lugens* (BPH) Whereas GNA binding studies to glycoproteins in gut tissues showed that BPH contained more "receptors" than GLH and that BPH had a stronger GNA binding affinity, particularly in the insects midgut. With no difference in toxicity between the two species, it is therefore unlikely that in this case GNA toxicity is directly proportional to the amount of lectin bound.

As demonstrated in chapters 4 & 5, lectins find their way into the insect haemolymph, and GNA binds to haemocytes of *M. persicae* (chapter 5) and to the granularcytes and plasmatocytes of *L. oleracea* (Raemaekers, personal communication), as well as to the haemolymph of *L. oleracea* in general (chapter 4) (Fitches *et al.*, 2001). Experiments in rats have demonstrated lectins not only act as powerful mitogens for the small intestine and immune system of animals, but can also survive the passage across the intestinal epithelium and affect remote organs such as the pancreas. (Pusztai 1991), as such it is probable that lectins would be capable of exerting similar effects in insects after transfer across the gut membrane. It is established that the main insect defence mechanism against foreign pathogens involves phagocytosis by haemocytes, and that haemocytes are the insect equivalent to blood cells and are involved in a number of processes such as melanisation, and intermediary metabolism (Gillespie *et al.*, 1997; Gupta 1991). As haemocytes play an important role in insects, interference with their function could have a severe detrimental effect on an insect. The

University of Waterloo, Ontario, Canada has filed a patent based on appearance or uncontrolled production of two peptides human Apob-100 molecule and/or Apolipophorin 1, as a means of developing insect resistant plants. With the premise being these peptides could cause perturbation of normal haemocyte function and result in death of the insect.

The effect of lectins on the polyphenoloxidase system (chapter 7) is not necessarily detrimental. However, it is likely that there would be an associated energy cost with such, which may go some way to explain the trends of retarded growth and development caused by lectins in feeding trials. Furthermore, a part of the polyphenoloxidase cascade involves the formation of quinone, which can react irreversibly with nucleophilic side chains of proteins (-SH $-NH_2$ –NH), leading to the modification of lysine, histidine and thiol amino acids. Therefore, this quinone reactivity leads to a potential loss of fuction (Felton *et al.*, 1992). Although the effect of the lectin on the polyphenoloxidase system of BPH has not been demonstrated in this work, it can be hypothesised that the known effect of lectins on the amino acid composition of honeydew (Powell *et al.*, 1998) may be at least in part due to high levels of quinone.

From the currently available evidence it appears that the most logical explanation for lectin toxicity is a combination of effects and events, such as blockage of the peritrophic membrane (Eisemann *et al.*, 1994), combined with a mitogenic effect (Powell *et al.*, 1998) and over-expression of polyphenoloxidase. However it must be remembered that in all probability not all mechanisms of lectin toxicity will be

exhibited in all species in which a toxic effect is observed. There is a possibility that a lectin may not be toxic to all insect species, and use of these proteins in plant protection will depend on which species a lectin is toxic to, and to what extent toxicity is exhibited, and what concentration of lectin is required for a toxic effect to be observed.

Ideas for future work

As the full role of insect haemocytes in lectin toxicity has yet to be determined, further investigation of the effect of lectins on such haematocytes would be an interesting avenue for future research. Initially it would be of interest to screen other lectins with different binding specificities for effects on the polyphenoloxidase system, and to look at lectin effects on the polyphenoloxidase systems of other insect species. Additionally it would be of interest to utilise different protease inhibitors to detect effects on induced levels of phenoloxidase, as this could also be a method of crop protection in addition to lectin use, by preventing upregulation of specific proteins in response to a immunogenic challenge to the insect in question. By, compromising the insects normal immune response the insects would be less able to respond to an immunogenic challenge.

To allow full understanding of the effect of lectins on the insect immune system a good avenue for future research would be to determine the precise pathways by which this system is initiated and to characterise the pathways by which the

insect immune response is produced in general. Initially a good approach would be to fully characterise the biochemical pathways of the polyphenoloxidase system following induction by lectins. As part of this a differential cDNA library could be made from insect haemolymph after exposure to PHA and GNA and perhaps other lectins such as Con A, to screen for induced mRNAs. In addition biochemical and immunological assays for protein and enzyme activities upregulated after exposure to various lectins could be carried out.
Potentially this could lead to determination of receptors which could be targeted by future pesticides, which could increase the potential use of lectins in the field of crop protection, whether in transgenic plants or as a tool in the more traditional field crop protection (agrochemicals).

Repeating the fluorescence experiment, following the development of new (and more expensive) fluorochromes such as Alexafluor (molecular probes California USA) would potentially allow generation of clearer results, allowing better visualisation of lectin binding, and therefore possible toxic mechanisms.

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Chapter 9

Concluding Discussion

After initial handling and technical problems, the of insect bioassays allowed a relatively quick assessment of the toxicity of the lectins tested. For practical reasons, in this work only the tomato moth larvae *L. oleracea* and the peach potato aphid *M. persicae* were used as models. Since lectins can be selective in their toxicity, toxic effects exhibited or not exhibited in these two species may not be duplicated in others. It would have been beneficial to test lectin toxicity on a wider range of species, but time and financial constraints prohibited this. The selectivity of lectin toxicity is demonstrated in chapter 3, where the black mulberry lectin *M. nigra* exerts no apparent toxicity to *L. oleracea* larvae, but exerts a detrimental effect on *M. persicae* by inhibiting normal growth.

The bioassays testing the effect of jacalin and *M. nigra* lectins on *M. persicae* indicated that both lectins exerted a similar effect. The main link between these two lectins is that they both exhibit a binding specificity for galactose (Gal) and N-acetylgalactosamine (GalNAc). The bioassays indicate a probable relationship between the binding specificity of two different lectins and adverse effect on an insect population. However, *M. nigra* lectin did not have an adverse effect *on L. oleracea*, perhaps because *L. oleracea* has less or no receptors for the *M. nigra* lectin (due to insufficient quantities of this lectin being available no binding studies using these two lectins could be carried out). The hypothesis is that by determining receptor specificity and lectin binding specificity, it could be possible to predict toxicity in a given species or family to a predetermined lectin. However,

as yet no link has been determined. WGA has no toxicity to the pea aphid *Acrythosiphon pisum* where as RSII does. Both lectins bind N-acetyl glucosamine but only the RSII lectin exhibits a toxic effect (Rahbe *et al.*, 1995).

The experimental design (described in chapter 3) for the testing of substances within an artificial diet is such that other substances such as protease inhibitors can be tested in the same manner as lectins. Indeed the effect of protease inhibitors on *L. oleracea* has been studied in this way (Gatehouse *et al.*, 1997), and (Gatehouse *et al.*, 1999).

In all instances where lectin toxicity occurs, both in animals and insects, there are no recorded cases where a toxic effect has not been shown to be accompanied by lectin binding (reviewed by, Van Damme *et al.*, 1998; Pusztai *et al.*, 1995, Pusztai 1991).

As seen in chapters 4-6 the lectin GNA binds to gut components in *L. oleracea* and *A. bipunctata* larvae as well as in *M. persicae.* The evidence of lectin binding is clear with the histological and non-histological evidence backing each other up. This is significant as the *in vitro* (non-histological) evidence alone is not proof of lectin binding *in vivo*, because the proteins the lectin binds to on Western blots may not actually be in contact with the gut lumen, and so binding to the gut surface *in vivo* may never occur. Therefore, histological work is vital in confirming that lectins do bind to the surface of the gut.

The histological evidence in chapters 4-6 was unable to point directly to a toxic effect caused by the GNA or Con A lectins tested, unlike the effect of PHA on rats in which histological evidence shows disruption of the epithelial membrane (King 1982).

As mentioned earlier GNA and Con A are known to have toxic effects on larvae of the Tomato moth *L. oleracea* (Gatehouse *et al.*, 1999; Fitches *et al.*, 1997) and on *M. persicae* (Gatehouse *et al.*, 1999; Sauvion *et al.*, 1996). However GNA does not affect *A. bipunctata* larvae (Down *et al.*, 2000) or the parasitic wasp *Eulophus pennicornis* (Bell *et al.*, 1999) indicating that lectin binding is not indicative of toxic effect.

Although lectin binding has not been determined in the adult form of *A. bipunctata*; GNA has been claimed to cause a toxic effect on adults (Birch *et al.*, 1999). However, a similar, but longer term bioassay by Down *et al* (2001) in which *A. bipunctata* were fed *M. persicae* raised on GNA expressing potato plants utilising a larger sample size than that used by Birch *et al.*, (1999), failed to indicate any toxic effects to either the adult or larval form of the two spot ladybird *A. bipunctata* (Down *et al.*, 2001). A recent paper studying tritrophic relationships between GNA expressing transgenic plants, *L. oleracea* and the parasitoid wasp *Eulophus pennicornis*, also determined that expression of GNA although effecting *L. oleracea* did not adversely effect the ectoparasitoid *E. pennicornis* (Bell *et al.*, 1999).

Currently there are a number of hypotheses regarding the mechanisms of toxicity caused by plant lectins, none of which are conclusive.

As discussed in chapter 1, it is known that GNA has a negative effect on a number of insect species. Obviously the size of the dose is important but the nature of negative effects caused by lectins is not fully known, although there are a number of theories, such as that lectins act as anti-feedants, or block areas of the gut from absorbing nutrients. As such all the current theories for toxicity must be considered when determining reasons for the toxicity of lectins.

One known toxic effect of lectins is to inhibit normal growth. Eisemann *et al* 1994 demonstrated that 3 different lectins, Con A, WGA and lentil lectin all inhibited the larval growth of *Lucilia cuprina,* and concluded that the reason normal growth was inhibited by lectins could be due to 3 or more mechanisms: 1) reduced intake of diet, 2) partial blockage of the pores of the peritrophic membrane, 3) direct binding of specific lectins to the midgut epithelial cells, which could affect various functions of the cell membrane. Within *Lucilia cuprina* mechanisms 1 and 2 would cause a restriction of nutrient availability, leading to a subsequent starvation effect, whereas mechanism 3 may seriously effect normal gut processes.

It has been determined that GNA acts as an antifeedant to BPH (seen by a drop in honeydew production), but the mechanism by which the BPH rejects GNA containing food is not known (Powell *et al.*, 1995). The controls, which included aphids fed on the lectin *Pisum sativum* agglutinin (PSA) (non toxic to aphids),

exhibited no antifeedant effect (Powell *et al.*, 1995). In addition to the antifeedant effect, lectins have also been seen to effect the amino acid composition of honeydew of BPH (Powell *et al.*, 1993). However, GNA does not always act as an antifeedant in all species, for example GNA does not cause an antifeedant effect on *L. oleracea* (Fitches *et al.*, 1998).

In chapter 4 it is demonstrated that both Con A and GNA bind to the peritrophic membrane of *L. oleracea*. As such one explanation for toxicity to the lepidopteran could be impaired functionality of the peritrophic membrane, perhaps by blockage of the pores as mentioned above with *Lucilia cuprina*.

The most studied effect of lectin toxicity is its effect on the epithelial membrane of the gut (Van Damme *et al.*, 1998; Pusztai *et al.*, 1995; Pusztai 1991). EM studies in chapter 4 determined that no structural differences were observed between the epithelial membranes from control and GNA or Con A lectins fed specimens. As no disruption of the brush border membrane was observed (as occurs in mammals when fed the lectin PHA [Pusztai *et al.*, 1990]), this is one mechanism of toxicity that can be discounted within *L. oleracea*. However GNA does cause disruption of the midgut epithelial layer in at least one insect, the rice pest BPH, probably resulting in expression of a toxic effect (Powell *et al.*, 1998).
Harper *et al.*, 1995 point out the possibility that the amount of binding of a lectin could be directly proportional to its toxic effect. However Foissac *et al.*, 2000 showed that transgenic rice plants expressing GNA had a similar level of

resistance to the green leafhopper *Nephotettix virescens* (GLH) and the brown planthopper *Nilaparvata lugens* (BPH) Whereas GNA binding studies to glycoproteins in gut tissues showed that BPH contained more "receptors" than GLH and that BPH had a stronger GNA binding affinity, particularly in the insects midgut. With no difference in toxicity between the two species, it is therefore unlikely that in this case GNA toxicity is directly proportional to the amount of lectin bound.

As demonstrated in chapters 4 & 5, lectins find their way into the insect haemolymph, and GNA binds to haemocytes of *M. persicae* (chapter 5) and to the granularcytes and plasmatocytes of *L. oleracea* (Raemaekers, personal communication), as well as to the haemolymph of *L. oleracea* in general (chapter 4) (Fitches *et al.*, 2001). Experiments in rats have demonstrated lectins not only act as powerful mitogens for the small intestine and immune system of animals, but can also survive the passage across the intestinal epithelium and affect remote organs such as the pancreas. (Pusztai 1991), as such it is probable that lectins would be capable of exerting similar effects in insects after transfer across the gut membrane. It is established that the main insect defence mechanism against foreign pathogens involves phagocytosis by haemocytes, and that haemocytes are the insect equivalent to blood cells and are involved in a number of processes such as melanisation, and intermediary metabolism (Gillespie *et al.*, 1997; Gupta 1991). As haemocytes play an important role in insects, interference with their function could have a severe detrimental effect on an insect. The

University of Waterloo, Ontario, Canada has filed a patent based on appearance or uncontrolled production of two peptides human Apob-100 molecule and/or Apolipophorin 1, as a means of developing insect resistant plants. With the premise being these peptides could cause perturbation of normal haemocyte function and result in death of the insect.

The effect of lectins on the polyphenoloxidase system (chapter 7) is not necessarily detrimental. However, it is likely that there would be an associated energy cost with such, which may go some way to explain the trends of retarded growth and development caused by lectins in feeding trials. Furthermore, a part of the polyphenoloxidase cascade involves the formation of quinone, which can react irreversibly with nucleophilic side chains of proteins (-SH $-NH_2$ –NH), leading to the modification of lysine, histidine and thiol amino acids. Therefore, this quinone reactivity leads to a potential loss of fuction (Felton *et al.*, 1992). Although the effect of the lectin on the polyphenoloxidase system of BPH has not been demonstrated in this work, it can be hypothesised that the known effect of lectins on the amino acid composition of honeydew (Powell *et al.*, 1998) may be at least in part due to high levels of quinone.

From the currently available evidence it appears that the most logical explanation for lectin toxicity is a combination of effects and events, such as blockage of the peritrophic membrane (Eisemann *et al.*, 1994), combined with a mitogenic effect (Powell *et al.*, 1998) and over-expression of polyphenoloxidase. However it must be remembered that in all probability not all mechanisms of lectin toxicity will be

exhibited in all species in which a toxic effect is observed. There is a possibility that a lectin may not be toxic to all insect species, and use of these proteins in plant protection will depend on which species a lectin is toxic to, and to what extent toxicity is exhibited, and what concentration of lectin is required for a toxic effect to be observed.

Ideas for future work

As the full role of insect haemocytes in lectin toxicity has yet to be determined, further investigation of the effect of lectins on such haematocytes would be an interesting avenue for future research. Initially it would be of interest to screen other lectins with different binding specificities for effects on the polyphenoloxidase system, and to look at lectin effects on the polyphenoloxidase systems of other insect species. Additionally it would be of interest to utilise different protease inhibitors to detect effects on induced levels of phenoloxidase, as this could also be a method of crop protection in addition to lectin use, by preventing upregulation of specific proteins in response to a immunogenic challenge to the insect in question. By, compromising the insects normal immune response the insects would be less able to respond to an immunogenic challenge.

To allow full understanding of the effect of lectins on the insect immune system a good avenue for future research would be to determine the precise pathways by which this system is initiated and to characterise the pathways by which the

insect immune response is produced in general. Initially a good approach would be to fully characterise the biochemical pathways of the polyphenoloxidase system following induction by lectins. As part of this a differential cDNA library could be made from insect haemolymph after exposure to PHA and GNA and perhaps other lectins such as Con A, to screen for induced mRNAs. In addition biochemical and immunological assays for protein and enzyme activities upregulated after exposure to various lectins could be carried out.
Potentially this could lead to determination of receptors which could be targeted by future pesticides, which could increase the potential use of lectins in the field of crop protection, whether in transgenic plants or as a tool in the more traditional field crop protection (agrochemicals).

Repeating the fluorescence experiment, following the development of new (and more expensive) fluorochromes such as Alexafluor (molecular probes California USA) would potentially allow generation of clearer results, allowing better visualisation of lectin binding, and therefore possible toxic mechanisms.

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Appendix

Common Reagents

All solutions made up in distilled water unless otherwise stated.

Appendix

Buffer A 300mM Mannitol

5mM EDTA

17mM Tris-HCL pH 7.5

50 μg/ml leupeptin

Solubilisation buffer = 20mM Tris/HCL pH 7.5, 1 mM EDTA, 1mM MgSO4, & 10% glycerol (added just prior to use) & leupeptin 50ug/ul.

	Control diet	Experimental diet
Freeze dried potato leaf	0.2525g	0.2525g
Casein	0.104g	0.101g
Test protein	0	3.02mg in 1ml
H_2O		
Distilled water	2ml	1ml
Vitamin C	0.0101g	0.0101g
Methylparabenzoate	0.005g	0.005g
Aureomycin	8.8mg	8.8mg
Agar	0.125g	0.125g

Water	3ml	3ml

SDS-PAGE gel electrophoresis solutions

Acrylamide	30g acrylamide, 0.8g bis-acrylamide/ 100ml
Destain	40% methanol 7% glacial acetic acid
Protein size markers	SDS7 (SIGMA)
10 X reservoir buffer	0.25M Tris pH 8.3 1.92M glycine 1% SDS
Resolving buffer	3.0M Tris pH 8.8
2X sample buffer	0.2M Tris pH 6.8 20% glycerol 2% SDS 0.002% Bromophenol blue
Stacking buffer	0.5M Tris pH 6.8
Stain	40% methanol 7% glacial acetic acid

	0.05% kenacid blue
Western blotting reagents	
	Anode buffer 1, 0.3M Tris. 20% methanol, pH 10.4
	Anode buffer 2, 25mM Tris. 20% methanol pH 10.4
	Cathode buffer 25mM Tris. 40mM
	6-aminohexanoic acid, 20% methanol pH9.4

Other reagents

10X PBS	2g KH_2PO_4
	11.5g Na_2HPO_4
	2g KCl
	80g NaCl
SM buffer	5.8g NaCl
	2.0g $MgSO_4$ $7H_2O$
	50ml of 1M Tris-HCl (pH7.5)
	5ml of 2% (w/v) gelatine
	Deionised water to final volume of 1 litre.
20x SSC (1 Litre)	175.3g NaCl
	88.2g sodium citrate
	800ml de-ionised H_2O
	Adjust pH to 7.0 with few drops of 10 N NaOH
	Top up to 1 litre with de-ionised H_2O

50X TAE (1litre) 242g TRIS

57.1ml Glacial acetic acid

100ml 0.5M EDTA pH 8

Make up to 1 litre with de-ionised H_2O

TBS (1 litre) pH 7.5 0.05M Tris-HCl

0.15M NaCl.

Western Blot Antisera (1L)

50g skimmed milk powder (Boots the Chemist)

1000ml TBS pH7.5

1ml Tween 20

Western Blot Sera As antisera except 10ml Tween 20

Prehybridisation/Hybridisation Solution

For 100ml

50% Formamide	50ml
6X SSC	30ml (10X)
0.05 X Blotto	5ml (1X)
100µg/ml Herring sperm DNA	1ml
Double distilled water	14ml

Media

(All media must be autoclaved before use)

LB broth (litre)	10g NaCl
	10g tryptone
	5g yeast extract
	Distilled water
NZY agar (litre)	5g NaCl
	2g $MgSO_4.7H_2O$
	10g NZ amine
	5g yeast extract
	15g agar
	Distilled water to 1 litre
NZY top agar (litre)	1L NZY broth
	0.7% (w/v) agarose
NZY broth	5g NaCl
	2g $MgSO_4.7H_2O$
	10g NZ amine
	5g yeast extract
	Distilled water to 1litre.

Blast search results for aminopepidase clone

BLASTP versus translated EMBL, alignments with probability values ≤ e-60
Duplicated sequences have been removed. Conservative substitutions are denoted by a (+).

Sequences producing significant alignments:
(bits) E Value

F Q9NHZ9 Q9NHZ9 AMINOPEPTIDASE 1.
406 e-112
F O76803 O76803 AMINOPEPTIDASE N.
394 e-109
F Q11001 AMPM_MANSE MEMBRANE ALANYL AMINOPEPTIDASE PRECURSOR (E... 389 e-107
F Q9U6W2 Q9U6W2 CRY1A TOXIN RECEPTOR A.
386 e-106
F O16851 O16851 AMINOPEPTIDASE N.
358 4e-98
F AAF99701 AAF99701 AMINOPEPTIDASE-N.
247 7e-65
F Q11000 AMPM_HELVI MEMBRANE ALANYL AMINOPEPTIDASE PRECURSOR (E... 245 4e-64
F O17484 O17484 AMINOPEPTIDASE.
243 1e-63
F Q9NHZ7 Q9NHZ7 AMINOPEPTIDASE 3.
236 2e-61
F O77046 O77046 AMINOPEPTIDASE N (EC 3.4.11.2) (FRAGMENT). 235 3e-61

Helicoverpa punctigera (Australian native budworm)
F Q9NHZ9 Q9NHZ9 AMINOPEPTIDASE 1.
Length = 1011

Score = 406 bits (1033), Expect = e-112
Identities = 192/257 (74%), Positives = 216/257 (83%), Gaps = 2/257 (0%)

Query: 4
LRHEVNSALVVGQQYIVRMSFTGILQSTMRGFYRSWYVDGNGDRRWMATTQFQPGHA
RQA 63
LR +AL + YI+ SF G LQ+ MRGFYRSWYVD
+G++RWMATTQFQPGHARQA
Sbjct: 131
LRIRTTAALALNTNYIITSSFRGNLQTNMRGFYRSWYVDSSGNKRWMATTQFQPGHA
RQA 190

Query: 64
FPCYDEPGFKATFDISITRDIEGFNPTLSNMPIRAFEEVGNGKIKETFYTTPRTSTY

```
LLA 123
            FPCYDEPGFKATFDI+I R+ + F+ TLSNMPIR    + +G+I
ETFYTTP TSTYL+A
Sbjct: 191 FPCYDEPGFKATFDITINRESD-
FSATLSNMPIRTQTPLASGRIAETFYTTPVTSTYLIA 249

Query: 124
FIVSGYKEVATNHLSTRPFHIYARGNIPAGSGDYSLRVGSPLLEVMERYTAIPYYTM
GTN 183
            FIVS YK VATN+   RPF IYAR N+   SG+++L +G
PLLEVMERYT IPYY M +N
Sbjct: 250 FIVSHYKSVATNNNQLRPFEIYARDNVGV-
SGNFALEIGMPLLEVMERYTEIPYYNMASN 308

Query: 184
MNMKQAAIPDFSAGAMENWGLLTYREALIIYDPENTNNFYKQRIANIISHEIAHMWF
GNL 243
            MNMKQAAIPDFSAGAMENWGLLTYREALI++DP
NTNNFYKQRIANIISHEIAHMWFGNL
Sbjct: 309
MNMKQAAIPDFSAGAMENWGLLTYREALILFDPVNTNNFYKQRIANIISHEIAHMWF
GNL 368

Query: 244 VTCAWWDNLWLNEGFAR 260
            VTCAWWDNLWLNEGFAR
Sbjct: 369 VTCAWWDNLWLNEGFAR 385


Bombyx mori (Silk moth)
>F O76803 O76803 AMINOPEPTIDASE N.
          Length = 986

 Score =  394 bits (1002), Expect = e-109
 Identities = 184/257 (71%), Positives = 214/257 (82%),
Gaps = 3/257 (1%)

Query: 4
LRHEVNSALVVGQQYIVRMSFTGILQSTMRGFYRSWYVDGNGDRRWMATTQFQPGHA
RQA 63
            LR     ALV+ ++YI++ +F G LQ+ MRGFYRSWYVD  G
RRWM TTQFQPGHARQA
Sbjct: 133 LRIRTTEALVLNREYIIKSTFRGNLQTNMRGFYRSWYVDSTG-
RRWMGTTQFQPGHARQA 191

Query: 64
FPCYDEPGFKATFDISITRDIEGFNPTLSNMPIRAFEEVGNGKIKETFYTTPRTSTY
LLA 123
            FPCYDEPGFKATFDI++ R+ E F+PT+SNMPIR    + NG++
ETF+TTP TSTYLLA
Sbjct: 192 FPCYDEPGFKATFDITMNRE-
ESFSPTISNMPIRTTNTLANGRVSETFWTTPVTSTYLLA 250
```

```
Query: 124
FIVSGYKEVATNHLSTRPFHIYARGNIPAGSGDYSLRVGSPLLEVMERYTAIPYYTM
GTN 183
            FIVS Y  V+TN+ + RPF IYAR N+   +GD+SL +G  LLE
ME YT IPYYTM  N
Sbjct: 251 FIVSHYTVVSTNNNALRPFDIYARNNV-
GRTGDWSLEIGEKLLEAMEAYTQIPYYTMAEN 309

Query: 184
MNMKQAAIPDFSAGAMENWGLLTYREALIIYDPENTNNFYKQRIANIISHEIAHMWF
GNL 243
            +NMKQAAIPDFSAGAMENWGLLTYREALI+YDP
N+N+FYKQR+ANI++HEIAHMWFGNL
Sbjct: 310
INMKQAAIPDFSAGAMENWGLLTYREALILYDPLNSNHFYKQRVANIVAHEIAHMWF
GNL 369

Query: 244 VTCAWWDNLWLNEGFAR 260
            VTCAWWDNLWLNEGFAR
Sbjct: 370 VTCAWWDNLWLNEGFAR 386


Manduca sexta (Tobacco hawkmoth) (Tobacco hornworm)

>F Q11001 AMPM_MANSE MEMBRANE ALANYL AMINOPEPTIDASE
PRECURSOR (EC 3.4.11.-)
            (AMINOPEPTIDASE N-LIKE PROTEIN) (CRYIA(C)
RECEPTOR)
            (FRAGMENT).
           Length = 990

 Score =  389 bits (989), Expect = e-107
 Identities = 182/257 (70%), Positives = 215/257 (82%),
Gaps = 3/257 (1%)

Query: 4
LRHEVNSALVVGQQYIVRMSFTGILQSTMRGFYRSWYVDGNGDRRWMATTQFQPGHA
RQA 63
            LR   ++ LV+ Q+YI+R +F G LQ+ MRGFYRSWYVD  G
+RWMATTQFQPGHARQA
Sbjct: 131 LRIRTSTPLVMNQEYIIRSTFRGNLQTNMRGFYRSWYVDRTG-
KRWMATTQFQPGHARQA 189

Query: 64
FPCYDEPGFKATFDISITRDIEGFNPTLSNMPIRAFEEVGNGKIKETFYTTPRTSTY
LLA 123
            FPCYDEPGFKATFDI++ R+ + F+PT+SNMPIRA   + NG+I
ETF+TTP TSTYLLA
Sbjct: 190 FPCYDEPGFKATFDITMNREAD-
FSPTISNMPIRATTTLTNGRISETFFTTPLTSTYLLA 248

Query: 124
```

```
FIVSGYKEVATNHLSTRPFHIYARGNIPAGSGDYSLRVGSPLLEVMERYTAIPYYTM
GTN 183
            FIVS Y+ ++ N+ + RPF IYAR N+    GD+SL +G  LL
ME YTAIPYYTM  N
Sbjct: 249 FIVSHYQVISNNNNAARPFRIYARNNV-
GSQGDWSLEMGEKLLLAMENYTAIPYYTMAQN 307

Query: 184
MNMKQAAIPDFSAGAMENWGLLTYREALIIYDPENTNNFYKQRIANIISHEIAHMWF
GNL 243
            ++MKQAAIPDFSAGAMENWGLLTYREALI+YDP N+N+
Y+QR+ANI+SHEIAHMWFGNL
Sbjct: 308
LDMKQAAIPDFSAGAMENWGLLTYREALILYDPLNSNHHYRQRVANIVSHEIAHMWF
GNL 367

Query: 244 VTCAWWDNLWLNEGFAR 260
            VTCAWWDNLWLNEGFAR
Sbjct: 368 VTCAWWDNLWLNEGFAR 384


Heliothis virescens (Noctuid moth) (Owlet moth)

>F Q9U6W2 Q9U6W2 CRY1A TOXIN RECEPTOR A.
          Length = 1010

 Score =  386 bits (981), Expect = e-106
 Identities = 183/257 (71%), Positives = 211/257 (81%),
Gaps = 3/257 (1%)

Query: 4
LRHEVNSALVVGQQYIVRMSFTGILQSTMRGFYRSWYVDGNGDRRWMATTQFQPGHA
RQA 63
            LR      L     YI++ ++ G LQ+ MRGFYRSWY+D +G
+RWMATTQFQPGHARQA
Sbjct: 129
LRIRTAGVLAENTDYIIQSTYRGNLQTNMRGFYRSWYIDSSGTKRWMATTQFQPGHA
RQA 188

Query: 64
FPCYDEPGFKATFDISITRDIEGFNPTLSNMPIRAFEEVGNGKIKETFYTTPRTSTY
LLA 123
            FPCYDEPGFKATFDI+I R+ + F+P+LSNMPIR+   VG  ++
ETFYTTP  STYL+A
Sbjct: 189 FPCYDEPGFKATFDITINREAD-FSPSLSNMPIRSTTNVG-
ARVAETFYTTPVMSTYLVA 246

Query: 124
FIVSGYKEVATNHLSTRPFHIYARGNIPAGSGDYSLRVGSPLLEVMERYTAIPYYTM
GTN 183
            FIVS Y +VAT+    RPF IYAR NI    G+++L VG
PLL+VMERYT IPYYTM TN
```

```
Sbjct: 247 FIVSHYTQVATHSNQQRPFAIYARNNI-
GNHGNHALDVGVPLLDVMERYTDIPYYTMATN 305

Query: 184
MNMKQAAIPDFSAGAMENWGLLTYREALIIYDPENTNNFYKQRIANIISHEIAHMWF
GNL 243
            MNMKQ AIPDFSAGAMENWGLLTYREALI+YDP+N+N+FY+QRI
NIISHEIAHMWFGNL
Sbjct: 306
MNMKQVAIPDFSAGAMENWGLLTYREALILYDPQNSNSFYRQRIPNIISHEIAHMWF
GNL 365

Query: 244 VTCAWWDNLWLNEGFAR 260
            VTCAWWDNLWLNEGFAR
Sbjct: 366 VTCAWWDNLWLNEGFAR 382


Plutella xylostella (Diamondback moth)
>F O16851 O16851 AMINOPEPTIDASE N.
          Length = 988

 Score =  358 bits (909), Expect = 4e-98
 Identities = 167/257 (64%), Positives = 207/257 (79%),
Gaps = 2/257 (0%)

Query: 4
LRHEVNSALVVGQQYIVRMSFTGILQSTMRGFYRSWYVDGNGDRRWMATTQFQPGHA
RQA 63
            LR     AL + Q+Y V +++ G LQ+ MRGFYRSWY D
+G++RWMATTQFQPGHAR+A
Sbjct: 136
LRVAPTQALQLNQEYTVNVTYRGNLQTDMRGFYRSWYRDSSGNKRWMATTQFQPGHA
RKA 195

Query: 64
FPCYDEPGFKATFDISITRDIEGFNPTLSNMPIRAFEEVGNGKIKETFYTTPRTSTY
LLA 123
            FPCYDEPGFKA F+I+I R+ + F P++SNMPIR    +GNG+
++FYTTP TS+YL+A
Sbjct: 196 FPCYDEPGFKALFNITINRE-
DDFKPSISNMPIRRTISLGNGRTADSFYTTPLTSSYLVA 254

Query: 124
FIVSGYKEVATNHLSTRPFHIYARGNIPAGSGDYSLRVGSPLLEVMERYTAIPYYTM
GTN 183
            FIVS Y++V +++ + RPF IYAR N+   +G +SL VG  LL
ME +T   YY+M
Sbjct: 255 FIVSHYEKVESSNNTLRPFDIYARDNVGV-
TGQWSLEVGEKLLAYMEGHTDYEYYSMAPF 313

Query: 184
MNMKQAAIPDFSAGAMENWGLLTYREALIIYDPENTNNFYKQRIANIISHEIAHMWF
```

```
GNL 243
           +NMKQAAIPDFSAGAMENWGLLTYREA I+Y
PEN+N+FYKQR+ANI++HEIAHMWFGNL
Sbjct: 314
LNMKQAAIPDFSAGAMENWGLLTYREANILYHPENSNHFYKQRVANIVAHEIAHMWF
GNL 373

Query: 244 VTCAWWDNLWLNEGFAR 260
           VTCAWWDNLWLNEGFAR
Sbjct: 374 VTCAWWDNLWLNEGFAR 390


Epiphyas postvittana.

>F AAF99701 AAF99701 AMINOPEPTIDASE-N.
          Length = 1007

 Score =  247 bits (625), Expect = 7e-65
 Identities = 129/246 (52%), Positives = 161/246 (65%),
Gaps = 3/246 (1%)

Query: 18  YIVRMSFTGILQSTMRGFYRSWYVDGNGDR-
RWMATTQFQPGHARQAFPCYDEPGFKATF 76
           Y + +SF   L++ M G YRSW+ +   D   WMATTQFQ
AR+AFPCYDEP FKATF
Sbjct: 156
YTLTISFEAPLRNDMYGIYRSWFRNQPNDPISWMATTQFQATSARKAFPCYDEPSFK
ATF 215

Query: 77
DISITRDIEGFNPTLSNMPIRAFEEVGNGKIKETFYTTPRTSTYLLAFIVSGYKEVA
TNH 136
           DI+I R     + + + +   A          + ++ TP
STYLLA IV+ Y  +  N+
Sbjct: 216
DITIRRPAAYRSWSCTRIASTAPSTTPPNYEDDIYHRTPIMSTYLLALIVAEYDSLT
VNN 275

Query: 137 LSTRP-FHIYARGN-
IPAGSGDYSLRVGSPLLEVMERYTAIPYYTMGTNMNMKQAAIPDF 194
              +  + + AR N I  G G Y+L VG  LL  M  +T   +YTM
N+ M QA+IPDF
Sbjct: 276
AQGQLIYEVIARPNAISTGQGQYALDVGQDLLAEMNDHTNYNFYTMNPNLKMTQASI
PDF 335

Query: 195
SAGAMENWGLLTYREALIIYDPENTNNFYKQRIANIISHEIAHMWFGNLVTCAWWDN
LWL 254
           SAGAMENWGLLTYREA I+YD  +TN+++KQ IA
I+SHEIAHMWFGNLVTC WWD LWL
Sbjct: 336
```

```
SAGAMENWGLLTYREAYIMYDEVHTNSYFKQLIAYILSHEIAHMWFGNLVTCDWWDV
LWL 395

Query: 255 NEGFAR 260
           NEGFAR
Sbjct: 396 NEGFAR 401


Heliothis virescens (Noctuid moth) (Owlet moth)
>F Q11000 AMPM_HELVI MEMBRANE ALANYL AMINOPEPTIDASE
PRECURSOR (EC 3.4.11.-)
           (AMINOPEPTIDASE N-LIKE PROTEIN) (CRYIA(C)
RECEPTOR)
           (BTBP1).
          Length = 1009

 Score =  245 bits (619), Expect = 4e-64
 Identities = 128/247 (51%), Positives = 159/247 (63%),
Gaps = 5/247 (2%)

Query: 18  YIVRMSFTGILQSTMRGFYRSWY--
VDGNGDRRWMATTQFQPGHARQAFPCYDEPGFKAT 75
           Y + + FT  ++  M G Y SWY  +  + + RWMATTQFQ   AR
AFPCYDEPGFKA
Sbjct: 156
YTLSIDFTAPMRDDMYGIYNSWYRNLPDDANVRWMATTQFQATAARYAFPCYDEPGF
KAK 215

Query: 76
FDISITRDIEGFNPTLSNMPIRAFEEVGNGKIKETFYTTPRTSTYLLAFIVSGYKEV
-AT 134
           FD++I R + G++          +      G  ++ ++TTP
STYLLA IVS Y  + AT
Sbjct: 216 FDVTIRRPV-
GYSSWFCTRQKGSGPSTVAGYEEDEYHTTPTMSTYLLALIVSEYTSLPAT 274

Query: 135 NHLSTRPFHIYAR-
GNIPAGSGDYSLRVGSPLLEVMERYTAIPYYTMGTNMNMKQAAIPD 193
           N        + AR G I  G   Y+ RVG  LL  M  +T   +Y
N+ M QAAIPD
Sbjct: 275
NAAGEILHEVIARPGAINNGQAVYAQRVGQALLAEMSDHTGFDFYAQDPNLKMTQAA
IPD 334

Query: 194
FSAGAMENWGLLTYREALIIYDPENTNNFYKQRIANIISHEIAHMWFGNLVTCAWWD
NLW 253
           F AGAMENWGLLTYREA ++YD ++TN+++KQ IA
I+SHEIAHMWFGNLVT AWWD LW
Sbjct: 335
FGAGAMENWGLLTYREAYLLYDEQHTNSYFKQIIAYILSHEIAHMWFGNLVTNAWWD
VLW 394
```

```
Query: 254 LNEGFAR 260
           LNEGFAR
Sbjct: 395 LNEGFAR 401


Plodia interpunctella (Indian meal moth)
>F O17484 O17484 AMINOPEPTIDASE.
          Length = 1016

 Score =  243 bits (615), Expect = 1e-63
 Identities = 129/248 (52%), Positives = 165/248 (66%),
Gaps = 5/248 (2%)

Query: 18  YIVRMSFTGILQSTMRGFYRSWYVDGNGDR--
RWMATTQFQPGHARQAFPCYDEPGFKAT 75
           Y++ ++F   L+  M G YRSW+ + N +    +MATTQFQ
AR+AFPCYDEP FKAT
Sbjct: 160
YVLTINFEAELRHDMYGIYRSWFRNDNYNATPNYMATTQFQATSARRAFPCYDEPSF
KAT 219

Query: 76  FDISITRDIEGFNPTLSNMPIRAFEEVGNGKIK-
ETFYTTPRTSTYLLAFIVSGYKEVAT 134
           FDISI R  +  + + + +   A  ++   + + +TFY TP
STYLLA IV+ YK V
Sbjct: 220
FDISIARRQDVKSWSCTRLAGTAPSDLYGPEFEVDTFYRTPIMSTYLLAIIVADYKS
VEF 279

Query: 135 NHLS-TRPFHIYAR-
GNIPAGSGDYSLRVGSPLLEVMERYTAIPYYTMGTNMNMKQAAIP 192
           N+      + + AR   I      Y+  VG  LL  M  +TAI
Y+++ +N+ M QAAIP
Sbjct: 280
NNTQGLLEYEVIARPAAIDNNQYQYAFDVGQELLAEMSDHTAIDYFSVDSNLKMTQA
AIP 339

Query: 193
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DVL 399

Query: 253 WLNEGFAR 260
           WLNEGFA+
Sbjct: 400 WLNEGFAK 407
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Gaps = 5/248 (2%)

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FKA 218

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Length = 953

Score = 235 bits (594), Expect = 3e-61

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Pergamon

Journal of Insect Physiology 46 (2000) 379–391

Journal of Insect Physiology

www.elsevier.com/locate/jinsphys

Snowdrop lectin (GNA) has no acute toxic effects on a beneficial insect predator, the 2-spot ladybird (*Adalia bipunctata* L.)

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Received 1 February 1999; accepted 20 April 1999

Abstract

Two-spot ladybird (*Adalia bipunctata* L.) larvae were fed on aphids (*Myzus persicae* (Sulz.)) which had been loaded with snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) by feeding on artificial diet containing the protein. Treatment with GNA significantly decreased the growth of aphids. No acute toxicity of GNA-containing aphids towards the ladybird larvae was observed, although there were small effects on development. When fed a fixed number of aphids, larvae exposed to GNA spent longer in the 4th instar, taking 6 extra days to reach pupation; however, retardation of development was not observed in ladybird larvae fed equal weights of aphids. Ladybird larvae fed GNA-containing aphids were found to be 8–15% smaller than controls, but ate a significantly greater number of aphids (approx. 40% to pupation). GNA was shown to be present on the microvilli of the midgut brush border membrane and within gut epithelial cells in ladybird larvae fed on GNA-dosed aphids, although disruption of the brush border was not observed. It is hypothesised that GNA does not have significant direct toxic or adverse effects on developing ladybird larvae, but that the effects observed may be due to the fact that the aphids fed on GNA are compromised and are thus a suboptimal food.

Keywords: Ladybird larvae; *Galanthus nivalis* agglutinin (GNA); Tritrophic level; Crop protection; Lectin binding

1. Introduction

Increased publicity and concerns over the widespread use of chemical insecticides have resulted in a greater amount of research into more environmentally friendly and sustainable methods of insect control (Poppy, 1997). As well as toxic effects on higher animals observed with some pesticides, chemical insecticides can have detrimental effects on beneficial insect species (Bozsik et al., 1996; Schmuck et al., 1997). Integrated Pest Management (IPM) combines several control systems, often including chemical pesticides, although biological control, using natural predators and parasitoids of crop pests, is usually the key element (Poppy, 1997). Biotechnology, particularly the use of transgenic crops expressing insecticidal proteins, is increasingly being investigated with the intention of playing a major role in IPM systems (Brough et al., 1995; Waage, 1997). With the production of transgenic plants containing constructs encoding specific plant-derived insecticidal proteins (Gatehouse and Gatehouse, 1998), it is imperative that possible adverse effects upon natural biological control agents resulting from use of these plants are investigated.

Recent popular press publications (Farmers Weekly, 1997; Gledhill and McGrath, 1997; Hawkes, 1997; Brookes and Coghlan, 1998) have created a great deal of wariness in the use of transgenic plants in crop protection, raising other issues besides the possible effects that transgenic plants will have on beneficial insect communities. However, it is important not to forget that whatever forms of insect control are employed, whether chemical or not, some adverse effect on natural predators of target pests will always be seen; this may not necessarily be a direct toxic effect, but could be indirectly due to ailing pest populations (resulting from successful control). The use of transgenic crop plants within IPM has the potential to overcome direct adverse effects on pest predators, providing that interactions between all the trophic levels are well researched.

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0022-1910/00/$ - see front matter
PII: S0022-1910(99)00121-3

As well as possible direct toxic effects of transgene products, indirect effects are also possible. Foraging behaviour of natural enemies can be both directly and indirectly affected by the plant through semiochemically, chemically and physically mediated mechanisms (reported in Poppy, 1997), and these interactions between the first and the third trophic level have often been neglected. Reports on these interactions are now emerging both in terms of direct and indirect effects between first and third trophic levels (Dogan et al., 1996; Hilbeck et al., 1998; Birch et al., 1999) and more complex interactions between first, second and third trophic levels involving pest adaptation (Johnson et al., 1997a,b).

The aims of this research are to investigate the effects of snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) on the biology of larval development, pupation and adult emergence of the 2-spot ladybird. The *gna* gene has been proposed as a means of protecting transgenic plants against insect attack, particularly for use against homopteran pests such as aphids and plant- and leafhoppers (Powell et al., 1993; Rahbé et al., 1995; Sauvion et al., 1996; Down et al., 1996; Gatehouse et al., 1996). Transgenic rice plants expressing GNA are partially resistant to a major pest of the crop, rice brown planthopper (*Nilaparvata lugens* (Stal); Rao et al., 1998), and potatoes expressing GNA have been shown to have enhanced resistance to both peach–potato aphids (*Myzus persicae*; Gatehouse et al., 1996) and glasshouse potato aphids (*Aulacorthum solani* (Kalt.); Down et al., 1996). Since ladybirds are an important resource for biological control of aphids, toxic effects of GNA on these predators would adversely affect the possibilities of integrating transgenic plants expressing this lectin into IPM strategies. We have therefore attempted to determine whether the protein is toxic to ladybirds by a direct feeding assay on larvae, which consume more aphids than adult forms of the insect. This work will complement research already carried out on the effects of GNA on adult 2-spot ladybirds (Birch et al., 1999), where the authors found that aphids reared on transgenic potatoes expressing GNA could reduce longevity and fecundity of the adult females, and adversely affected egg viability. However, no direct toxic effects were suggested by assays using adult ladybirds, since short-term survival was not decreased. As well as assessing the toxicity of GNA to ladybird larvae, the present paper investigates possible mechanisms by which GNA may affect aphid predators, which are not directly exposed to transgenic plants expressing the protein.

2. Materials and methods

2.1. Materials

GNA was obtained from Drs W. Peumans and E. van Damme (Catholic University, Leuven, Belgium). Antibodies against recombinant GNA (Longstaff et al., 1998) in rabbit were supplied by Dr Christine Newell of Pestax, Cambridge, UK. Antibodies against GNA purified from snowdrop bulbs were raised in rabbits using standard procedures (supplied by Dr L.N. Gatehouse and Dr R.D.D. Croy, University of Durham). LR white resin, nickel grids (150 mesh, hexagonal), goat anti-rabbit 10 nm gold-conjugated IgG and goat serum were all obtained from Agar Scientific, Stansted, Essex. The ECL detection kit was supplied by Amersham, Bucks, UK. TRI reagent was obtained from the Sigma Chemical Company. All other chemicals were from either Sigma Chemical Company or BDH (Poole, Dorset) and were of analytical grade unless otherwise stated.

2.2. Insect cultures

Stocks of the peach–potato aphid (*M. persicae*) were continuously reared on Chinese cabbage plants in environmentally-controlled incubators at 21±2°C under a L16:D8 lighting regime. Mating 2-spot ladybirds (*Adalia bipunctata*) were either supplied by Dr M. Majerus, University of Cambridge, where they had previously been reared on the pea aphid (*Acyrthosiphon pisum* (Harris)), or were collected from the wild. Once obtained, ladybirds (both adults and hatching larvae) were reared on *M. persicae* in an environmentally-controlled incubator at 21±2°C under an L16:D8 lighting regime.

Artificial diet, capable of sustaining the growth and parthenogenetic reproduction of *M. persicae* was prepared according to Febvay et al. (1988). Aphid feeding chambers were used for artificial diet studies, and were prepared according to Down et al. (1996); diet sachets were replaced with fresh ones on alternate days.

2.3. Accumulation of GNA within the pest species (M. persicae)

Seven aphid feeding chambers, each containing 10 newly mature, apterous, adult *M. persicae* feeding on an artificial diet containing 0.1% w/v GNA were set up. Aphids were collected from the replicate feeding chambers at 1-day intervals for 7 days. Samples were flash frozen on collection and stored at −20°C for further analysis. A control containing aphids feeding on an artificial diet with no added GNA was sampled after 24 h. The aphid samples were homogenised in 50 mM Tris–HCl, pH 9.5 containing 1% phenylmethylsulfonylfluoride (PMSF; 36 mg/ml in ethanol), using 5 μl of buffer per aphid. Extractions were performed overnight at 4°C, with shaking. Samples were centrifuged at 13,000*g* for 10 min. 35 μl of supernatant was added to 35 μl of 2x SDS sample buffer containing 2% 2-mercaptoethanol before denaturing by boiling for 3 min. 30 μl of each sample was analysed by electrophoresis on a 12.5%

SDS-PAGE minigel, using a known amount of GNA as a standard. Western blotting was performed according to the method of Kyhse-Andersen (1984); blots were probed with anti-GNA antisera as primary antibody and peroxidase-coupled anti-rabbit IgG as secondary antibody. Specifically bound antibody was detected by the ECL protocol as described in the manufacturer's instructions (Amersham, Bucks, UK). To directly visualise lectin accumulation in the aphids, *M. persicae* were fed on FITC-labelled GNA (prepared as described in Cuello, 1985) for 48 h, followed by a 48-h feeding period on a diet without added GNA, and viewed using a fluorescence microscope. FITC-labelled casein was used as a control.

2.4. Investigating the effects of GNA on 2-spot ladybird larvae

Aphids (*M. persicae*) were divided into two groups, controls, and aphids dosed with GNA prior to feeding them to ladybird larvae. On a daily basis, 12 chambers containing approximately 50 neonate to mid-instar aphids, picked from the stocks, were prepared; six were supplied with an artificial diet with 0.1% w/v GNA added and six were supplied a diet without GNA incorporated. Aphids were fed on the diet for 4 days, before feeding to the ladybird larvae, with the diet sachets being replaced on day 2 to limit fungal contamination of the diet. To assess the effects of GNA on aphid development, 50 aphids from each treatment were selected at random, and subjected to image analysis to measure length (from the tip of the head to the end of the cauda) and width (across the metathorax). Image analysis was carried out using a Nikon Type 104 microscope (×4 lens) fitted with a standard video camera; images were acquired by connecting the camera to the S-video input of an Apple Power Macintosh 7600/120 computer, and using the Apple Video Player software to capture freeze-frames. Images were analysed using the NIH Image software package; the system was calibrated using an image of a millimetre scale.

To feed aphids to ladybird larvae, the larvae were individually placed in containers made from the lid of a 3.5-cm petri dish lined with a piece of dry filter paper; two layers of parafilm were stretched over the top. Aphids were supplied to the larvae on a daily basis.

In the first bioassay, ladybird larvae in control and GNA-fed groups were fed an equal number of aphids. Larvae emerging from each egg batch were equally divided between the control (fed aphids dosed on a control diet) and GNA-fed groups (fed aphids dosed on a diet with added GNA) such that there were 25 larvae per group. On most days, 10 aphids per larva per day were supplied; any remaining uneaten aphids were removed from the ladybird pots on a daily basis and replaced with fresh aphids. During ladybird larval development, survival, number of aphids consumed, and instar duration were recorded daily. All surviving larvae were weighed 24 h after the moult into the 4th instar. Numbers of ladybirds reaching pupation and successfully emerging into adults were also recorded.

A second bioassay was performed essentially following the same procedure as for bioassay 1, with 25 ladybird larvae per control and treatment group, but with feeding being carried out on the basis of equal weights of aphids being supplied to control and GNA-fed groups. This resulted in a greater number of aphids being supplied to the GNA-fed group, since these aphids were smaller. The aphid weight supplied per ladybird larva varied on a daily basis according to availability of dosed aphids, although all larvae, whether control or treatment, were fed the same weight of aphids on any one day. Ladybird larval weight was measured after hatching and again 24 h after the exoskeletons were sloughed between 2nd/3rd and 3rd/4th instars. Other parameters were measured as in bioassay 1.

2.5. Immunohistochemical studies

Adult *M. persicae* were dosed on artificial diet with the addition of 0.1% w/v GNA for at least 24 h prior to supplying to ladybird larvae, which were fed from egg hatch until reaching the 4th instar. Larval guts were dissected (five in total), on ice, from the ladybird larvae on reaching the 4th instar, immediately placed in fixative (a solution of 2.5% paraformaldehyde, 2.5% gluteraldehyde, 2% sucrose in Sorensens buffer at pH 7.4; buffer prepared as described in Glauert, 1975) and then cut into smaller sections. After 3 h of fixation, at room temperature and spinning on a wheel, the pieces of gut were washed for 3× 5–10 min in Sorensens buffer pH 7.4, then briefly rinsed in distilled water before incubating in a 2% aqueous solution of uranyl acetate for 30 min. The gut pieces were again briefly rinsed in distilled water before dehydration by incubation in 50% ethanol for 2× 5–10 min followed by 70% ethanol for 3× 10 min. Gut pieces were then placed in a 70% ethanol/LR white resin mix for 30 min before transferring to pure LR white resin for 3–4 h. Sections were incubated with fresh resin overnight, then again with fresh resin for 3–4 h, and embedded in capsules containing LR white resin at 50–55°C for 24 h.

Ultra thin sections (60–80 nm thick) were cut on a microtome (Leica Ultracut) and mounted on formvar and carbon coated nickel grids (150 mesh, hexagonal). For immunohistochemical labelling, sections were incubated for 10 min in heat inactivated goat serum before incubating overnight at 4°C in a 1:100 dilution (in Tris–HCl buffer pH 7.5) of antibody raised against recombinant GNA. Sections were washed for 4× 5 min in Tris–HCl buffer pH 7.5, and 1× 5 min in Tris–HCl buffer pH 8.2, before incubating for 1 h in anti-rabbit 10 nm gold-con-

jugated IgG (1:20 dilution in Tris–HCl buffer pH 8.2). The above Tris-buffers were prepared according to the guidelines sent with the gold conjugate (Agar Scientific, Stansted), except that the sodium azide was omitted. Sections were then washed for 1× 5 min in Tris–HCl pH 8.2 and 4× 5 min in distilled water, then stained for 10 min in 1% aqueous uranyl acetate and 10 min in lead citrate (Reynolds, 1963); sections were washed between and after staining for 5× 5 min in distilled water. Sections were then viewed using a transmission electron microscope (Philips EM400T). This procedure was repeated on midguts dissected from larvae which had been reared on *M. persicae* fed an artificial diet in the absence of GNA.

2.6. GNA binding to ladybird larval gut proteins

Total gut proteins were extracted from dissected larval ladybird guts in TRI reagent. After removal of insoluble material by centrifugation, the solution was partitioned with chloroform. The coloured organic (phenol) phase was removed, and 0.3 ml ethanol was added per ml to precipitate DNA. The precipitate was collected by centrifugation at 12,000*g* for 5 min. Proteins were then precipitated from the phenol–ethanol supernatant with isopropanol (1.5 ml per ml solution). After standing for 10 min at room temperature, the precipitate was collected by centrifugation at 12,000*g* for 10 min. After removal of the supernatant, the precipitated proteins were resuspended in SDS sample buffer and electrophoresed on a 12.5% SDS-PAGE minigel, using 5 μg of carboxypeptidase-Y as a control. Proteins were blotted onto PVDF membrane, which had been wetted with methanol and then incubated in water for 2 min before use. Once blotting had been performed, the membrane was incubated in 0.5% blocking solution in TBS (Blocking reagent; supplied by Boehringer-Mannheim) for 30 min. Membranes were washed for 2× 10 min in TBS and equilibrated in buffer 1 (1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ in TBS pH 7.5) for 5 min before incubating with recombinant GNA (1 μg/ml) in buffer 1 for 1 h. Membranes were washed for 2× 10 min in TBS before incubating in blocking buffer (5% fat-free milk in PBS, 10 ml/l Tween 20) for 1 h, followed by a 5-min wash in antisera buffer (5% fat-free milk in PBS, 1 ml/l Tween 20). Membranes were incubated with anti-recombinant GNA antibody (1:10,000 dilution in antisera buffer) for 1 h, washed for 3× 5 min in antisera buffer and incubated in HRP-anti-rabbit secondary antibody (1:10,000 dilution in antisera buffer) for 1 h. The membrane was washed for 2× 5 min and 1× 15 min in PBS/Tween 20, once in water for 5 min before detection was performed as described above.

2.7. Statistical analyses

All statistical analyses were performed using the Statview v. 4.5 software package for Macintosh computers (Abacus Concepts, CA, USA), and include the use of the unpaired *t*-test, non-parametric Mann-Whitney *U*-test and Kaplan-Meier Logrank χ^2 test.

3. Results

3.1. Effects of GNA on aphids and accumulation in body tissues

In agreement with previous results, GNA retarded the growth of *M. persicae*. Aphids fed on an artificial diet containing GNA for 4 days were shorter in length (mean length 1.11±0.02 mm) compared to those fed on a control diet (mean length 1.19±0.02 mm) for the same period (significant at $p<0.01$, unpaired *t*-test). The GNA-fed aphids also had a slightly reduced width compared to control aphids (means of 0.443±0.008 mm compared to 0.458±0.007 mm), although this difference was not statistically significant. The reduction in aphid size caused by GNA was clearly seen in the mean aphid weight, which decreased from 0.14±0.002 mg (control) to 0.13±0.003 mg (GNA-fed; difference significant at $p<0.01$; unpaired *t*-test).

Accumulation of GNA within aphid bodies was shown by two methods. Protein extracts from GNA-fed aphids were analysed by SDS-PAGE followed by Western blotting with anti-GNA antibodies. As shown in Fig. 1, GNA could be detected within the aphids after feeding on a GNA-containing diet for 24 h, and accumulated in the aphids up to the end of the experiment (7 days feeding). Amounts of GNA per aphid were estimated by comparison with GNA standards as approx. 0.08 μg after 4 days, and 0.13 μg after 7 days. The accumulation of GNA shows that the lectin detected in the aphids cannot only be the material present in the diet, but that binding of lectin to components in the aphid must occur. Accumulation of GNA in the aphid body was also visualised directly by feeding FITC-labelled GNA, and observing the labelled protein directly by fluorescence microscopy. Although labelled GNA was observed to be excreted in aphid honeydew, significant amounts accumulated in the aphid, initially predominantly in the gut region (Fig. 2). The fluorescent material was shown to be not only gut contents, or transiently associated with the gut, by carrying out a two-phase feed and chase experiment, where FITC-labelled lectin was fed for 2 days, followed by a control diet for a further 2 days; after the 4-day feeding period, significant amounts of fluorescence were still present throughout the aphid body (data not shown). Aphids fed on FITC-labelled casein as a control showed only weak and transient fluorescence

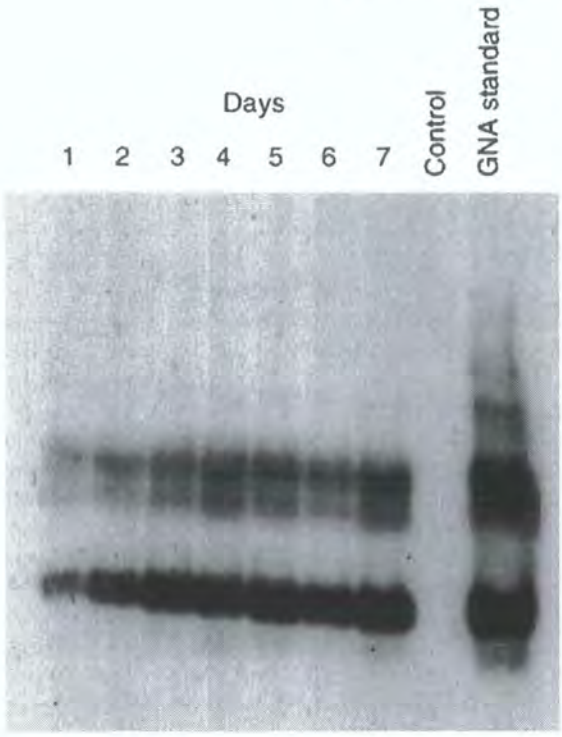

Fig. 1. Western blot showing accumulation of GNA within aphids with time. Aphids were fed on an artificial diet containing 0.1% GNA for 1–7 days (lanes 1–7); lane 8 contains the control sample where aphids were fed for 24 h on an artificial diet which did not contain GNA; lane 9 contains a known amount of GNA (1.5 μg).

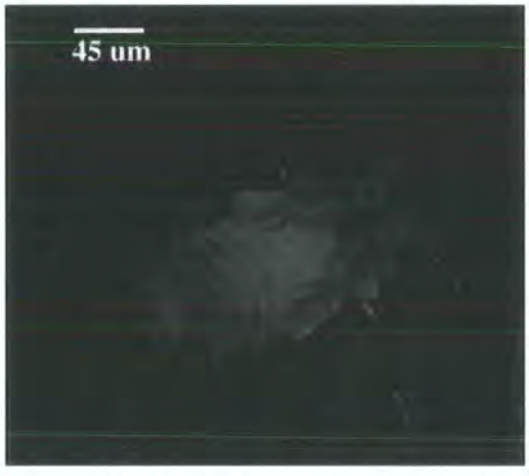

Fig. 2. An aphid fed on artificial diet containing 0.1% w/v FITC-labelled GNA for 2 days and viewed under UV light. Fluorescent regions indicate the presence of GNA within and around the gut region.

in the insect, and no accumulation of fluorescent material was observed.

3.2. Effects of GNA on 2-spot ladybird larvae

Two separate bioassays were carried out to estimate the effects of GNA on ladybird larvae. In the first bioassay, ladybird larvae in control and GNA-fed groups were offered equal numbers of aphids. Because of the difference in weights between aphids fed on control and GNA-containing diets, this meant that the group of ladybird larvae exposed to GNA were offered a lower total weight of aphids.

Exposure to GNA in this bioassay had no effect on the survival of ladybird larvae, as shown in Fig. 3. Survival analyses were performed at two time points during the trial. The first, on day 21, represents the time point when no control larvae remained (i.e. they had either died or entered the pupal stage); at this point not all GNA treatment larvae had reached pupation. Assuming those in the pupal stage were alive, no significant differences were found in survival between the two ladybird groups (p=0.56, Kaplan-Meier, Logrank χ^2 test). The second time point analysed was the day the trial was terminated and assumes that any pupae remaining were dead (since they had remained in the pupal stage for much longer than 7 days — the approximate length of pupation of this species when reared under our conditions); this analysis therefore reflects the numbers of adults alive at day 35. No significant differences in survival to this time point were found (Kaplan-Meier, Logrank χ^2, p=0.30) although a much greater number of the control group had reached adulthood and were still alive on this day compared to the treatment group.

The effects of GNA on instar duration of the ladybird larvae were monitored as a measure of development. No significant differences in the lengths of the 1st, 2nd and 3rd instars were found (Fig. 4(A)), with mean cumulative instar duration being approximately 3, 6 and 8 days from the 1st to 3rd instars inclusive. However, it was observed that larvae fed on GNA-dosed aphids spent a significantly greater number of days (approximately 6 extra days) in the 4th instar, before progressing to pupation, compared to those fed on control-dosed aphids (see Fig. 4(A)). The control larvae had taken a mean of 16 days to reach pupation compared to an average 22 days taken by the larvae fed on GNA-dosed aphids; this difference proved to be statistically significant (Mann-Whitney U-test, $p<0.01$). The number of adults successfully emerging from pupae was too small to be able to draw any meaningful conclusions as to possible effects of GNA on length of pupation and time taken to reach adulthood. As a measure of growth, the weights of surviving ladybird larvae were determined 24 h after moulting between the 3rd and 4th instars. Larvae fed on GNA-dosed aphids weighed less (mean 3.7±0.1 mg) than the control larvae (mean 4.4±0.1 mg); this difference was significant (unpaired t-test, $p<0.01$).

The numbers of aphids eaten by the ladybird larvae

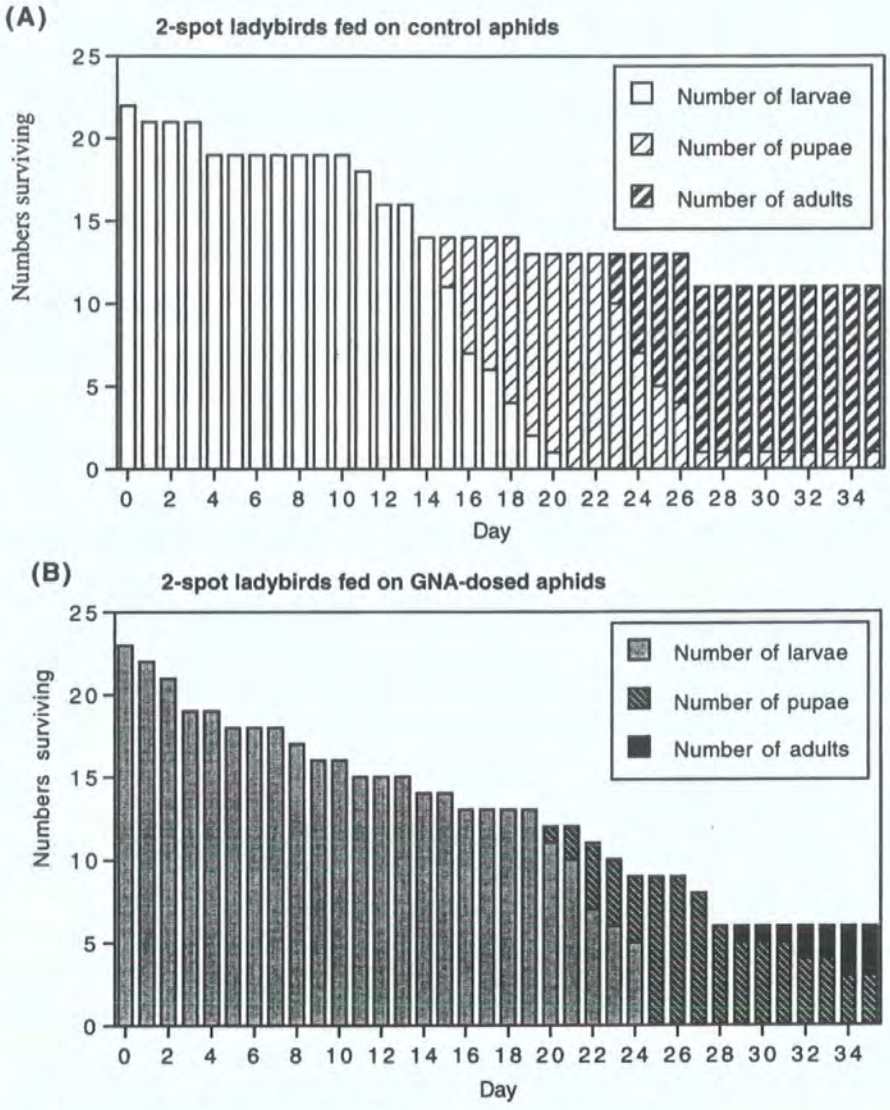

Fig. 3. Bioassay 1. Graphs showing the numbers of surviving larvae, numbers in pupation and numbers successfully emerging as adults of 2-spot ladybirds when fed on (A) control diet-dosed aphids and (B) GNA-dosed aphids.

throughout development, are shown in Fig. 5(A). Approximate cumulative means of aphids eaten during the 1st, 1st+2nd, 1st–3rd and 1st–4th instars are 13, 28, 50 and 127 for the control larvae compared to 15, 32, 59 and 194 for larvae feeding on the GNA-dosed aphids. No significant differences in cumulative number of aphids eaten, were observed between the two groups of larvae during the 1st–2nd instar period. By the end of the 3rd instar the differences in numbers of aphids eaten became significant (unpaired t-test, $p<0.05$). The significance proved even greater by pupation, due to the longer period spent in the 4th instar by larvae exposed to GNA (unpaired t-test, $p<0.01$).

In the second bioassay, ladybird larvae in control and GNA-fed groups were offered equal weights of aphids instead of equal numbers, because of the weight difference between control and GNA-fed aphids, as noted earlier. As with bioassay 1, no significant differences in the survival of ladybird larvae were observed (Fig. 6), as confirmed by survival analyses carried out at equivalent time points to those selected for bioassay 1 (end of control larval period, and end of assay; $p=0.53$ on day 27 and $p=0.33$ on day 38; Kaplan-Meier, Logrank χ^2 test). Although in this bioassay fewer numbers of controls successfully emerged as adults compared to the previous assay, by the end of the trial similar numbers of adults had successfully emerged from pupation and were still alive for both the control and treatment groups.

The effects of GNA exposure on duration of the final instar observed in bioassay 1 were not observed in this

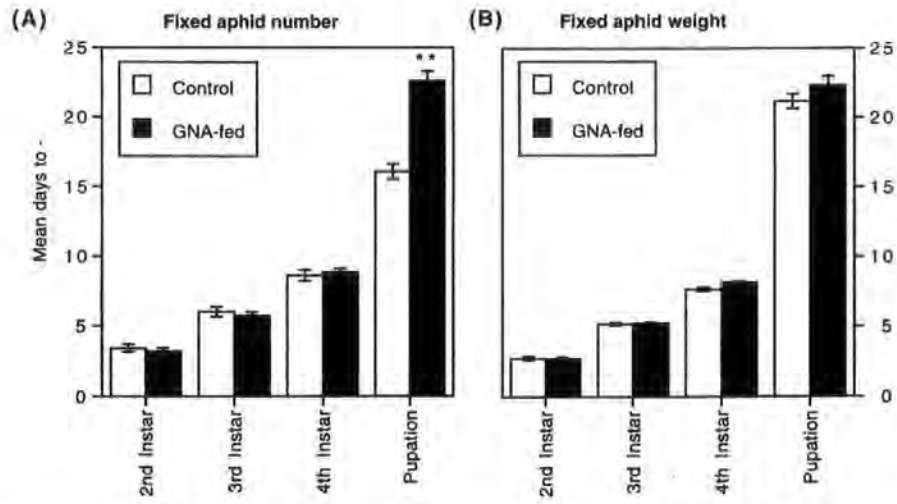

Fig. 4. Graphs showing the mean cumulative number of days taken to develop through the larval instars by 2-spot ladybirds fed on GNA-dosed and control diet-dosed *M. persicae* when fed on (A) a fixed aphid number (bioassay 1) and (B) a fixed aphid weight (bioassay 2) basis. **Significant difference (Mann-Whitney *U*-test, $p<0.01$).

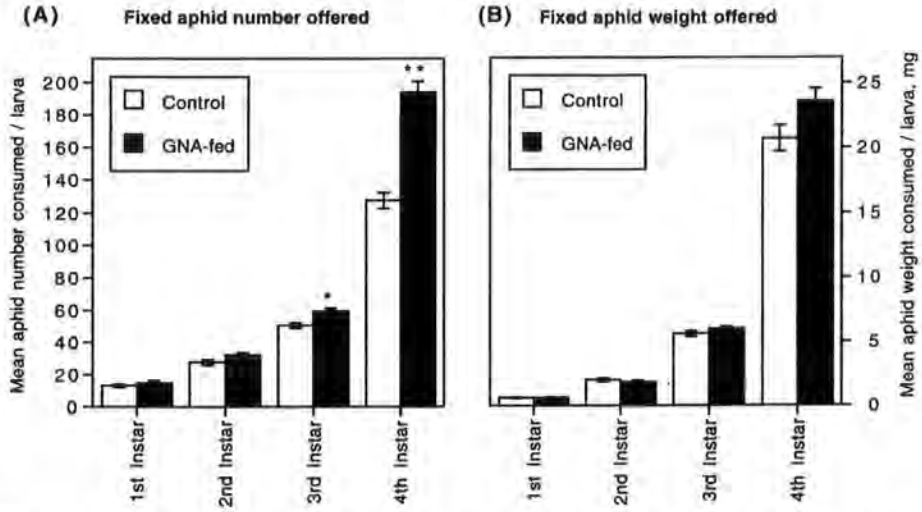

Fig. 5. Graphs showing the mean cumulative number of aphids eaten throughout the larval instars by 2-spot ladybird larvae fed on GNA-dosed and control-dosed *M. persicae* when offered (A) a fixed aphid number (bioassay 1) and (B) a fixed aphid weight (bioassay 2). *Significant difference at the $p<0.05$ level; **Significant difference at the $p<0.01$ level (unpaired *t*-tests).

bioassay (see Fig. 4(B)). As in the earlier bioassay, no significant differences in instar duration were observed for 1st and 2nd instars; mean cumulative number of days taken to 2nd instar were approximately 2.7 and to 3rd instar approximately 5.2 for both groups. However, a significant difference (Mann-Whitney *U*-test, $p<0.05$) was observed in the mean cumulative number of days to the 4th instar; the control group taking an average of 7.6 days whereas those fed on GNA-dosed aphids took an average of 8.1 days. By the time pupation had been reached, this difference had been lost with the control larvae taking an average of 21 days to reach pupation compared to the 22 days taken by the treatment group (see Fig. 4(B)).

Ladybird larval weights were measured initially and 24 h after the exoskeleton sloughed between 2nd/3rd instars and 3rd/4th instars had been observed. Significant differences in mean increase in larval weights, from hatch, were observed at both time points, with larvae fed on GNA-dosed aphids weighing less than the controls. In the 3rd instar, mean increase in control larval weight was 2.3±0.1 mg compared to 2.1±0.04 mg for the treatment group (unpaired *t*-test, $p<0.05$); and 4th instar mean control larval weight was 4.0±0.1 mg compared

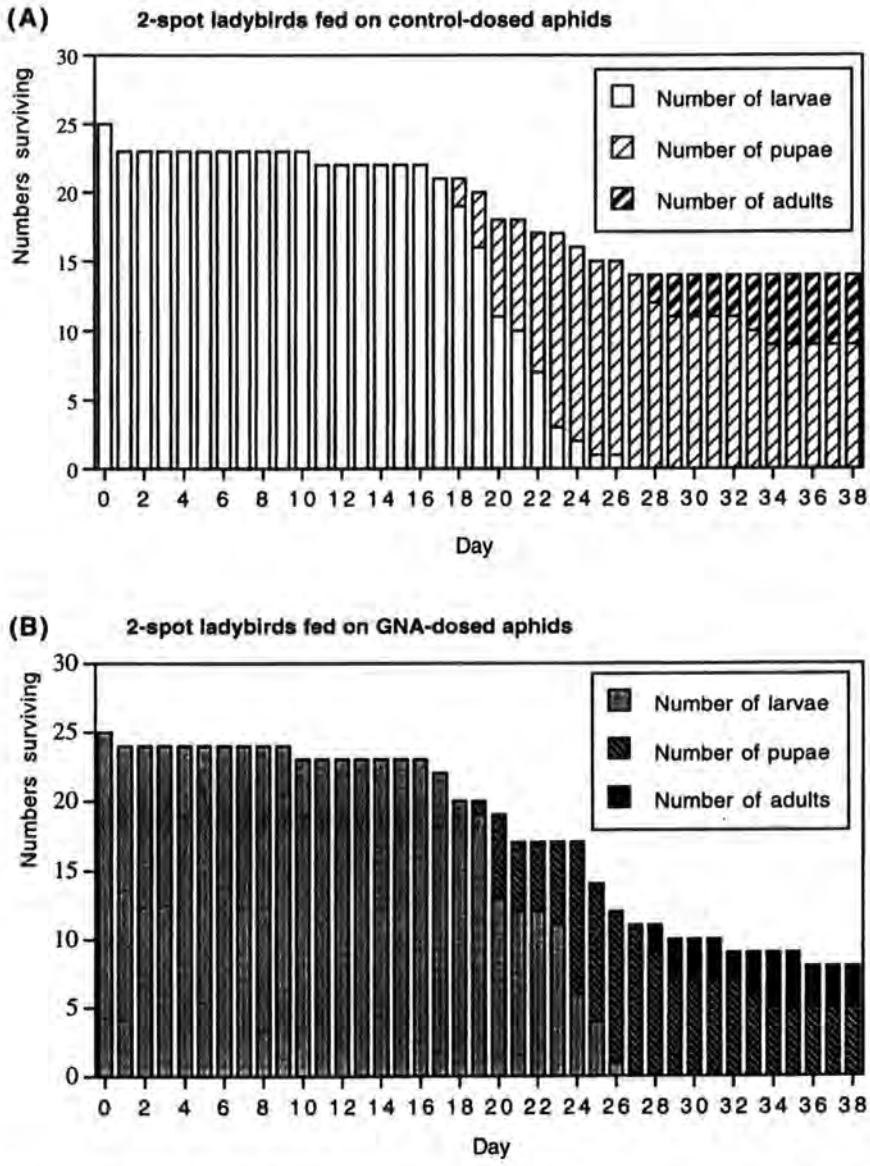

Fig. 6. Bioassay 2. Graphs showing the numbers of surviving larvae, numbers in pupation and numbers successfully emerging as adults of 2-spot ladybirds when fed on (A) control diet-dosed aphids and (B) GNA-dosed aphids.

to 3.7±0.1 mg for the treatment group (unpaired *t*-test, $p<0.05$). No significant differences were found in the initial weights of the ladybird larvae (data not shown).

No significant differences in the cumulative weight of aphids eaten were observed at any point during larval development (unpaired *t*-test). Total mean cumulative consumption throughout the larval stages ranged from 0.021 g for the control larvae compared to 0.024 g for the GNA-fed larvae (see Fig. 5(B)). However, from the 2nd instar the mean cumulative numbers of aphids eaten by larvae in the GNA-fed group are consistently and increasingly more than the control group; differences in means were 2.9 for 1st+2nd instars, 9.1 for 1st+2nd+3rd instars and 32.9 during total larval development. This once again indicates that the GNA-dosed aphids are smaller than the control-dosed aphids and ladybird larvae must eat greater numbers of GNA-dosed aphids to obtain the same weight of food.

3.3. Immunohistochemical studies on ladybird larval guts

Electron micrographs showing sections of the ladybird larval guts can be seen in Fig. 7. Due to the fact that an osmium fixative destroyed antigenicity of the samples, a non-osmium fixative was employed and hence ultras-

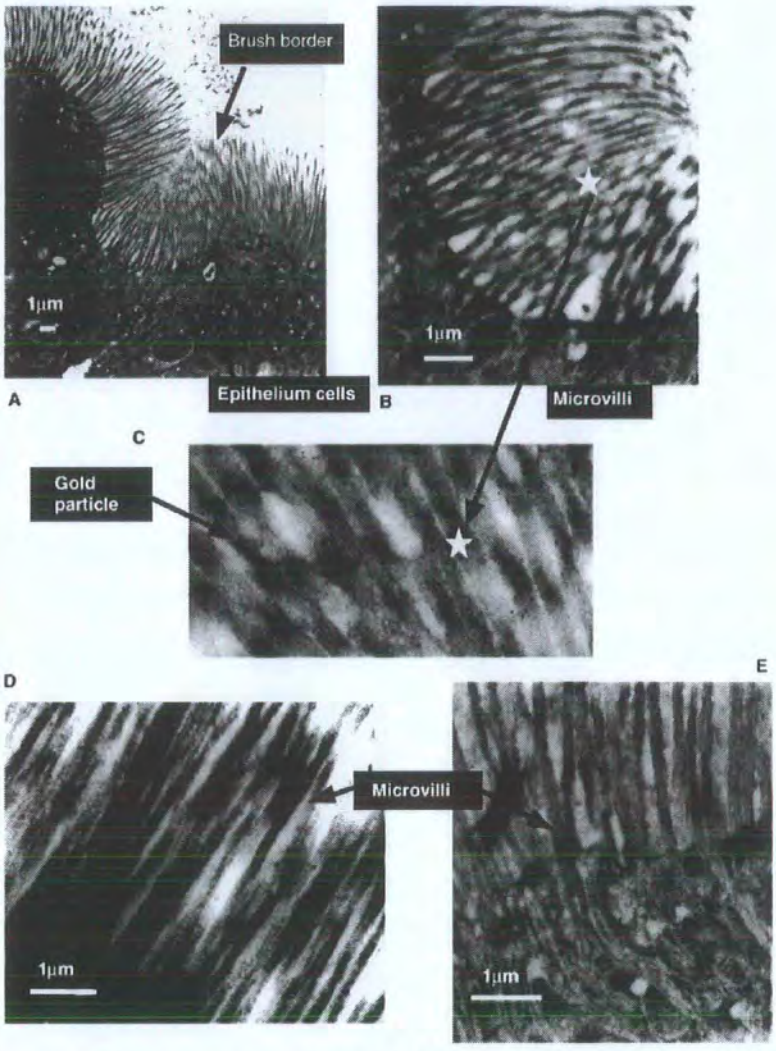

Fig. 7. Shows electron micrographs of the midgut region of 2-spot ladybird larvae: (A) at a magnification of 4600 shows some of the cell ultrastructure; (B) magnification 22,000, shows the presence of GNA binding to the microvilli and presence within the cells lining the gut lumen (as depicted by black dots representing the gold conjugated secondary antibody); (C) an enlargement of (B) to aid visualisation; (D) magnification 28,000, shows a section from a control fed and immunohistochemically stained ladybird larvae; (E) magnification 28,000, shows a section of GNA-fed larvae where the primary GNA-antibody was omitted from the staining procedure (note the absence of gold particles).

tructure is not so clear. However, the midgut ultrastructure shows normal cellular components in the epithelial cells, with a clearly visible brush border on the luminal surface. Exposure to GNA, through feeding on GNA-dosed aphids, had no discernable effects on the gut ultrastructure (Fig. 7(A)). GNA present in these ladybird larval guts was visualised by treatment with anti-GNA antibodies (raised against GNA expressed in *E. coli*; recombinant GNA) and gold-labelled secondary antibodies. In GNA-fed larvae, the lectin was observed binding to the microvilli of the brush border membrane and was also present within the cells lining the gut lumen (Fig. 7(B, C); enlargement to aid visualisation). Sections from control ladybird larvae (fed on control aphids), or sections of GNA-fed larvae where the primary antibody was omitted, showed no positive staining (gold particles) for GNA (Fig. 7(D, E), respectively).

3.4. GNA binding to ladybird larvae gut proteins

The presence of GNA-binding glycoproteins within the ladybird gut was investigated by probing a Western blot of ladybird gut protein with recombinant GNA. Polyclonal rabbit anti-GNA primary antibodies were used to detect bound GNA. Fig. 8 shows the presence of at least four different glycoproteins within the ladybird gut extract, which are capable of binding GNA.

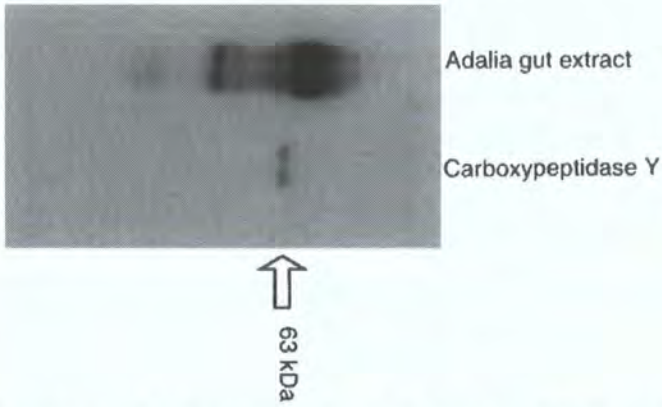

Fig. 8. Shows a Western blot of 2-spot ladybird larvae gut extract, incubated with recombinant GNA. Bands represent glycoproteins within the gut extract to which GNA will bind.

4. Discussion

Dosing aphids on an artificial diet containing 0.1% w/v GNA for 4 days, prior to feeding to the ladybird larvae, will deliver significant amounts of GNA to the aphid predator. However, the estimated value for the GNA content per aphid will include food present in the gut lumen. In the feeding trials, this may result in some of the GNA being excreted out in the aphid honeydew and therefore not entering the ladybird larvae when the aphids are consumed, especially if the aphids are not eaten soon after being offered to the ladybird larvae. This is more likely to be the case early in the feeding trial, when the ladybird larvae are small and do not consume aphids so quickly. Various lectins, including GNA can be detected in the honeydew of the pea aphid, *A. pisum*, when incorporated into an artificial diet (Rahbé et al., 1995). Thus, the dose of GNA per aphid determined by Western blotting may overestimate how much GNA is delivered to the ladybirds.

The accumulation of GNA within aphids shown in the present paper is in agreement with previous work, which has suggested that lectins are capable of binding to the gut epithelium within homopteran species, with internalisation occurring. For example, lectin binding to the gut surface was reported in the pea aphid, *A. pisum* when fed the lectin from jackbean, Con A (Sauvion, 1995). GNA is able to bind to midgut epithelial cells in other homopteran sap-suckers such as the rice brown planthopper, *Nilaparvata lugens*, and can be detected in the fat bodies, ovarioles and haemolymph, indicating that it is able to cross the midgut membrane (Powell et al., 1998). Binding of lectins to the gut surface has also been observed in Lepidopteran species (Law and Kfir, 1997), Dipterans (Eisemann et al., 1994), as well as Coleopteran species (Gatehouse et al., 1984). Feeding aphids on FITC-labelled GNA in the present paper also clearly showed that the lectin was internalised within the insect. The nature of the glycoproteins to which GNA binds in homopteran insects remains unknown.

The GNA-fed aphids deliver GNA to ladybird larvae feeding on them, as shown by immunohistochemical evidence of the presence of GNA in ladybird larval guts. GNA is resistant to proteolytic digestion (Van Damme et al., 1987) and thus its survival in the ladybird gut is not unexpected. Perhaps less expected is the observation that GNA binds to the microvilli of the brush border membrane, and is internalised into the epithelial cells, with a mechanism apparently similar to that observed for GNA uptake in the rice brown planthopper (Powell et al., 1998). However, the Western blot binding studies clearly show that glycoproteins which bind GNA are present within the ladybird gut. At least four different glycoproteins interacting with GNA were detected, one of which gave a strong signal; this could either be due to a high abundance within the ladybird gut and/or a high affinity towards GNA. Unlike the situation in the brown planthopper, disruption of the ladybird larval midgut brush border membrane was not observed during these investigations, indicating that perhaps GNA does not cause morphological damage to the epithelium. However, such damage could be dose-dependent, and so the absence of disruption may be because ladybird larvae used for sectioning had not consumed sufficient quantities of GNA. The larvae encountering GNA in the feeding trials will have consumed a greater amount of GNA (aphids were dosed for 4 days prior to feeding to the ladybirds) than those used for the binding studies (aphids were dosed for a maximum of 48 h). Other lectins have been observed to cause disruption of the gut in insect species; for example, Con A caused accelerated cell loss and increased shedding of the striated border in *A. pisum* (Sauvion, 1995). Whether or not morphological damage could be observed, the lack of toxicity of GNA towards

ladybird larvae observed in the present study is clearly not a consequence of the protein failing to bind to the gut epithelium.

From the data presented within this paper it is evident that GNA is not acutely toxic to ladybird larvae at the tritrophic level (i.e. via aphids fed on artificial diet), although some marginal effects on the developmental biology of the larvae were observed. In both bioassays no significant differences were observed in the survival of the ladybirds from egg to the end of larval development, but there was some evidence that GNA-fed insects showed poorer survival to adults, particularly in bioassay 1, although the overall survival curves were not significantly different. In both control and GNA-fed groups, most of the larvae were lost either during the early days of the trial or during the pre-pupal stage, when successful pupation did not always occur; this may be a consequence of the bioassay system used. The results compare well with work by Birch et al. (1999) where it was reported that GNA (fed via aphids feeding on transgenic plants expressing GNA) was not acutely toxic to adult ladybirds, although the longevity of the female adults was reduced by up to 51%.

Estimations of growth and development parameters for ladybird larvae do, however, suggest that the GNA-fed group suffer some adverse effects compared to the control group. In both bioassays 1 and 2 significant differences in ladybird larval weight were observed after ecdysis between the instars, with those feeding on the GNA-dosed aphids weighing less than those fed on the control-dosed aphids. There is also some evidence, especially from bioassay 1, that development times may be slightly extended in the GNA-fed group. However, two factors could contribute to these effects. First, they could be a direct effect of the GNA which the aphid prey delivers to the ladybird larva; second, they could be an indirect effect — a result of the effects of GNA on the aphid itself, rendering it a suboptimal food source for the predator. The effects of GNA on *M. persicae* observed in this work are comparable to previous data showing that GNA-fed aphids were significantly smaller than control-fed ones, as a result of slower development (Down et al., 1996). In bioassay 1 ladybird feeding was based on identical numbers of aphids, and thus the GNA group were actually receiving less food on a daily basis compared to the control group. This difference could easily account for the lower weight of larvae, and the delay of approximately 5 days in the onset of pupation. If pupation is occurring when larvae have reached a threshold weight, then those fed a smaller weight of aphids will take longer to reach that threshold. Significantly, in bioassay 2 where the ladybird larvae were fed on a weight basis so that both GNA and control groups were fed equal weights, this delay in the onset of pupation was not seen. Although a significantly longer 3rd instar was observed, any deleterious effect on development had been lost by the time pupation was reached. However, larvae in the GNA-fed group were still approx. 10% smaller in terms of weight in the 3rd and 4th instars in bioassay 2, although this could still be a result of the indirect effects of GNA.

The data on aphid consumption in these bioassays supports the conclusions that any deleterious effects of GNA are indirect. In bioassay 1, from the 3rd instar onwards significantly more aphids were consumed by the ladybirds feeding on GNA-dosed aphids. In the early part of the trial, the amount of aphids supplied to the larvae exceeds the number that are required, so not all are eaten. However, because the GNA-dosed aphids are significantly smaller than the control aphids, ladybird larvae in the GNA-treatment group are having to consume a greater proportion of the aphids provided in order to obtain the same food intake compared to the control group. By the time the ladybird larvae reach the 4th instar, the number of aphids provided to either group on a daily basis is less than the number which would be consumed if fed *ad libitum* (although, obviously, not so much less as to be detrimental to normal development), since all the aphids provided are consumed. Once again, the fact that the GNA-dosed aphids are smaller and probably a suboptimal food source, leads to the GNA-fed group receiving less nutritional resources than the control group; as a consequence, the GNA-fed larvae take an extra 5 days to reach pupation, and thus consume more aphids during this period. In bioassay 2 ladybird larvae were offered equal weights of aphids; however, the GNA-fed larvae would have had to have eaten more aphids (and thus use more energy) to obtain the same nutritional resource. As expected, no significant differences in consumption by weight were observed between the GNA-fed and control groups, but the GNA-fed group consumed more aphids, since they were offered more in order to equalise the weights of food in the two groups. The necessity to eat more aphids in order to obtain the organism's nutritional requirements could account for the small reduction in ladybird larval weight observed in the GNA-fed group in this assay; it is noteworthy that the weight reduction in this assay was only approx. 10%, half that observed in bioassay 1. Later in the assay the control and GNA-fed larval groups become equally limited by the supply of aphids, and overall development is very similar between the two groups.

These assays give a valuable insight as to the possible effects of GNA, if it were to be used in transgenic crops to enhance aphid resistance, against predatory ladybirds at the tritrophic level. From the data presented within this paper, it is unlikely that GNA has significant direct effects upon the survival or development of ladybird larvae. The marginal indirect effects on growth and development that have been observed in the bioassays are likely to be a result of aphids feeding on GNA being compromised and nutritionally sub-optimal, thus provid-

ing a poorer diet for the ladybirds compared with aphids which have not fed on GNA. The artificial diet method for loading aphids with GNA employed in these investigations did not allow aphids to be provided *ad libitum* to the ladybird larvae throughout the trial period. However, in a field situation, aphids are not likely to be in short supply and it is hypothesised from this data that no effects (direct or indirect) will be seen on ladybird larvae feeding on plants expressing GNA, so long as aphid populations are at a level sufficient to sustain them. Further work will be required to test this hypothesis; however, from the present data it may be concluded that in this example of a potential transgenic plant–pest–predator tritrophic interaction, no evidence for deleterious effects of the transgene-encoded resistance factor (GNA) on the predator, over and above its effect on the pest, can be found. This is in contrast to the direct harmful effects seen against ladybird predators seen with the use of most chemical pesticides.

Acknowledgements

The authors thank the European Commission (Project BIO4-CT96-0365), the Scottish Office (SOAEFD Programmes FF821 and FF818) and the Department of Biological Sciences, University of Durham for funding which supported the work described in this paper. We also thank Dr Christine Newell (Axis Genetics) for supplying antibodies; Dr Mike Majerus (University of Cambridge) and Dr G. Marris (Central Science Laboratories, MAFF, York) for supplying 2-spot ladybirds; and Mrs C. Richardson (University of Durham) for technical help with the electron microscopy and immunohistochemical methods.

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Pergamon

Journal of Insect Physiology 47 (2001) 777–787

Journal of Insect Physiology

www.elsevier.com/locate/jinsphys

In vitro and in vivo binding of snowdrop (*Galanthus nivalis* agglutinin; GNA) and jackbean (*Canavalia ensiformis*; Con A) lectins within tomato moth (*Lacanobia oleracea*) larvae; mechanisms of insecticidal action

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Received 18 August 2000; received in revised form 23 December 2000; accepted 10 January 2001

Abstract

When fed in semi-artificial diet the lectins from snowdrop (*Galanthus nivalis*: GNA: mannose-specific) and jackbean (*Canavalia ensiformis*: Con A: specific for glucose and mannose) were shown to accumulate in vivo in the guts, malpighian tubules and haemolymph of *Lacanobia oleracea* (tomato moth) larvae. Con A, but not GNA, also accumulated in the fat bodies of lectin-fed larvae. The presence of glycoproteins which bind to both lectins in vitro was confirmed using labelled lectins to probe blots of polypeptides extracted from larval tissues. Immunolocalisation studies revealed a similar pattern of GNA and Con A binding along the digestive tract with binding concentrated in midgut sections. Binding of lectins to microvilli appeared to lead to transport of the proteins into cells of the gut and malpighian tubules. These results suggested that both lectins are able to exert systemic effects via transport from the gut contents to the haemolymph across the gut epithelium. The delivery of GNA and Con A to the haemolymph was shown to be dependent on their functional integrity by feeding larvae diets containing denatured lectins. Con A, but not GNA, was shown to persist in gut and fat body tissue of lectin-fed larvae chased with control diet for three days. Con A also shows more extensive binding to larval tissues in vitro than GNA, and these two factors are suggested to contribute to the higher levels of toxicity shown by Con A, relative to GNA, in previous long term bioassays.

Keywords: GNA; Con A; *Lacanobia oleracea*; Lectin-binding; Immunohistochemical localisations

1. Introduction

In recent years genetic engineering has made a growing contribution within integrated pest management systems towards global agricultural production. Insect resistant transgenic crops have now been produced for most of the world's economically important crops including maize, cotton, rice and potatoes. Although most attention has focussed on insect resistance genes of bacterial origin, primarily those encoding *Bacillus thuringiensis* toxins, other sources of potentially insecticidal gene products have also been examined, including the defensive proteins of plants. Although a full explanation of the roles of lectins (carbohydrate-binding proteins or agglutinins) in plants has not yet been made, it is clear that some lectins, at least, have insecticidal properties, and thus genes encoding lectins have been suggested for protection of transgenic crops against insect pests. A transgenic crop has to meet both grower and public approval to be economically viable. Thus in order to allow full exploitation of the potential of this technology a thorough understanding of the mechanisms of action of insecticidal proteins such as lectins is essential.

The snowdrop lectin GNA (*Galanthus nivalis* agglutinin) has been shown to be insecticidal to a range of economically important pests (Powell et al., 1993;

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0022-1910/01/$ - see front matter.
PII: S0022-1910(01)00068-3

Rahbé et al., 1995; Hilder et al., 1995; Sauvion et al., 1996; Gatehouse et al., 1996, 1997; Down et al., 1996; Rao et al., 1998; Stoger et al., 1998). As such, it has obvious potential as an insect control agent although knowledge as to the mechanisms of GNA action is limited. In mammals toxic lectins such as *Phaseolus vulgaris* (PHA) bind to suitably glycosylated targets in the gut causing damage to the integrity of the intestinal brush border (BB) membrane (Pusztai et al., 1990, 1995). Similarly, ultrastructural studies have shown insecticidal lectins to be bound to midgut epithelial cells in a variety of pest species (Powell et al., 1993, 1998; Habibi et al., 1998, 2000). In some cases damage to the microvilli and structure of the BB epithelium, such as that observed by Powell et al. (1998) in the Homopteran *Nilaparvata lugens* fed on a toxic dose of GNA, have led to suggestions that binding may be a causative factor in the toxicity of a wide range of lectins to insects. However, whilst a correlation between the strength of lectin binding to the brush border epithelium and antinutritive effects has been demonstrated in rats (Pusztai et al., 1990) no such relationship has been observed in insects. The sensitivity of different insect species to the effects of lectin ingestion is variable, and binding of a particular lectin to the gut of a given species does not necessarily facilitate insecticidal activity (Harper et al., 1995). The peritrophic matrix (PM) may act as an additional target in many insects for lectin binding. Eisemann et al. (1994) concluded that blockage of the PM pores by wheatgerm, lentil and jackbean lectins upon blowfly larvae (*Lucina cuprina*) was at least in part responsible for observed starvation effects. Wheatgerm lectin has also shown to bind to the PM of the Lepidopteran *O. nubilalis*, resulting in reduced larval growth (Harper et al., 1998). Furthermore, the potential of lectins to exert direct and/or indirect effects in insects, as they do in mammals, has recently been suggested by immunolocalisation studies showing GNA to be present in the haemolymph, fat bodies and ovarioles of GNA-fed rice brown planthopper (*N. lugens*) (Powell et al., 1998).

GNA, strictly specific for α-D-mannose and jackbean lectin (*Canavalia ensiformis*; Con A) which binds both glucose and mannose residues, have been shown to exert detrimental effects upon larvae of the tomato moth (*Lacanobia oleracea*), a member of the economically important noctuid group of Lepidopteran pests (Fitches et al., 1997; Gatehouse et al., 1997, 1999). Comparable bioassays have shown Con A to be the more toxic of the two lectins towards *L. oleracea* larvae. Both lectins have previously been shown to affect the activities of soluble and BBM enzymes in the midguts of *L. oleracea* larvae (Fitches and Gatehouse, 1998). In addition Con A was shown to bind to many BBM and PM proteins in vitro whereas GNA showed more specific binding. Correspondingly higher levels of Con A compared to GNA were found to be present in vivo in gut tissues of insects chronically exposed to lectin containing diets.

The aims of this study were to compare, at the ultrastructural level, the ability of GNA and Con A, with differing sugar specificities and degrees of toxicity, to bind to the alimentary tract of *L. oleracea* larvae. To further our understanding of possible modes of lectin action, the ability of GNA and Con A to bind, accumulate and persist in various sites within the insect, including the circulatory system, has been examined and compared. Possible explanations for the increased toxicity of Con A as compared to GNA towards *L. oleracea* are discussed.

2. Materials and methods

2.1. Materials

Snowdrop lectin (GNA) used for immunocytochemistry and insect bioassay 1 was supplied by Drs. W. Peumans and E. van Damme, Catholic University of Leuven, Belgium. The purity of GNA was estimated by spectrophotometry to be >90% and shown to be functionally active by haemagglutination assays. GNA used in insect bioassays 2 and 3, and jackbean lectin Con A was supplied by Vector Laboratories Inc, 30 Ingold Rd, Burlingame, CA 94010, USA. Secondary gold conjugated antibodies, goat serum, nickel grids (150 mesh, hexagonal) and LR White resin were purchased from Agar Scientific, British Biocell Int, Essex, UK. Polyclonal anti-Con A antiserum was supplied by Sigma Chemicals Co, St. Louis, USA, and polyclonal anti-GNA antiserum was raised by Genosys Biotechnologies, Cambridge, UK. Antibodies were tested for specificity prior to immunolabelling by Western blotting. Analytical reagents for gel electrophoresis were obtained from Northumbria Biologicals, UK, the DIG (digoxigenin-labelled) Glycan Differentiation Kit from Boehringer Mannheim, Biochemica and the ECL (enhanced chemiluminescence) kit from Amersham, UK. All other chemical reagents, except when referenced otherwise, were supplied by B.D.H. Chemicals Ltd, Poole, Dorset, UK, or Sigma Chemicals Co, and were of analytical or best available grade. Potato leaf powder was prepared by freeze-drying leaf material from plants raised under standard growth room conditions (20°C; 12 h day); leaves were frozen in liquid air immediately after harvesting.

2.2. Insect cultures

Larval cultures of *L. oleracea*, originally obtained from Central Science Laboratory (Sand Hutton, MAFF, York, UK,), were reared continuously, at the University of Durham (Science Laboratories, South Rd, Durham,

UK), on optimal artificial diet (Bown et al., 1997) at 25°C under a 16:8 L:D regime. In all assays larvae were placed on a potato leaf based artificial diet (Fitches et al., 1997) with or without either lectin. Although suboptimal this diet does not contain additional lectins or sugars that might mask the effects of GNA or Con A. In all instances GNA or Con A was incorporated at a single concentration of 2% total dietary protein dry weight (3.6 mg of GNA/Con A per 0.5 g dry weight of diet). The control diet was supplemented with an equivalent weight of casein to account for the extra protein (i.e. GNA or Con A) added to experimental diets.

2.3. Methods

2.3.1. In vitro detection of lectin binding proteins

Pre-chilled fifth instar larvae were dissected over ice. Intact guts were extracted following the removal of contents and peritrophic membranes (PM) by flushing with ice-cold extract buffer (50 mM Tris–HCl pH 7.5, 1 mM dithiothreitol (DTT)). Foregut, midgut and hindgut tissue was removed from 20 larvae under a dissecting microscope (model Nikon SM 2–1). Malpighian tubules were teased from the hindgut and the layer of fat bodies lying beneath the larval cuticle was removed using a scalpel. Following washes with extract buffer to remove contaminating tissue and haemolymph, samples were placed in extract buffer and stored at −20°C. Crude extracts were prepared by homogenisation using a TRI-R (Model S63C) and centrifugation for 15 mins at 4°C (Beckmann 12 000 rpm). Supernatants were removed and stored at −20°C. Haemolymph samples were obtained from pre-chilled fifth instar larvae (blotted with ethanol and dried prior to extraction) by piercing the cuticle with a fine needle. Droplets of extruded haemolymph were placed into pre-chilled eppendorfs dusted with phenylthiocarbamide–phenol oxidase inhibitor (PPO) to prevent melanisation. The protein content of all samples was analysed using a microtitre plate-based Bradford Assay (Biorad), using BSA as the standard protein. Duplicate samples were boiled in the presence of 4×SDS and β-mercaptoethanol prior to separation by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins (20 μg aliquots) on one gel were visualised by Coomassie staining, and proteins Proteins (10 μg aliquots) on the second were electroblotted onto nitrocellulose filters (Schleicher and Schuell BA85) and probed for the presence of GNA and Con A binding proteins using the DIG Glycan Differentiation kit according to the manufacturers instructions.

2.3.2. Immunocytochemistry for electron microscopy

Fourth instar larvae were exposed for 48 hours to control diet or diet containing GNA or Con A. Foregut, midgut, hindgut and malpighian tubules were subsequently dissected from pre-chilled larvae as previously described. Sections were placed into heptane-loaded fixative of 3% (v/v) paraformaldehyde, 1.25% gluteraldehyde (v/v) in 50 mM phosphate buffered saline (PBS) for 30 mins and then placed in fixative without heptane overnight at room temperature. Samples were then dehydrated using a graded series of ethanol embedded in LR-White resin according to the manufacturers instructions. Ultra-thin sections (60–80 nm) were cut with a Leica Ultratome and mounted on formvar and carbon coated nickel 150 hexagonal grids. Sections were labelled in the following manner: 10 mins room temperature incubation in heat inactivated serum; overnight incubation at 4°C in anti-GNA or anti-Con A antibodies (dilution 1:100) in Tris buffer (20 mM) pH 7.5; 4×5 mins washes with Tris buffer pH 7.5 and 1×5 mins wash with Tris buffer (20 mM) pH 8.2; 1 hour incubation at room temperature in anti-rabbit gold conjugated secondary antibody (dilution 1:20) in Tris buffer pH 8.2; 1×5 mins wash with Tris buffer pH 8.2 and 4×5 mins washes with distilled water. Sections were then stained for 10 mins each with uranyl acetate and lead citrate, with 5×5 mins washes with distilled water between and after staining. Sections were air-dried in a dust free environment and viewed on a Phillips 400T transmission electron microscope.

2.3.3. Short term bioassay 1: in vivo detection of lectin binding proteins

Newly eclosed fourth instar larvae were placed on control potato leaf based artificial diet for 24 hours prior to placing on experimental diets. Larvae were exposed to control diet or diet containing GNA or Con A for five days with daily changes of diet. For each treatment four larvae were placed into five clear plastic pots to give 20 larvae per treatment and five replicates per treatment. Larval wet weights (±0.1 mg) were recorded prior to the onset of the trial and prior to dissection. Larval consumption was analysed per replicate on a dry weight basis as previously described (Fitches et al., 1997).

Midgut, hindgut, malpighian tubule, fat body and haemolymph samples were dissected and prepared for SDS–PAGE as described in Section 2.3.1. Dissected tissues were pooled (four larvae per sample) to give five replicates per treatment and analysed for the in vivo presence of either lectin by running equal concentrations of protein on SDS–PAGE gels. Proteins Proteins (20 μg aliquots) were electroblotted onto nitrocellulose and probed for the presence of lectins by ECL detection, carried out according to the manufacturer's protocol, using polyclonal anti-GNA (1:3300 dilution) or anti-Con A antibodies (1:10 000 dilution). Simultaneously run replicate gels were run to check equal loading of proteins. Levels of lectin were estimated visually.

2.3.4. Short term bioassay 2: dynamics of lectin uptake and persistence in vivo

To investigate the uptake and persistence of GNA and Con A in various larval tissues a short term feeding trial

was carried out. Newly eclosed fifth instar larvae were starved overnight (to encourage immediate consumption of diet) prior to placing on control diet or diet containing GNA or Con A. For each lectin treatment four larvae were placed into eight clear plastic pots to give 24 larvae per treatment. Following exposure for 2 hours; 6 hours; 24 hours and 48 hours to the diets, guts, malpighian tubules, fat bodies and haemolymph samples were dissected from four larvae per lectin treatment (at each time point). The remaining eight larvae were exposed to lectin containing diets for a total of 72 hours and then placed on control artificial diet. After 24 hours and 72 hours of exposure to control diet samples were dissected, as before, from four larvae per lectin treatment. Larval weights and diet consumption (wet weight) were analysed to allow estimates of the amount of lectin ingested by each larvae at each time point to be calculated. Pooled samples (four larvae per time point) were prepared and analysed for the presence of GNA or Con A by SDS–PAGE and ECL detection as described in Section 2.3.1. Faecal samples were collected, prepared as described for larval tissue samples, and analysed for the presence of GNA or Con A. Diet samples were also taken and similarly analysed to confirm the comparability of dietary lectin concentration by Western blotting and ECL detection.

2.3.5. *Short term bioassay 3: exposure to denatured lectins*

The dependancy of ingested GNA and Con A delivery to larval haemolymph upon lectin binding to glycosylated gut proteins was investigated by exposing larvae to diets containing denatured GNA and Con A (2% total dietary protein dry weight). Lectins were denatured by boiling for 1 hour; Con A was boiled in the presence of 1% (w/v) SDS. Prior to diet preparation boiled GNA was shown to be non functional by microtitre haemagglutination assay (results not shown). Larval midgut and haemolymph samples were dissected from larvae exposed to the boiled lectin treatments for 48 hours (eight per treatment) and analysed for the presence of each lectin as previously described.

2.3.6. *Statistical analysis*

Data analysis was carried out using the Statview (v. 4.5, Abacus Concepts, Berkely, Ca USA) software package on Apple Macintosh computers. The acceptance level of statistical significance was $P<0.05$ in all instances. ANOVA analysis and subsequent Fishers PLSD (probability least significant difference) tests were carried out to determine any significant differences between larval weights and consumption between treatments.

3. Results

3.1. *In vitro detection of larval GNA and Con A binding glycoproteins*

Proteins were extracted from foregut, midgut, hindgut, fat body, malpighian tubules and haemolymph of *L. oleracea* larvae, separated by SDS–PAGE and blotted. Glycopolypeptides that interacted with GNA and Con A were detected by probing with digoxigenin labelled lectins. Results presented in Fig. 1(A) and (B) show that

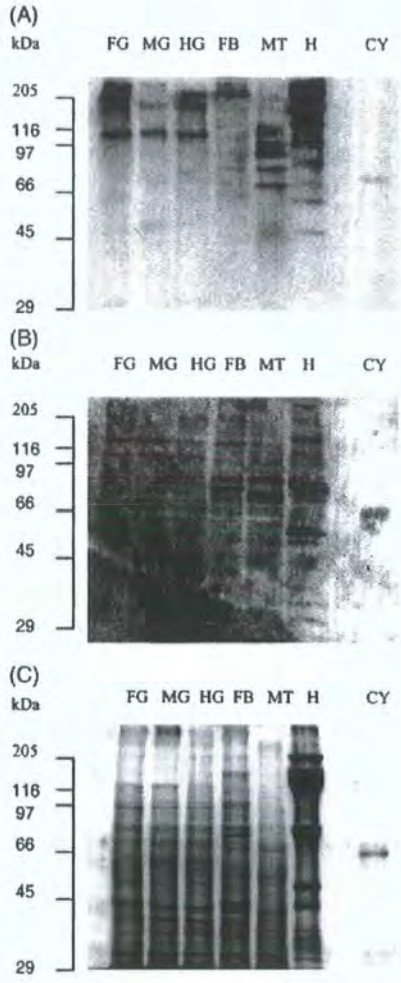

Fig. 1. Binding of GNA (A) and Con A (B) to *L. oleracea* proteins in vitro. Protein extracts, pre-boiled in the presence of (10%) β-mercaptoethanol, were analysed by SDS–PAGE (10% acrylamide gels), blotted onto nitrocellulose and probed with digoxigenin labelled GNA (A) or Con A (B). Bound lectin was detected using alkaline phosphatase-labelled anti-dioxigenin antibodies followed by incubation in CSPD (chemiluminescent substrate) and exposure to X-ray film. (C) shows a replicate Coomassie stained SDS–PAGE gel. 10 μg aliquots of protein were loaded in (A), (B) and (C) as follows: Lane 1 (FG) fore-gut, Lane 2 (MG) mid-gut, Lane 3 (HG) hind-gut, Lane 4 (FB) fat body, Lane 5 (MT) malpighian tubule, Lane 6 (H) haemolymph, Lane 7 control (CY) glycoprotein carboxypeptidase Y (A and B 0.25 μg; C 1.0 μg).

both GNA and Con A exhibit binding to glycoproteins present in all of the analysed samples, with Con A showing more extensive binding in all samples than GNA. This indicated that all analysed tissues are potential targets for GNA and Con A binding in vivo. Membrane glycosylation, with respect to glucose and mannose moieties, did not appear to differ significantly along the digestive tract as both lectins exhibited similar binding characteristics to fore-, mid-, and hindgut extracts. GNA bound predominantly to two uncharacterised glycopolypeptides (approx. 100 kDa and 200 kDa) in all gut samples and to a third high molecular weight glycoprotein of the same size (>205 kDa) in foregut and midgut samples but to a smaller species (approx. 205 kDa) in the hindgut sample. Con A also bound predominantly in all gut samples to a 100 kDa protein but also exhibits binding to a 120 kDa protein previously characterised as aminopeptidase (Fitches, 1999) and an abundant uncharacterised glycoprotein of approx. 62 kDa. Differences in the binding potential of the two lectins are most apparent in the fat bodies where Con A shows binding to at least three glycoproteins and GNA predominantly to a single high molecular weight glycoprotein.

3.2. Localisation of lectin binding in larval tissue by electron microscopy

Immunohistochemical localisations of GNA and Con A at the cellular level were carried out on foregut, midgut, hindgut and malpighian tubule sections prepared from fourth instar larvae fed for 48 hours on either control diets or diets containing GNA or Con A. Localisations were detected as electron-dense gold particles on sections from GNA and Con A fed insects after treatment with anti-GNA and anti-Con A primary antibodies and gold-labelled secondary antibodies. Identically processed samples from insects fed on control diet were used as controls.

Ultrastructural studies revealed a similar pattern of GNA and Con A binding along the digestive tract of *L. oleracea* larvae, with the greatest amount of binding observed in midgut sections (Figs. 2 and 3). Both lectins exhibited binding to the microvilli. An absence of staining observed in control midgut sections (exemplified in Fig. 4) demonstrated the specificity of antibodies used to detect the bound lectins. Binding of both GNA and Con A was also apparent in malpighian tubule sections (Figs. 5 and 6). Again, control sections showed no binding of either the anti-GNA or anti-Con A antibodies. Neither lectin appeared to have a detrimental effect on the ultrastructure of any of the gut regions; in particular no disruption of the integrity of the microvilli was observed. However, gold particles were reproducibly observed to be present in the contents of cells lining the gut and (to a lesser extent) the malpighian tubules, and their distribution implied a transport process by which lectin bound to the surface of the cell adjacent to the gut lumen was internalised, and moved across the cell to the surface opposite the gut lumen, from which it could potentially be released into the haemolymph.

3.3. Detection of lectins in insect tissues after short term feeding

Larvae in the second day of the fourth instar were transferred to experimental diets and exposed to GNA and Con A diets for five days. Under these conditions, the inclusion of lectins at 2% total dietary protein had no effect on survival, growth or consumption. Controls and larvae fed on GNA or Con A exhibited increases of 91.1 mg, 97.8 mg and 98.9 mg, in mean wet weight (n=20), respectively. Similarly control, GNA- and Con A-fed larvae consumed respectively 1.106 g, 1.166 g, and 1.180 g total dry weight of artificial diet.

The presence of GNA and Con A in polypeptides extracted from midgut, hindgut, malpighian tubules, fat bodies and haemolymph, of larvae exposed to the lectins for five days was detected by Western blotting. For each treatment five replicates were analysed. Both lectins were detected in all of the replicate samples analysed and no major differences in the levels of lectins were observed between replicate samples (results not presented; see Figs. 7 and 8 for comparable data). GNA and Con A were most abundant (on a per μg protein basis) in midgut extracts and least abundant in haemolymph samples. Notably higher levels of Con A were detected in fat body extracts from Con A-fed larvae than the levels of GNA present in equivalent samples prepared from GNA-fed larvae. Lower levels of Con A were detected in haemolymph samples compared to equivalent GNA samples. These results suggested that comparable amounts of the two lectins were transported to the circulatory system but that Con A has a greater binding affinity for fat body glycoproteins whereas GNA has a greater affinity for haemocyte and/or plasma glycoproteins.

3.4. Dynamics of lectin binding and persistence in larval tissues

The rate at which lectins were accumulated in insect tissues, and their persistence, was investigated by a pulse-chase experiment. GNA and Con A were fed to insects for varying times (2–48 hours) and the presence of the lectins in polypeptides extracted from midgut, malpighian tubules, fat bodies and haemolymph was analysed by Western blotting. The persistence of lectin bound to tissues was investigated by sampling larvae that had been fed for 72 hours on lectin-containing diets and subsequently chased with control diet for 24 and 72 hours. For each treatment, at each time point, pooled

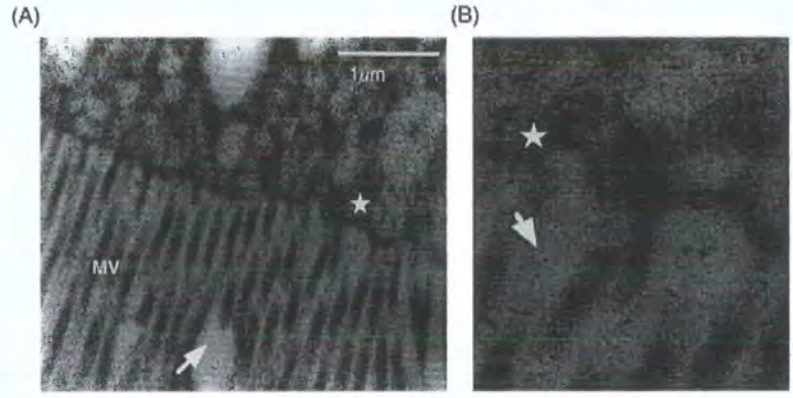

Fig. 2. Localisation of GNA in *L. oleracea* larvae fed for 48 hours on diet containing GNA by immunolabelling at the electron microscope level. (A) Midgut section showing intact microvilli (MV) and cytosol, labelling by gold-conjugated antibodies is observed along the microvilli and within the epithelial cytosol. (B) Shows an enlarged region of (A) denoted by a white arrowhead.

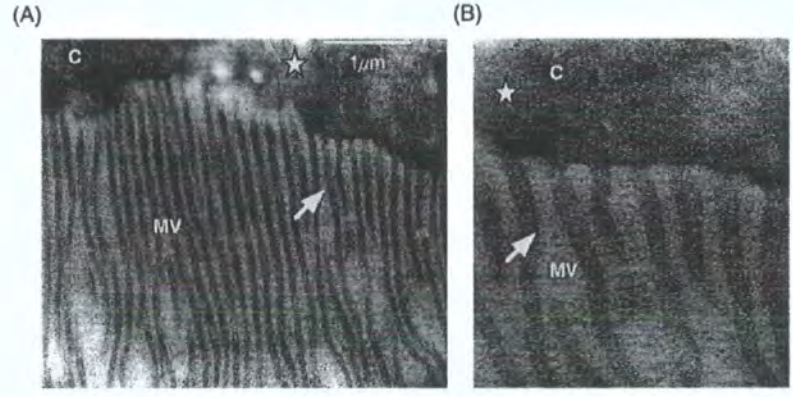

Fig. 3. Localisation of Con A in *L. oleracea* larvae fed on diet containing Con A by immunolabelling at the electron microscope level. (A) Midgut section showing intact microvilli (MV) and cytosol (C), labelling by gold-conjugated antibodies is observed along the microvilli and within the epithelial cytosol. (B) shows an enlarged region of (A) denoted by a white arrowhead.

samples (dissected from four larvae) were analysed for the presence of GNA and Con A. Summarised results for polypeptides extracted from tissues of larvae fed on GNA-containing diet are presented in Fig, 7(A) and (B), and for polypeptides extracted from tissues of larvae fed on Con A-containing diet in Fig. 8(A) and (B). No significant differences were observed in the levels of lectin present in larval tissues after 6 hours exposure to GNA or Con A, or between samples collected from larvae chased for 24 hours and 72 hours with control diet. Thus results are presented, for clarity, only for tissue samples collected after 2 hours and 48 hours feeding with lectins, and after 72 hours of chase with control diet.

Both GNA and Con A were detectable in midgut, malpighian tubule, fat body and haemolymph, samples dissected from larvae fed lectin-containing diet for just 2 hours. Estimates of the amount of lectin consumed at this time point were similar for the two lectins (14.4 μg (SE±1.9) for GNA and 18.6 μg (SE±2.9) for Con A fed larvae). Following 48 hours of exposure to lectin-containing diets comparable levels (approx. 0.25% of total proteins) of GNA (Fig. 7(A) and (B)) and Con A (Fig. 8(A) and (B)) are seen to be present in midgut and malpighian tubule extracts. Higher levels of GNA were detected in haemolymph samples extracted from larvae exposed to the lectin for 48 hours (Fig. 7(A)) as compared to equivalent Con A samples (Fig. 8(A)). Also notable is the very low level of GNA present in fat body (<2.5 ng/20 μg protein; Fig. 7(B)) extracts of larvae exposed to the lectin for 48 hours as compared to levels of Con A (10–50 ng/20 μg protein; Fig. 8(B)). These results were in agreement with those obtained in the short-term feeding experiment.

Larvae fed lectin-containing diets for 72 hours consumed similar amounts of GNA and Con A (347 μg (SE±15.6) and 328 μg (SE±45.6) for GNA and Con A fed larvae respectively). When chased with control diet for a further 72 hours total mean consumption of the control diet was also similar for the two lectins (0.399 g (SE±0.05) and 0.491 g (SE±0.03) wet weight for GNA

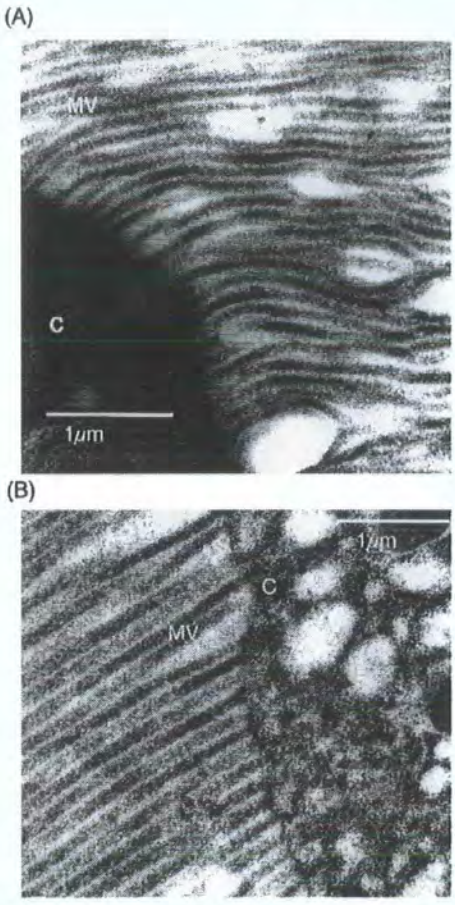

Fig. 4. Control midgut section from *L. oleracea* larvae fed on control diet. No labelling by gold-conjugated antibodies was observed in control sections.

and Con A fed larvae respectively). Fig. 7(A) shows that levels of GNA were reduced in midgut and haemolymph samples extracted from larvae chased with control diet for 72 hours, and only trace amounts of GNA remained in malpighian tubules with no GNA detectable in fat body extracts. In contrast, no reduction in levels of Con A were observed in midgut (Fig. 8(A)) and fat body (Fig. 8(B)) samples prepared from larvae chased with control diet for 72 hours, although there was a reduction in Con A levels in haemolymph and malpighian tubule samples dissected from larvae chased with control diet (Fig. 8(A) and (B)). These results indicated that Con A but not GNA was sequestered in fat body tissue of larvae exposed to lectin-containing diets.

Both lectins were present at levels of approx. 20% total soluble proteins in faecal samples collected from larvae exposed to the lectin diets for 48 hours (results not presented). In faecal samples collected from Con A-fed larvae chased with control diet for 24 hours and 72 hours Con A accounted for $<1\%$ total soluble proteins. In contrast, GNA continued to be excreted during the chase period, with equivalent faecal samples from larvae fed on GNA-containing diet and chased exhibiting a reduction in GNA level to approx. 10% of total faecal proteins after 24 hours, and to 1% of total faecal proteins after 72 hours of lectin-free chase diet. This difference in excretion of the two lectins corresponded with the persistence of Con A in midgut and fat body samples of control chased larvae (Fig. 8(A) and (B)) and indicated that Con A was purged less readily, presumably due to a greater binding affinity for these tissues, than GNA.

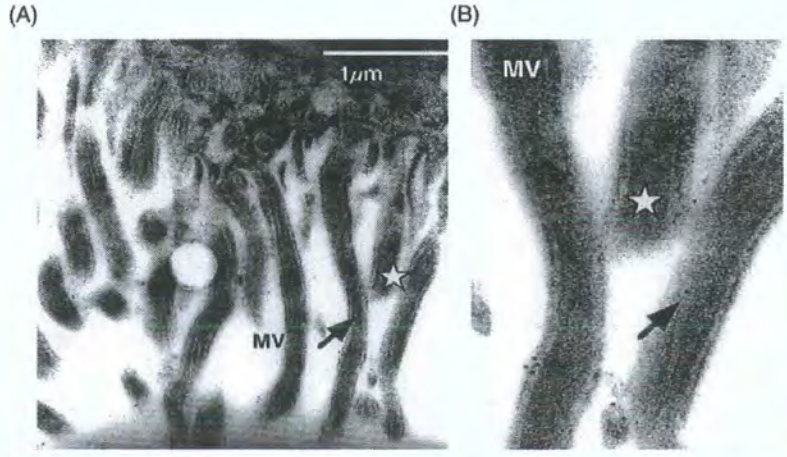

Fig. 5. Localisation of GNA in *L. oleracea* larvae fed on diet containing GNA by immunolabelling at the electron microscope level. (A) malpighian tubule section showing intact microvilli (MV), labelling by gold-conjugated antibodies is observed within and along the microvilli, (B) shows an enlarged region of (A) denoted by a black arrowhead.

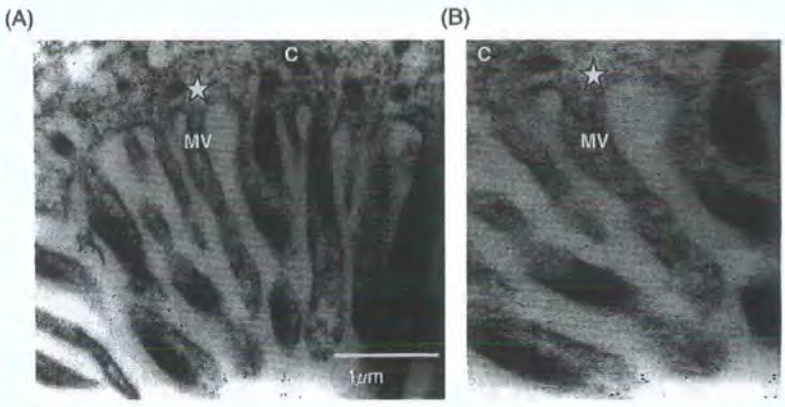

Fig. 6. Localisation of Con A in *L. oleracea* larvae fed on diet containing Con A by immunolabelling at the electron microscope level. (A) malpighian tubule section showing intact microvilli (MV) and cytosol (C), labelling by gold-conjugated antibodies is observed within and along the microvilli and within the cytosol. (B) shows an enlarged region of (A) denoted by a white star.

3.5. Uptake of lectins into the systemic circulation of larvae

The dependency of ingested GNA and Con A delivery to larval haemolymph upon the ability of lectins to bind to gut glycopolypeptides was investigated by exposing larvae to diets containing denatured GNA and Con A. After exposure for 48 hours to experimental diets, midgut and haemolymph samples were dissected and analysed for the presence of bound lectin by Western blotting. Neither lectin was detectable in either gut or haemolymph samples extracted from these larvae (results not presented). This confirmed that lectin uptake into the systemic circulation was dependent upon the ability of the lectin to bind to suitably glycosylated proteins present in the guts of exposed larvae.

4. Discussion

Earlier studies of the effects of GNA and Con A on *L. oleracea* larvae have indicated that, as in mammals, there is a correlation between the degree of lectin binding to the BB epithelium and subsequent toxicity effects. Con A, shown to be the more toxic of the two lectins in artificial diet assays and trials using transgenic plants (Fitches et al., 1997; Gatehouse et al., 1999) binds in vitro to far more BBMV and PM proteins than GNA and correspondingly shows greater accumulation in vivo in guts of lectin-fed larvae (Fitches and Gatehouse, 1998). In contrast Harper et al. (1995) who screened 38 lectins found no such correlation between binding to BB epithelium of *Ostrina nubilalis* and *Diabrotica undecimpunctata* and insecticidal activity. This paper provides evidence to suggest that lectin-gut interactions constitute only one of many factors involved in determining toxicity. Both GNA and Con A were shown to be delivered to the circulatory system of *L. oleracea* larvae and to accumulate in peripheral tissues. This was considered to be indicative of the potential for multiple mechanisms of lectin action and is analogous to the situation in mammals (Pusztai, 1991).

Ultrastructural studies revealed a similar pattern of GNA and Con A antibody binding along the digestive tract of lectin-fed larvae with the greatest amount of binding observed in midgut sections. This was in agreement with in vitro studies which indicated that both lectins had the potential to bind to all sections of the gut. In mammals, damage to the microvillus membrane of epithelial cells as a consequence of the binding of toxic lectins has been well documented (Pusztai, 1991; Pusztai et al., 1990, 1995). Comparable pathological effects have been reported in a number of insects (Powell et al. 1993, 1998; Sauvion et al., 1996; Habibi et al., 1998, 2000) supportive of the gut as the primary target for lectin toxicity. In this study neither GNA or Con A was shown to cause morphological damage or changes to the gut epithelial membrane of fifth instar larvae exposed to lectin diets for 48 hours. As larvae were not exposed to a toxic lectin dosage this does not rule out the possibility for damage to the integrity of the gut following long term exposure. The absence of effects and detection of both lectins within epithelial cells does, however, demonstrate that transport to the systemic circulation does not arise simply as a consequence of gut leakiness due to cellular damage. Furthermore the dependancy of delivery to the circulatory system upon lectin binding to gut glycoproteins was demonstrated by the absence of either lectin in gut and haemolymph samples extracted from larvae fed for 48 hours on diets containing denatured lectins.

Both Con A and to a lesser extent GNA exhibited binding in vitro to glycoproteins extracted from fat body tissue, malpighian tubules and haemolymph. This indicated that both lectins have the potential to exert systemic effects and to act directly or indirectly upon organs

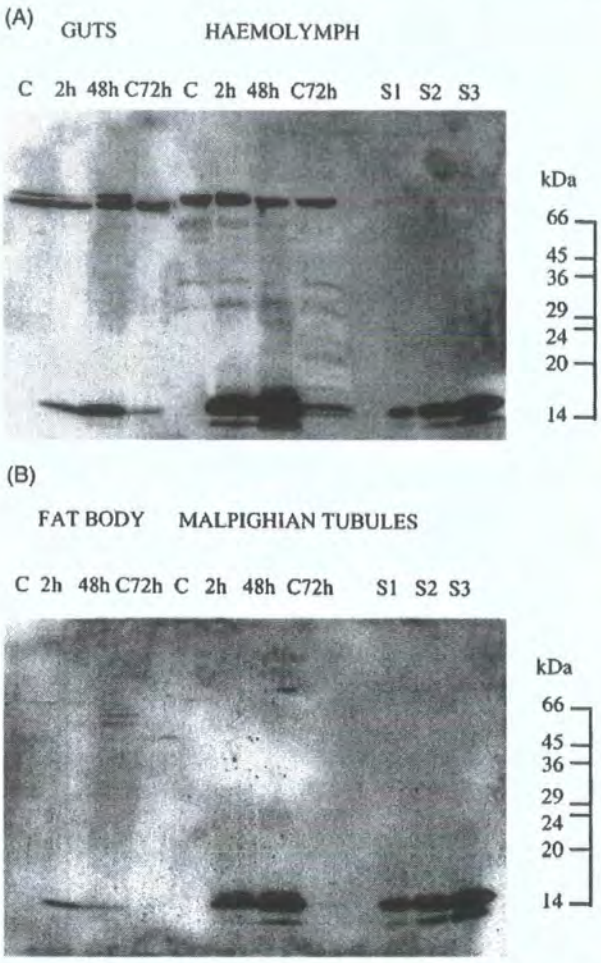

Fig. 7. Presence of GNA in polypeptides extracted from (A) larval gut and haemolymph and (B) fat body and malpighian tubules after feeding insects on lectin containing diets. 20 µg aliquots of protein were loaded in all lanes unless otherwise specified. Samples were prepared and analysed by SDS–PAGE (15% acrylamide gels) as in Fig. 1. Gels were blotted onto nitrocellulose and probed with primary anti-GNA antibodies, secondary peroxidase-labelled secondary antibodies, followed by treatment with ECL reagents and exposure to X-ray film. Loadings in (A) and (B) are as follows: Lane 1 (C) control gut, Lanes 2 (2h) and 3 (48h) guts from larvae fed for 2 hours and 48 hours, respectively, on GNA containing diets, Lane 4 (C 72h) guts from larvae fed for 72 hours on GNA containing diet and subsequently chased for 72 hours with control diet, Lane 5 (C) control haemolymph, Lanes 6, 7, and 8 are haemolymph samples collected from larvae at time points specified for lanes 2, 3, and 4. Lanes 9,10, and 11 (S1, S2, S3) are GNA standards of 2.5 ng, 10 ng, and 50 ng, respectively.

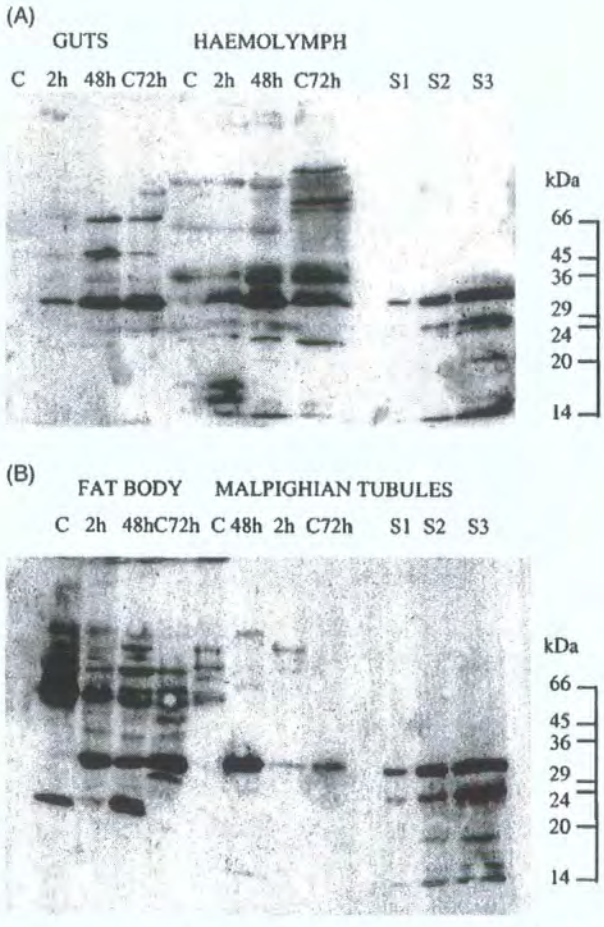

Fig. 8. Presence of Con A in polypeptides extracted from (A) larval gut and haemolymph and (B) fat body and malpighian tubules after feeding insects on lectin containing diets. 20 µg aliquots of protein were loaded in all lanes unless otherwise specified. Samples were prepared and analysed by SDS–PAGE (15% acrylamide gels) as in Fig. 1. Gels were blotted onto nitrocellulose and probed with primary anti-Con A antibodies, secondary peroxidase-labelled secondary antibodies, followed by treatment with ECL reagents and exposure to X-ray film. Loadings in (A) and (B) are as follows: Lane 1 (C) control gut, Lanes 2 (2h) and 3 (48h) guts from larvae fed for 2 hours and 48 hours, respectively, on Con A containing diets, Lane 4 (C 72h) guts from larvae fed for 72 hours on Con A containing diet and subsequently chased for 72 hours with control diet, Lane 5 (C) control haemolymph, Lanes 6, 7, and 8 are haemolymph samples collected from larvae at time points specified for lanes 2, 3, and 4. Lanes 9, 10, and 11 (S1, S2, S3) are Con A standards of 2.5 ng, 10 ng, and 50 ng, respectively.

other than the insect gut. A potential for systemic effects of lectins in insects has previously been suggested by Powell et al. (1998) who observed immunolabelling of GNA in the fat bodies, ovarioles and haemolymph of the homopteran (*N. lugens*) exposed to lectin containing artificial diet.

The in vivo presence of GNA and Con A, bound to polypeptides extracted from midgut, hindgut, malpighian tubules, fat bodies and haemolymph of larvae exposed to lectin diets for five days was detected by Western blotting. Both lectins were found to be most abundant in the midgut and least abundant in the haemolymph. Notably higher levels of Con A, compared to GNA, were detected in the fat body. This difference was also observed in a subsequent trial investigating lectin accumulation over a period of 48 hours. When lectin-fed larvae were chased for 72 hours with control diet very little reduction in levels of Con A in gut and fat

body extracts were observed indicating that Con A was less easily purged by the larvae compared to GNA. These results suggested that Con A not only binds to a greater number, but may also bind more avidly, to gut and fat body glycoproteins as compared to GNA. The accumulation, and persistence of Con A, but not GNA, in fat body extracts of lectin-fed larvae suggested that Con A may additionally act by disrupting the multiple metabolic processes carried out by this tissue.

In mammals toxic lectins act as gut immunogens (de Aizpurua and Russel-Jones, 1988). Uptake into the systemic circulation facilitates a humoral antibody response and a local IgE based allergenic immune reaction contributes to the overall poor nutritional state of the body thereby increasing the nutritional toxicity of PHA (Pusztai and Bardocz, 1996). The detection of GNA and Con A in the circulatory system of *L. oleracea* larvae demonstrated that both lectins have the potential to disrupt the immune system of exposed insects and this requires further investigation. Whilst the data presented does not provide evidence for the functionality of lectins delivered to the haemolymph, the detection of intact non-truncated lectins in Western blots (Figs. 7 and 8) was indicative of functionality. In addition recent studies (data not presented) have shown conclusively that GNA in the haemolymph is functional. That GNA may, to some extent, compromise the immune system of *L. oleracea* larvae has previously been indicated by a study which examined the ability of the gregarious ectoparasitoid *Eulophus pennicornis* to parasitise control and GNA-fed larvae (Bell et al., 1999). Larvae exposed to GNA-containing diets appeared more suitable than control-fed larvae as hosts for *E. pennicornis* and this was thought to be indicative of a compromised immune system.

The insecticidal activity of both GNA and Con A has been shown to be most apparent when fed to newly hatched *L. oleracea* larvae (Fitches et al., 1997; Fitches and Gatehouse, 1998; Gatehouse et al., 1997, 1999). In this study fourth instar larvae exposed for five days to a relatively high dose of Con A or GNA exhibited no reduction in weight or consumption compared to control fed larvae. In the long term GNA does not effect survival but does delay larval development and cause a significant reduction in growth and consumption. In contrast, Con A has been shown to have a significant effect on survival and to cause a greater delay in development and greater reductions in growth and consumption than GNA. Obviously young larvae with immature immune systems and limited energy reserves are more vulnerable to the impact of dietary lectins. Thus far studies indicate that toxicity may arise due to cumulative effects with ingested lectins detectable in a number of sites other than the gut in exposed larvae. A greater binding affinity of Con A for gut glycoproteins together with binding to fat body glycoproteins may be responsible for its greater toxicity, as compared to GNA, upon this Lepidopteran pest. Similar differences in the binding of other lectins, to tissues other than the gut, may provide an explanation for the extreme variability that has been observed in the toxicity of different lectins to various insect species.

Acknowledgements

The authors acknowledge support from the Pesticides Safety Directorate of MAFF. Work reported in this paper was also supported by a CASE studentship provided by MAFF (Central Science Laboratory) to E.Fitches, and by a Scottish Office studentship to S. Woodhouse (Research Project FF821), both of which are acknowledged with gratitude.

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