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

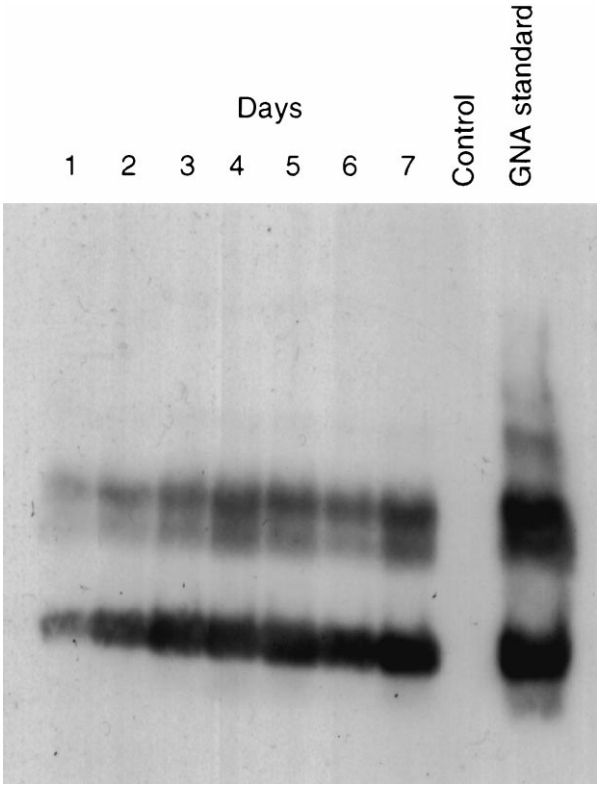

Fig. 1. Western blot showing accumulation of GNA within aphids with time. Aphids were fed on an artificial diet containing 0.1% GNA for 1–7 days (lanes 1–7); lane 8 contains the control sample where aphids were fed for 24 h on an artificial diet which did not contain GNA; lane 9 contains a known amount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

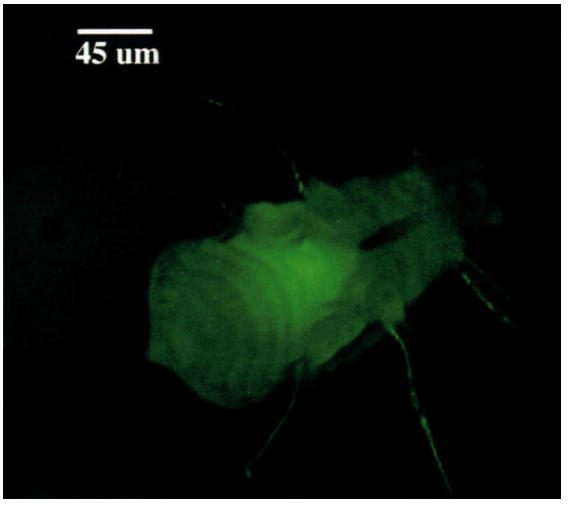

Fig. 2. An aphid fed on artificial diet containing 0.1% w/v FITC-labelled GNA for 2 days and viewed under UV light. Fluorescent regions indicate the presence of GNA within and around the gut region.

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

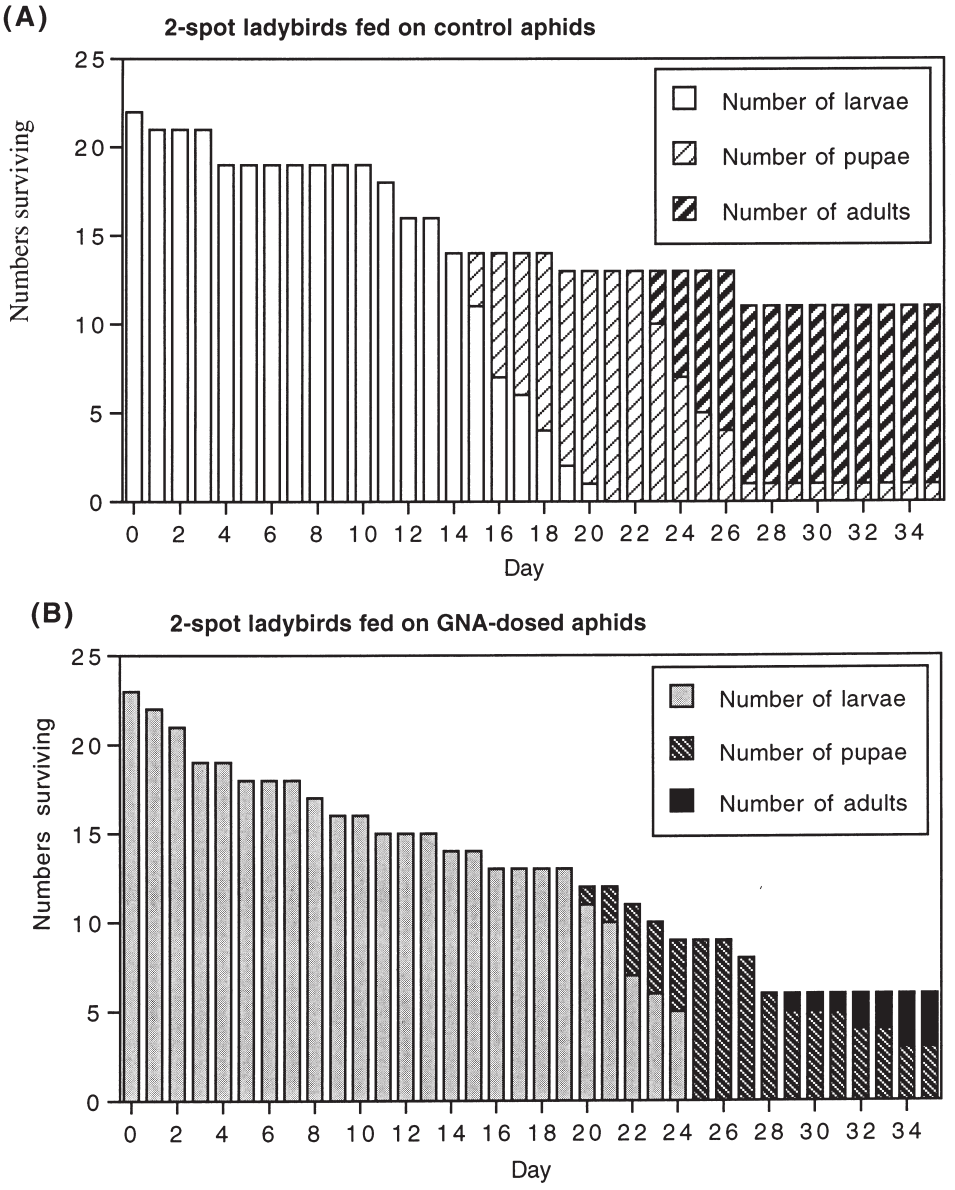

Fig. 3. Bioassay 1. Graphs showing the numbers of surviving larvae, numbers in pupation and numbers successfully emerging as adults of 2-spot ladybirds when fed on (A) control diet-dosed aphids and (B) GNA-dosed aphids.

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

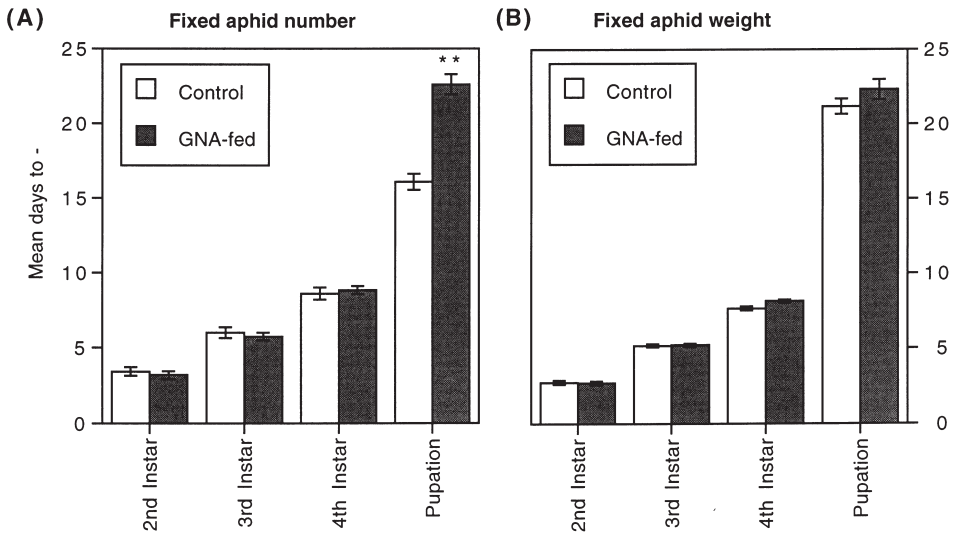

Fig. 4. Graphs showing the mean cumulative number of days taken to develop through the larval instars by 2-spot ladybirds fed on GNA-dosed and control diet-dosed *M. persicae* when fed on (A) a fixed aphid number (bioassay 1) and (B) a fixed aphid weight (bioassay 2) basis. **Significant difference (Mann-Whitney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

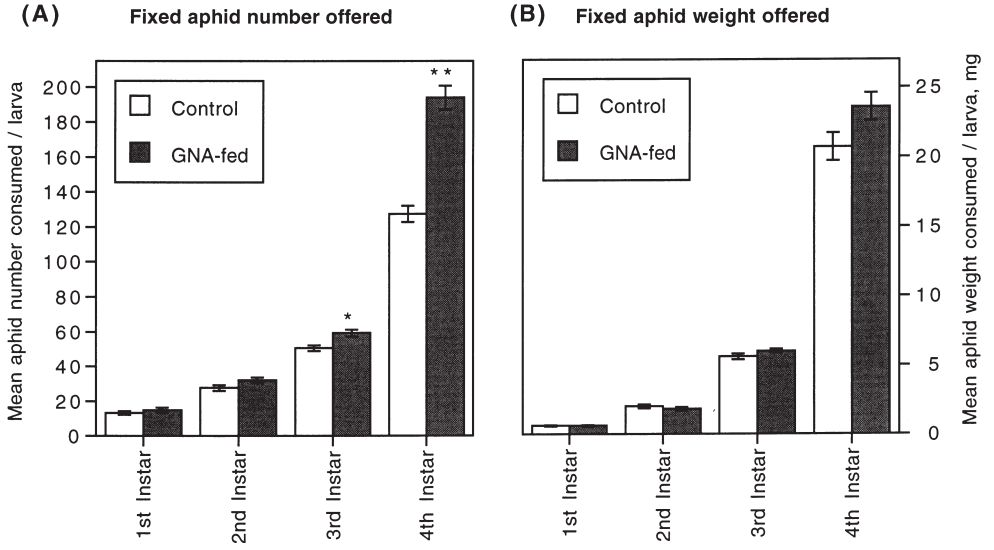

Fig. 5. Graphs showing the mean cumulative number of aphids eaten throughout the larval instars by 2-spot ladybird larvae fed on GNA-dosed and control-dosed *M. persicae* when offered (A) a fixed aphid number (bioassay 1) and (B) a fixed aphid weight (bioassay 2). *Significant difference at the $p<0.05$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

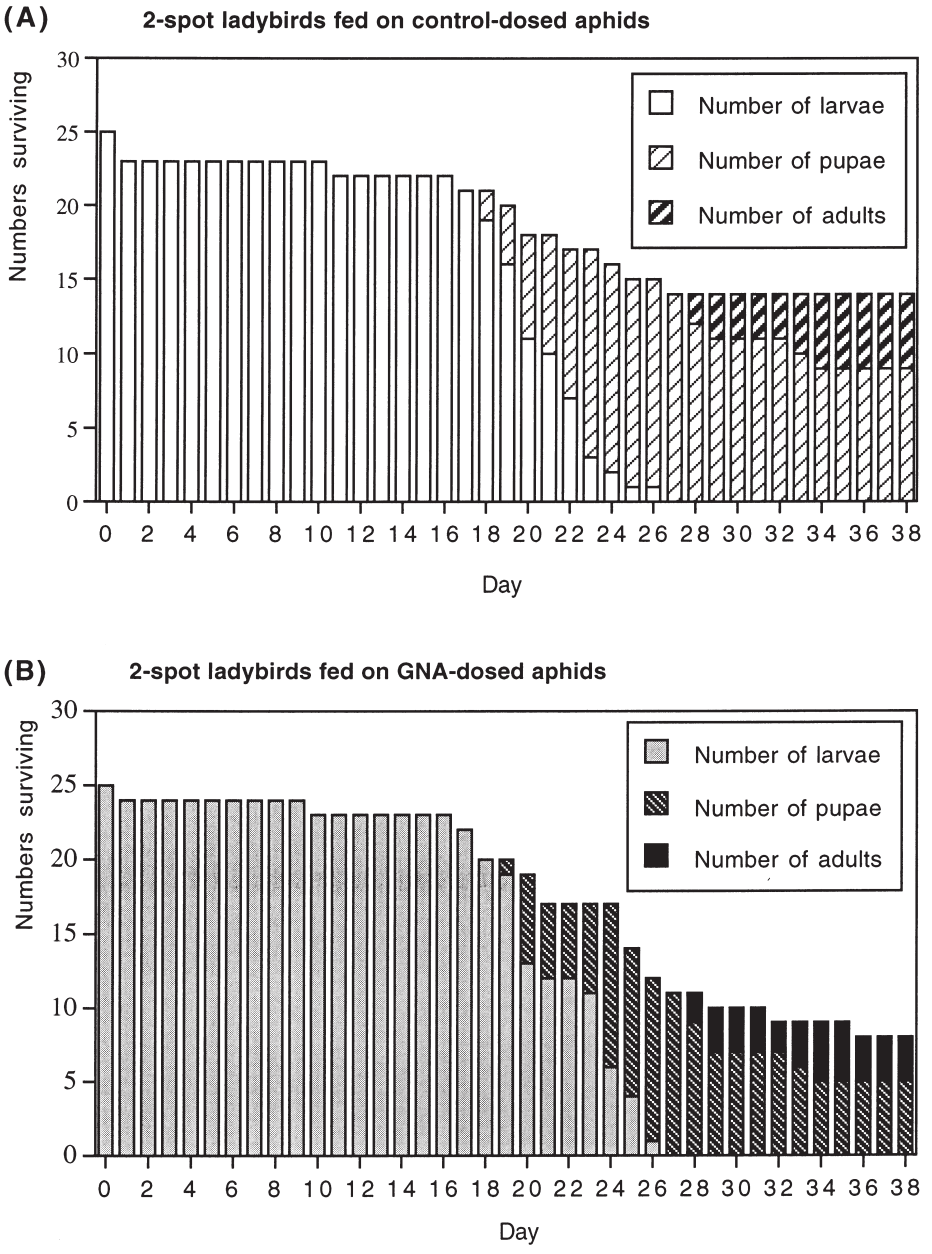

Fig. 6. Bioassay 2. Graphs showing the numbers of surviving larvae, numbers in pupation and numbers successfully emerging as adults of 2-spot ladybirds when fed on (A) control diet-dosed aphids and (B) GNA-dosed aphids.

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-

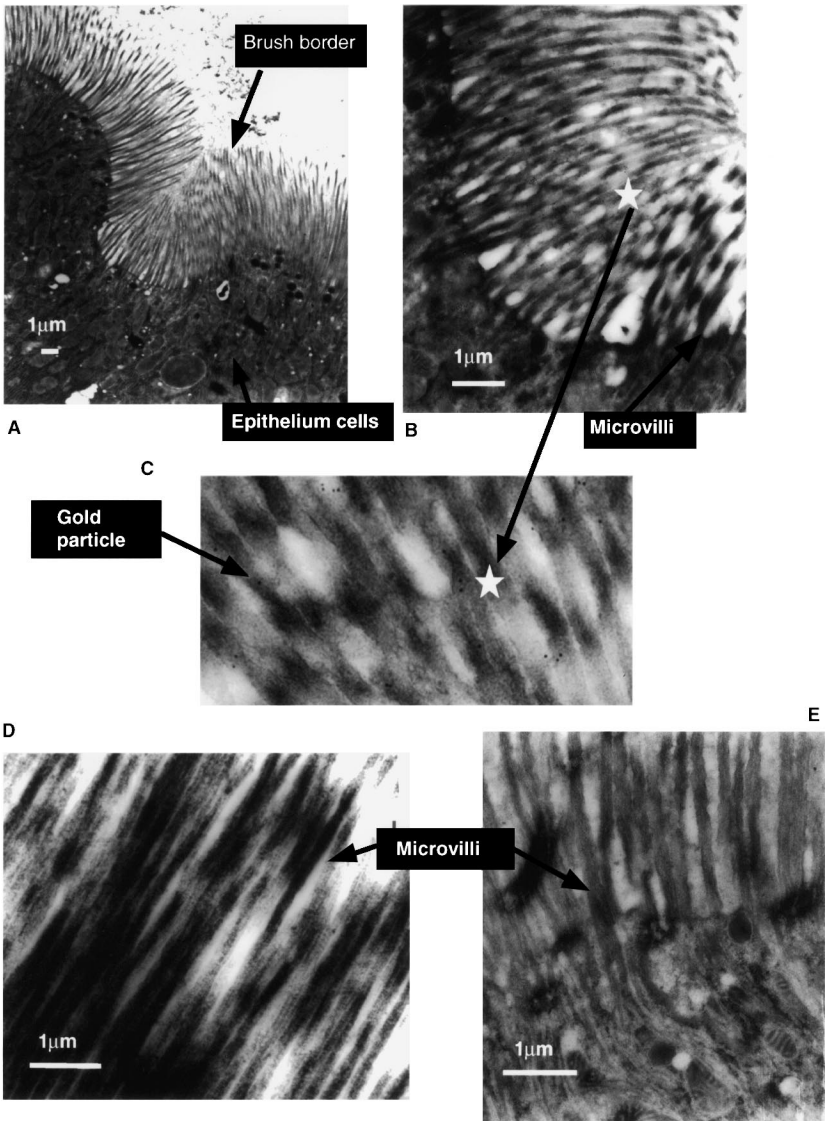

Fig. 7. Shows electron micrographs of the midgut region of 2-spot ladybird larvae: (A) at a magnification of 4600 shows some of the cell ultrastructure; (B) magnification 22,000, shows the presence of GNA binding to the microvilli and presence within the cells lining the gut lumen (as depicted by black 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.

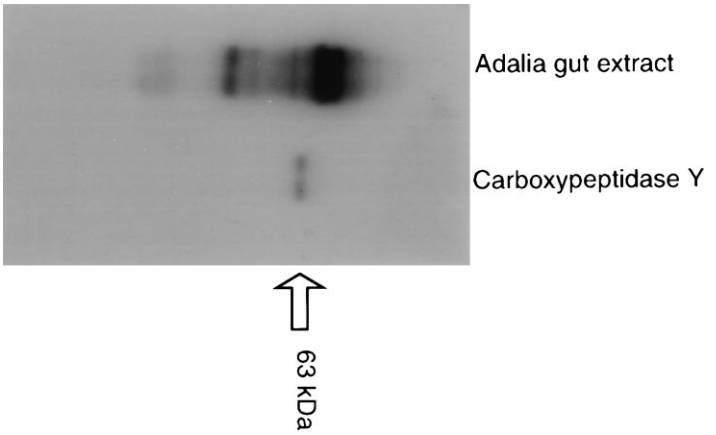

Fig. 8. Shows a Western blot of 2-spot ladybird larvae gut extract, incubated with recombinant GNA. Bands represent glycoproteins within the gut extract to which GNA will bind.

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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