HORTICULTURE RESEARCH INTERNATIONAL
EAST MALLING

PREDATION ON VINE WEEVIL IN SOFT FRUIT PLANTATIONS

Project SF 15b

Final Report (September 1997)

Previous reports:	First Annual Report 1995
	Second Annual Report 1996
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Date Project Commenced:	January 1994
Date of Completion :	April 1997

Key Words: Vine Weevil, Biological Control, Strawberry, Blackcurrant, Polyphagous Predators, Carabid Beetles, Natural Enemies.

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Scope And Objectives Of The Project

The vine weevil feeds on more than 150 species of plants, and is a serious pest of strawberry, blackcurrant, and protected and hardy ornamentals. Adult vine weevils feed on leaves, causing characteristic notching damage, but in soft fruit it is the feeding of larvae on roots that is more damaging. Although vine weevil has long been recognised as a pest, it has become more serious in recent years. A number of different factors has contributed to this, including the withdrawal of the broad spectrum persistent insecticide aldrin ten years ago, changing husbandry practices, such as the use of polythene sheeting in strawberry growing, and increased trade in potted and containerised plants which has aided the distribution of this flightless weevil. The pest is particularly difficult to control chemically in field grown crops, because the soil dwelling larvae are a technically challenging target.

Several earlier studies in the UK and elsewhere have provided evidence, mostly circumstantial, of vine weevils being consumed by predators. Real evidence of this was scarce, however, because of the difficulties of conducting field observations of the nocturnal adults or the larvae living concealed in the soil.

The objectives of this project were

- to determine the kinds of predator that occur naturally in blackcurrant and strawberry plantations
- to determine which of these predators (if any) consume vine weevils
- to assess the possibility of reducing vine weevil numbers by manipulating predator numbers.

HDC took the view that the seriousness of the vine weevil problem, and the absence of any previous thorough investigation of natural predators, made this somewhat strategic research topic a priority for funding. It was recognised from the outset that the findings would provide the leads to enable future research on vine weevil rather than produce practical guidelines suitable for immediate implementation by growers.

Summary Of Results

The occurrence of polyphagous ground dwelling predators in strawberry and blackcurrant plantations was assessed by a programme of pitfall trapping. Over twenty species of carabid and staphylinid beetles were caught, some of them in large numbers, such as *Pterostichus melanarius, Pterostichus madidus, Harpalus rufipes, Nebria brevicollis,* and *Calathus fuscipes.* Culturing techniques were devised to maintain populations in the laboratory until required for predation experiments.

It was important to be able to analyse the gut content of predators from the field to detect what they had eaten prior to capture. A serological method based on antibodies was developed in preference to alternative methods of predator gut analysis such as electrophoretic isoenzyme analysis, because of its higher sensitivity in detecting very low concentrations of

vine weevil material, and its convenience as a system for screening large numbers of predators. A polyclonal antiserum was raised against vine weevil larvae. In fact, it recognised egg, larva and adult vine weevil stages but was unable to distinguish between them.

A panel of fifteen monoclonal antibodies (MAbs) was subsequently developed. These MAbs had specificities to different developmental stages of vine weevil. None of the MAbs recognised predator antigens. It was intended that both these MAbs and the IgG (the fraction of the antiserum that contains antibodies) purified from the polyclonal antiserum would be used in large-scale screening of predators caught in vine weevil-infested crops by an indirect sandwich ELISA. However, the MAbs were of sufficient sensitivity and specificity to vine weevil proteins that the assay finally developed was a simplified indirect ELISA which did not require the extra step in the protocol using the IgG.

Collection of predators from vine weevil infested crops, and their subsequent gut analysis by ELISA using the MAbs, identified nine key predator species, and defined the extent to which they fed on each stage. The three predators which consumed the highest numbers of each stage are presented in the table below.

Predators of Eggs	Predators of Larvae	Predators of Adults
Notiophilus biguttatus	*Notiophilus biguttatus*	*Carabus violaceus*
Bembidion lampros	*Pterostichus madidus*	*Calathus fuscipes*
Ocypus olens	*Harpalus rufipes*	*Harpalus rufipes*

To investigate the ability of various predators to control vine weevil populations, a preliminary field trial was set up. Individual strawberry plants were enclosed within plastic barriers to prevent insects from climbing in or out of the enclosures, and various combinations of vine weevils and predators were placed within the enclosures. However, it became apparent that the barriers did not extend to a sufficient depth in the soil to prevent carabids from burrowing under them. A second field experiment was then established with a different design of barrier. Polythene barriers, sunk to a depth of 45 cm, were used to enclose plots of twenty strawberry plants. Two different densities of vine weevil were established, to simulate low and high density infestations, both with and without predators. In this field trial the presence of predators was found to have a significant impact on the vine weevil population at the lower weevil density.

Points For Growers

Rather than specific action points emerging at this stage, there are some general points that should be considered when making crop management decisions in situations where vine weevil is an actual or potential problem:-

- some predatory carabids (ground beetles) and staphylinids (rove beetles) consume vine weevil and can contribute to vine weevil pest management; these predators should therefore be encouraged

- the species shown to be the greatest consumers of vine weevil are *Harpalus rufipes, Nebria brevicollis, Pterostichus madidus, Carabus violaceus, Ocypus olens,* and *Notiophilus biguttatus,* (See HDC Project News Nos. 30 and 45 for illustrations of some of these)

- broad spectrum pesticides, particularly organophoshorus compounds and pyrethroids, are damaging to these predatory beetles; this should be borne in mind when making decisions on pesticide use.

- populations of predatory carabids and staphylinids benefit from ground cover in fruit plantations such as grass or other non-crop plants in the rows or alleys.

Introduction

Pest Status

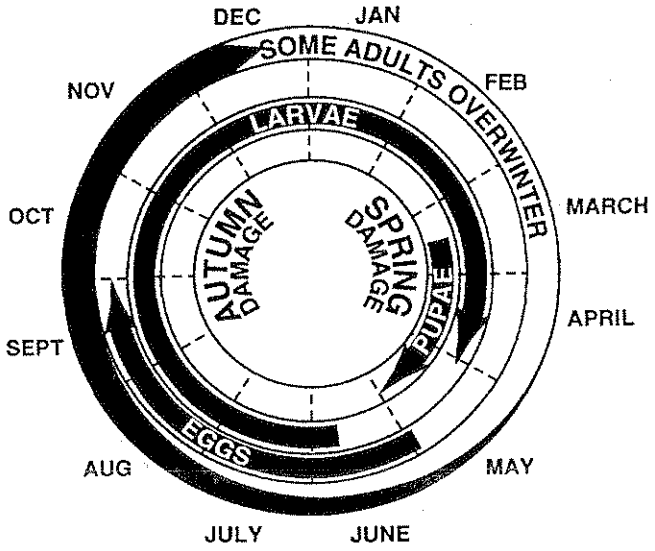
The vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae), is known to feed on around one hundred and fifty plant species, both wild and cultivated, including soft fruit, ornamentals and coniferous trees (Masaki *et al.*, 1984; Warner & Negley, 1976; Smith, 1932). It is endemic to the temperate regions of Europe, and is now also commonly found in Australasia, Japan and the eastern and western seaboards of North America. The vine weevil is flightless and much of its spread has been due to the sale and use of contaminated plant material. Horticultural practices such as the widespread use of black polythene mulches and peat-based potting composts have also increased the incidence of infestations. Vine weevil populations are hard to control, particularly the larval and pupal stages which are hidden amongst the roots.

The most severe damage results from the larvae feeding on the root system, causing the plant to wilt and die. In particularly susceptible species, such as *Cyclamen*, a single vine weevil larva is sufficient to kill a plant (Moorhouse, 1990). Moderate damage to strawberries reduces fruit set while more severe damage may result in complete loss of a planting. The degree of damage is dependent on crop age and the position of the larvae in the root system (Foster, 1982; Evenhuis, 1978). Adults are more polyphagous than the larvae (Nielsen & Dunlap, 1981) and feeding by adults on leaves results in characteristic notches. Although generally less damaging to the plant than the root-feeding, leaf-feeding causes serious economic losses where the appearance of the plant is important, as with potted ornamentals and cut flowers. The presence of adults in a crop also results in contamination of mechanically harvested soft fruit (Shanks, 1981).

Lifecycle Of Vine Weevil

Vine weevils are parthenogenetic and no males have ever been found (Feytaud, 1918, Moorhouse *et al*, 1992). Vine weevils are prolific egg layers, a single adult being able to lay around 500 eggs outdoors and 1200 in the laboratory, so small populations can develop rapidly into large infestations, elevating their status as a pest. There is one generation per year, (Figure 1), (see Moorhouse *et al*, 1992 for review) although there is considerable overlap between developmental stages (Schread, 1972). Vine weevils generally overwinter as half-grown larvae, which resume feeding in spring before pupating around May or June. Some adults also overwinter and recommence oviposition during the early part of their second summer. The size of this overwintering adult population is not known. In glasshouses, all stages may be present at the same time (Evenhuis, 1982). Development is greatly influenced by temperature and relative humidity, as is the size of the overwintering adult population.

Figure 1: Lifecycle of vine weevils- timing of developmental stages



For more information, see Moorhouse *et al.*, (1992), which gives a comprehensive review of the biology and control of vine weevil. Oakley (1994) also reviews the limited range of chemical control options currently available.

Polyphagous Predators

The suggestion that some predators consume vine weevil was made in early studies (Feytaud, 1918; Smith, 1932). These species included carabid and staphylinid beetles, poultry and several other birds, lizards, toads and frogs, mammals such as hedgehogs, moles and shrews, as well as the fungi *Fusarium* spp. and *Metarhizium anisopliae*. Thiem (1922) reported that *Pandelleia sexpunctata* Pand. (Diptera: Tachinidae) was a major parasite of vine weevil larvae in German vineyards. Evenhuis (1982 & 1983) found significantly reduced carabid populations and higher vine weevil numbers in strawberry plots treated with some insecticides, particularly ethoprophos, compared to untreated plots. In his laboratory trials, a number of carabids were found to feed on vine weevil; larger carabids ate larvae and eggs, and smaller species fed on eggs only, including *Bembidion ustulatus* which was found to be a voracious egg-predator (Evenhuis, 1982). Carabid species and the earwig *Forficula auricularia* L. were also recorded as predators of vine weevil eggs and larvae in glasshouses in the USA (Garth & Shanks, 1978).

Although it has long been thought that predatory carabid beetles and other polyphagous predators influence the community structure of phytophagous arthropods (*e.g.* Dempster, 1960; Crook & Sunderland, 1984), direct field observation is difficult due to the small size and cryptic nature of both the pest and many of the predators. Dissection and physical examination of the gut contents of these predators is difficult and laborious, and impossible in the case of predators

that consume only liquid parts of their prey. Immunological techniques involving polyclonal antibodies have been employed previously in a number of predation studies but the antisera often cross-reacted with non-target species, partially restricting their use (for reviews see Boreham & Ohiagu, 1978; Sunderland, 1988; Hagler *et al.*, 1991). The use of monoclonal antibodies minimises the problem of lack of specificity. Recently, MAbs have been used to identify species-, stage-, and even instar-specific prey (Ragsdale *et al.*,1981; Greenstone and Morgan,1989; and Hagler *et al.*,1991 & 1994).

Experimental Section

Introduction

In this work, pitfall traps have been used to assess the range of predator species present in blackcurrant and strawberry fields. Two trials were carried out in an attempt to assess the potential impact of some of these predators on vine weevils in the field. A laboratory method (ELISA) was developed to identify vine weevil remains within predator guts, initially using a polyclonal antiserum. However, subsequent development of monoclonal antibodies permitted the remains to be identified as egg, larva or adult vine weevil material. For the purpose of the methods and results chapters of this report, the work has been divided into field-based and laboratory work.

Materials And Methods -Fieldwork

Initial Survey Of Polyphagous Predators (Rock's Farm)

An existing field trial designed to examine the impact of different wild flower plots on populations of flying beneficial insects provided an opportunity to pitfall sample the ground-dwelling predators as an identification exercise and to note patterns of activity.

The field trial was located at Rock's Farm, HRI East Malling (grid reference: TQ705558), where each of 14 species of plant were sown in individual 4m x 4m plots on 20/5/1994. Each species was planted in two non-adjacent plots.

List of Plant Species Sown

Selfheal (*Prunella vulgaris*)	Corn Chamomile (*Anthemis arvensis*)
Wild Carrot (*Daucus carota)*	Phacelia (*Phacelia tanacetifolia)*
Corn Cockle (*Agrostemma githago)*	Corn Marigold (*Chrysanthemum segetum*)
Field Poppy (*Papaver rhoeas*)	Bellflower (*Campanula glomerata*)
Cornflower (*Centaurea cyanus*)	Buckwheat (*Fagopyrum nesculentum*)
Coriander (*Coriandrum sativum*)	Oxeye Daisy (*Leucanthemem vulgare*)
Cow Parsley (*Anthriscus sylvestris*)	Bird's Foot Trefoil (*Lotus corniculatus*)

A single pitfall trap was set in the centre of each of the 4m x 4m plots on 28/8/1994, after the plants had become established, and was emptied through the summer until 10/10/1994. The pitfall traps consisted of 7.3 cm diameter polypropylene beakers, sunk into the ground so that they were level with the soil surface. An inner cell consisting of a disposable plastic cup was placed inside the outer container, so that the contents could be emptied without disturbing the ground around the trap. Both containers had drainage holes in their base.

The Calves Ley Field Trial: Enclosure & Exclusion Plots

A field trial was established in a crop of strawberries at HRI East Malling to assess the effect of carabid predators on vine weevil early larval stages, by use of enclosure and exclusion

barriers around individual strawberry plants. This trial was intended as a preliminary experiment to examine whether this type of barrier was capable of enclosing the carabids, prior to full field trials later in the study. The trial was established in September 1994, and assessed in April 1995.

Twenty modified 72 litre round plastic storage containers of 55 cm diameter were used as barriers around the strawberry plants. The bases were sawn off so that they could be sunk into the ground. Fluon (a 'non-stick' PTFE suspension) was painted beneath the lip of the containers to prevent insects entering the plot. The barriers were sunk to an average depth of 7 cm, and all weeds and dead strawberry material were removed. The barriers extended 35 cm above ground level.

Pitfall traps were set up inside the barriers for two days to remove any predators that were present initially. The plants were then inoculated with 50 vine weevil eggs and 10 first instar larvae taken from cultures.

The pitfall traps were removed and the following carabids were added to each of plots 1-10: 2 *Harpalus rufipes*; 1 *Pterostichus madidus*; 2 *Calathus fuscipes* and 2 *Nebria brevicollis*. No carabids were added to plots 11-20 which served as predator-free controls.

When assessing the trial, pitfall traps were placed inside containers 1-20 for 3 days to remove any predators present and a ground search located any carabids which had not fallen into the traps. Aerial parts of the plants were examined for evidence of adult vine weevils which may have emerged earlier than expected. A 25 cm cube of soil was cut around each strawberry plant and removed to a labelled plastic bag; the contents were then examined by hand. These samples contained the entire root system. The soil below this was examined in the field for the presence of larval/pupal vine weevils and adult carabids.

The East Egham Field Trial: Enclosure And Exclusion Plots

Sixteen enclosures measuring approximately 5 m x 1 m were established in a strawberry crop at HRI East Malling in September 1995. Each contained two rows of ten strawberry plants on a single strip of polythene, without sub-soil irrigation. Barriers made of polythene sheeting were sunk to a depth of 45 cm using a sub-soiler trailed behind a tractor. Wooden batons supported the sheeting 60 cm above ground level.

In eight plots, all predators were removed by continuous pitfall trapping throughout the winter. Those predators caught were used to build up the predator populations in the other eight enclosures. Vine weevil adults were then added to enclosures at two densities in July 1996 to simulate large (425 per plot) and small (60 per plot) populations.

Fluon, painted around the inner rim of the barriers, prevented the vine weevils climbing over the top of the polythene sheeting. The predator populations were augmented throughout the summer. The trial was assessed in March 1997 in the same way as the previous field trial.

1996 Survey Of Commercial Crops And Collection Of Samples For Predator Gut Analysis

A number of sites on commercial farms in Kent were selected for further investigation of vine weevil predators, using monoclonal antibodies (MAbs) (Table 1). Dry pitfall traps were used to collect polyphagous predators and other fauna in infested fields between May and October 1996. Predators caught overnight were transferred to the laboratory in ice boxes, identified and stored in individual vials at -80 °C until screened. The extract from each predator was divided into three portions and each portion was screened against a MAb with a different specificity in an ELISA. If the trap had not been emptied in the previous 24 hours, the predators caught were recorded as part of the general survey of which species were present at each site, but their gut contents were not analysed as any meal may no longer be detectable. Temperature loggers situated in each field recorded temperatures at 30 minute intervals throughout the sampling period. A record of all spray applications was kept.

Table 1: Commercial farm sites for predator collection in 1996.

Site	Map reference	Crop	Details
Baldwin's Field, Upper Horton Farm, Upper Horton.	TR121543	Baldwin Blackcurrant	20 year old plantation, no irrigation
Andrew's Field Upper Horton Farm, Upper Horton	TR121543	Ben Alder Blackcurrant	9 year old plantation, no irrigation
Hare Field, Norham Farm, Selling.	TR051564	Ben Lomond Blackcurrant	20 year old plantation, no irrigation
Dogleg Field, Norham Farm, Selling.	TR051564	Ben Lomond Blackcurrant	9 year old site invaded by wild grasses. No irrigation.
Store Field, Wilford Court Farm, Tonge.	TQ945650	Elsanta Strawberry	Planted May 1994 on black polythene covered raised beds. Single T-tape irrigation. Dursban 4 drenched Autumn 1994 & 1995.
Westfield-Northwest Gore Farm, Upchurch.	TQ855666	Pegasus Strawberry	Planted Autumn 1994 in raised beds, no polythene, irrigation with double T-tapes

Materials And Methods - Laboratory Work

Introduction

This introduction is designed to explain the methods used in the laboratory work and rationale behind them, before describing the experiments carried out.

ELISA (enzyme-linked immunosorbant assay)

Direct observation of predator feeding and behaviour in the field is difficult because of their small size and nocturnal habit. Laboratory feeding trials permit a large number of potential predators to be screened to see if they will feed on vine weevils, but it is a highly artificial situation, and only indicates that a particular predator will feed on vine weevil if the alternative is starvation. Such tests do not indicate if a predator would normally encounter vine weevils or be able to catch and kill them, nor do they account for the presence of alternative prey. An enzyme-linked immunosorbant assay (ELISA) provides a convenient method for screening large numbers of different predators for the presence of vine weevil material in their digestive tracts. This technique is preferable to alternative methods such as manual dissection of the gut and visual identification of vine weevil remains, or gel electrophoresis, because of its greater sensitivity to minute quantities of vine weevil and the relative speed with which numerous potential predators can be screened.

The development of the ELISA is detailed below. First, a polyclonal antiserum that recognised vine weevil material was produced by standard immunological technology. Following purification, the titre of the antiserum was calculated to determine the smallest quantities of vine weevil material detectable. The antiserum was also screened against extracts prepared from predators and alternative prey species present in the same environment as the vine weevil to determine if the antiserum cross-reacted, recognising proteins common to vine weevil and any of these non-target species. Cross-reactions were removed by incubating the antiserum with extracts of species known to cross-react, so that any proteins the antiserum recognised bound to it and precipitated out of solution. The resulting cross-absorbed antiserum no longer recognised these non-target species, although its sensitivity to vine weevil antigens was slightly reduced. This polyclonal antiserum recognised the remains of vine weevil eggs, larvae and adults, but was unable to distinguish between them. Results of electrophoretic tests indicated that each developmental stage contained proteins which were specific to that stage. This meant that it was possible to produce monoclonal antibodies which were able to recognise a single developmental stage. By using these monoclonal antibodies in an ELISA it was possible to pinpoint which were the key predator species feeding on each stage.

Standard protocols were used throughout and are described in detail in the Appendix and in the first annual report of this project. To summarise, an ELISA protocol is as follows: each well of a 96-well plate is coated with a crude extract made by grinding a potential vine weevil predator in a buffer. After incubation, the plate is washed and then the antiserum is added. If the antibodies in the antiserum recognise the antigenic material on the plate they will bind together, whereas if they do not recognise the antigen, ie. there are no vine weevil proteins present, the antibodies will not bind and will be simply washed off when the plate is rinsed. An

enzyme is then added which will bind to any antiserum that has not been washed off. After another incubation period, a substrate is added which is broken down by the enzyme, if present, causing a colour change in the wells. The reaction is then stopped by addition of concentrated acid, and the degree of colour change analyzed using a spectrophotometer.

Electrophoresis (SDS-PAGE)

The production of a polyclonal antiserum raised against an extract prepared from vine weevil larvae has been described above. However, this antiserum was found to also recognise vine weevil adults and eggs. To identify why the polyclonal antiserum recognised all stages, their proteins were separated out by electrophoresis (SDS-PAGE) and probed with the polyclonal antiserum (Western blotting) to determine whether the recognition was based on a few common proteins or many. The rationale of SDS-PAGE is as follows. When proteins are heated to 100 °C for a few minutes in the presence of SDS (sodium dodecyl sulphate) and reducing agents, they unfold and bind in a uniform ratio of one SDS molecule to two amino acids. SDS binding imparts a very strong negative charge to the protein, dominating its native charge. Therefore, the charge to mass ratio becomes virtually constant for all proteins, with the consequence that proteins separate on size alone, as small molecules travel more rapidly through an electrophoretic gel than large ones (Weber & Osborne, 1969). A comparison was made of the protein spectra of the different stages by separating the proteins by electrophoresis and then staining with Coomassie Blue. This identified any stage-specific proteins, with a view to the subsequent development of stage-specific monoclonal antibodies (MAbs) against them. This would produce a predator gut analysis system capable of distinguishing between predation on vine weevil eggs, larvae and adults.

Western blotting

During the procedure known as Western blotting (Burnette, 1981), vine weevil proteins, which had been previously separated in a polyacrylamide gel, were eluted electrophoretically by a transverse electrical field onto a nitrocellulose membrane which bound the proteins tightly. The membrane was then probed with the polyclonal antiserum and visualised by immunoperoxidase staining. This identified which proteins were recognised by the polyclonal antiserum.

Development Of An Indirect Elisa Protocol For Predator Gut Analysis

All recipes for stock reagents are given in the Appendix.

Polyclonal Antiserum Production

Using a standard protocol (Harlow & Lane, 1988), two polyclonal antisera, AS_1 and AS_2, were raised against an immunogen of crude extract of soluble vine weevil larval proteins prepared with Titermax™ adjuvant. In order to prevent fungal growth, 0.05% sodium azide was added to each antiserum. Antisera were then stored at 4 °C.

The titres of the antisera were determined in a number of indirect ELISA experiments

using dilution series of both antigen and antisera. Two vine weevil larvae, starved for 24-48 hours, together weighing 0.10 g were homogenised in 10 ml PBS to form a 1:100 w/v solution. The material was centrifuged at 5000 rpm and 10 °C for 15 minutes, before the supernatant was removed (including the floating tissue). A dilution series was produced from this (1 ml 1:100 stock solution + 4 ml PBS = 1:500; 1 ml 1:500 solution + 4 ml PBS = 1:2500 etc...)

Indirect ELISA Protocol Summary

1. 100 µl of antigen solution was used to coat each of the 96 wells of an immuno plate.
2. After overnight incubation of the immuno plates, the plates were washed three times with PBS-Tw on an automatic plate washer.
3. The antiserum was diluted in PBSTPO buffer, and 100 µl was added to each well.
4. The plates were incubated for two hours at 30 °C, and then washed as before.
5. Protein A/HRP (Horseradish peroxidase) was diluted 1000-fold in PBSTPO and 100 µl added to each well.
6. The plates were incubated again for two hours at 30 °C, and then rinsed.
7. 100 µl of freshly-prepared substrate was added to each well. (Positive reactions were accompanied by a blue colour change).
8. 50 µl of 15% sulphuric acid stop solution was added after 15-20 minutes, or sooner if the colour stopped changing. (Addition of the stop solution produced a colour change from blue to yellow).
9. The plates were then read on a plate-reader at 450 nm, a digital print-out obtained and the results recorded on disk.

Determination of Antibody Cross-reactivity with Non-target Species

In order to screen field collected predators to detect feeding on vine weevil, it was necessary to determine if the antisera would cross-react with predator proteins and give false positives. Two concentrations of the AS_1 antiserum were used (1:5000 & 1:10,000) to screen four dilutions of non-target antigens (1:100, 1:1000, 1:10,000 & 1:100,000) and compare them with vine weevil. Initial screening was against adults of three carabid species (*Trechus quadristriatus*, *Notiophilus biguttatus* and *Amara eurynota*), 2 larval carabids (*Pterostichus melanarius* and *Nebria brevicollis*) and 3 non-carabid species (Salticid spider, *Coccinella septempunctata* and a millipede). All individuals were starved for a minimum of 24 hours prior to the start of the experiment.

Cross-reactions were observed with the beetles, and weaker ones with the non-Coleoptera species. It was, therefore, necessary to cross-absorb the antiserum against predator proteins to remove the cross-reactions.

Cross-Absorption of the Antiserum

Since it had already been established that the two antisera were similar, only one of them, AS_1, was cross-absorbed initially to try to reduce the cross-reactions observed with non-target species.

A *Pterostichus madidus* adult, two *Trechus quadristriatus* adults, two *Notiophilus*

biguttatus adults and three *Nebria brevicollis* larvae (total combined weight 0.2g) were homogenised in 1.0ml PBS to produce a 1:5 solution which was spun at 13,000 rpm for 5 minutes. The supernatant was removed immediately and 250 µl added to 1ml of the AS_1 antiserum, mixed and incubated for two hours at room temperature in an eppendorf tube. The sample was centrifuged at 13,000 rpm for 5 minutes and the supernatant drawn off. The procedure was repeated twice more, one incubation for two hours at room temperature and the final one overnight at 4 °C. This precipitated out all proteins that cross-reacted. Distilled water was added to the final supernatant to make a volume of 10ml.

Manufacture & Purification of IgG from Cross-absorbed & Non Cross-absorbed Antisera

Immunoglobulin (IgG) is the concentrated antibody fraction of the antiserum. It was purified from the cross-absorbed AS_1 antiserum as its use in the final ELISA would make the system more sensitive than if using whole antiserum. This was performed by the ammonium sulphate precipitation method, based on the fact that immunoglobulins are precipitated at lower concentrations of ammonium sulphate than most other serum proteins. This method enriches the concentration of immunoglobulins in the final fraction, although it does not provide complete purity. IgG was also purified from non cross-absorbed antiserum to allow the true effect of cross-absorption to be assessed. The precise protocol is given in the Appendix.

Comparison of Cross-absorbed IgG & Non Cross-absorbed IgG

The effect of cross-absorbing the antiserum was determined by a direct ELISA. Eleven species were screened for cross-reactions with the IgG. They were adult *Pterostichus madidus*, *Amara eurynota*, *Notiophilus biguttatus*, *Trechus quadristriatus*, *Tachyporus hypnorum*, larvae of *Pterostichus melanarius* and *Nebria brevicollis* and the following species from other taxonomic groups; *Coccinella septempunctata*, *Forficula auricularia*, a salticid spider and a millipede. In addition, vine weevil larval antigen was tested to determine how much of the polyclonal's activity had been lost during the cross-absorption.

The antigens were all applied at a concentration of 1:1000 w/v in PBS. IgG was applied in a 5-fold dilution series (at 10, 2, 0.4 & 0.08 µg/ml). Duplicate cells of each treatment were set up.

Cross-reactions between Developmental Stages of Vine Weevil

Both antisera were originally raised against larval vine weevil antigens, but it was necessary to check the degree of cross-reactivity to adult and egg material. Therefore, starved adult, starved larva, and egg antigen preparations were made at four concentrations (1:100, 1:1000, 1:10,000 & 1:100,000 w/v) in PBS by the usual method. Cross-absorbed IgG was applied at 2 µg/ml and 10 µg/ml.

Comparison of Results given by Different Batches of Cross-absorbed IgG and the Effects of Storage

Six months after the original batch of IgG was prepared, a second batch was produced.

The AS_1 antiserum was cross-absorbed against one *Pterostichus madidus*, one *Notiophilus biguttatus* and two *Harpalus aeneus* adults. Since the two batches had been cross-absorbed against different materials, they were compared on a single ELISA plate against seven different dilutions of vine weevil egg, larva and adult antigens. This experiment also determined if IgG deteriorated during storage. IgG was applied at 10 µl/ml.

Analysis Of The Protein Composition Of Vine Weevil By Electrophoresis (Sds-Page), Western Blotting And Immunoperoxidase Staining.

All recipes for stock reagents and the precise protocol used in this chapter are to be found in the Appendix.

Separate developmental stages of vine weevils were homogenised and spun at 13,000 rpm to remove any particulate matter. A discontinuous gel was run with 1:150, 1:300 and 1:600 dilutions of egg, larva and adult samples. Broad range molecular markers were used to identify the size of the proteins. The gel was then stained with Coomassie Blue.

An identical unstained gel, which had been run simultaneously, was blotted onto nitrocellulose paper and probed with IgG prepared from AS_1 to determine whether the recognition was based on a few or several proteins in each stage.

PRODUCTION AND SELECTION OF MONOCLONAL ANTIBODIES (Mabs) FOR USE IN DETECTING PREDATION ON VINE WEEVIL

This section reports the production and selection of a number of MAbs specific to different developmental stages of vine weevil. The protocol is given in greater detail in the Appendix. In three separate fusions, stage-specific MAbs were produced to egg and adult antigens, as well as those recognising egg-plus-adult, larva-plus-adult, and all three stages using the standard protocol of Galfre & Milstein, 1975. Supernatant screening of hybridoma cells was performed using an indirect ELISA protocol, originally described by Voller *et al.* (1976). These MAbs were initially screened with various life stages of seven predatory arthropod species (Table 2), to ensure that they did not cross-react. The selected MAbs were subsequently screened against other arthropod species (Table 3).

Table 2: Predator Species Initially Screened for Cross-reactivity

Predator Species	Stage Screened
Amara similata	Adult
Calathus fuscipes	Adult
Harpalus latus	Adult
Harpalus rufipes	Adult
Nebria brevicollis	Adult & Larva
Pterostichus madidus	Adult
Pterostichus melanarius	Adult & Larva

Table 3: Species Subsequently Screened for Cross-Reactivity

Species	Stage Screened
Carabidae:	
Abax parrallelopipedus	Adult
Agonum dorsale	Adult
Asaphadion flavipes	Adult
Badister bipustulatus	Adult
Bembidion lampros	Adult
Carabus violaceus	Adult
Harpalus aeneus	Adult
Harpalus rufipes	Egg
Leistus spinibarbis	Adult
Notiophilus biguttatus	Adult
Pterostichus niger	Adult
Trechus quadristriatus	Adult
Staphylinidae:	
Ocypus olens	Adult & Egg
Tachyporus hypnorum	Adult
Curculionidae: (Weevils)	
Anthonomus rubi	Adult
Apion miniatum	Adult
Phyllobius pomaceus	Adult
Polydrosus sericeus	Adult & Pupa
Dermaptera:	
Forficula auricularia (European Earwig)	Adult

Antibody class and sub-class (isotype) were characterised using an ISOStrip mouse monoclonal antibody isotyping kit. This information was needed because each isotype requires a different protocol to purify it. Monoclonal antibodies are more stable when stored after they are purified and freeze-dried.

Results And Discussion - Fieldwork

Survey Of Polyphagous Predators - Rock's Farm 1994

A number of large and medium sized predatory beetle species were found to be common. The number of predators caught daily was standardised by dividing the catch size by the number of nights since the trap was last emptied. Figure 2a illustrates how the number of predators caught changed throughout the season. Beetle catches were highly variable during the trial, although numbers decreased in September.

The relative number of each species caught in the traps altered during the experiment (Figures 2b-g). Highest numbers of *Pterostichus melanarius* and *Pterostichus madidus* were caught before the end of August, whereas *Nebria brevicollis* and *Ocypus olens* did not appear in the traps until late August. Numbers of these species then increased to a maximum two weeks later before tailing off again by mid-October. *Calathus fuscipes* was caught consistently throughout the trial. This demonstrated that although most species were only active for part of the summer, there was a high degree of overlap between species, so that there was always a number of species active in any period.

Pitfall trap catches do not provide an absolute index of population size, but rather reflect the activity of the species at the time they are caught as well as the size of the population. The more active an individual is, the more likely it is to encounter a pitfall trap. Periods of activity may correspond with mating, or intensive feeding prior to egg-laying or overwintering. A number of factors affect the efficiency of pitfall traps, including their size and the material from which they are constructed (Luff, 1975). In general, designs that are best for catching medium to large carabids tend to be poor at catching small species, and *vice versa*. These factors must be taken into account when analysing their contents. This survey may underestimate the true population of the smaller species which are of interest as potential predators of eggs and young larvae.

The influence of different plant species is summarised in Table A2 of the Appendix. Plants fell into three categories; those with high, medium or low catches of predators. Although there was a large difference between species such as Coriander and Buckwheat which had high catches, and Carrot and Marigold which had relatively few, the other ten plant species were fairly similar. The total numbers of predators caught on each plant species were analysed by the chi-squared (χ^2) test using Genstat 5 software to determine if there were significant differences between them. The catches of six individual predator species were also analysed for significant differences in abundance on different plants. The results are summarised in Table 4. They indicate that the abundance of each species varied significantly between plant species. For example, catches of *Calathus fuscipes* varied from 11 found on Carrot to 62 on Selfheal, a difference that was significant at $p<0.001$. However, if the analysis is repeated after the removal of those plant species with extremely high or low catches (Coriander, Buckwheat, Bellflower, Phacelia, Oxeye Daisy, Corn Marigold and Wild Carrot), as shown in Table 5, each of the remaining seven species have similar abundances of predators. There are similarities in their total catches (chi-squared = 3.9775 at $p= 0.6797$ with 6 df), and between the populations of some individual predator species (eg. numbers of *Nebria brevicollis* are similar at the $p= 0.0478$

Figure 2a: Adjusted Daily Pitfall Trap Catches at Rock's Farm, 1994.

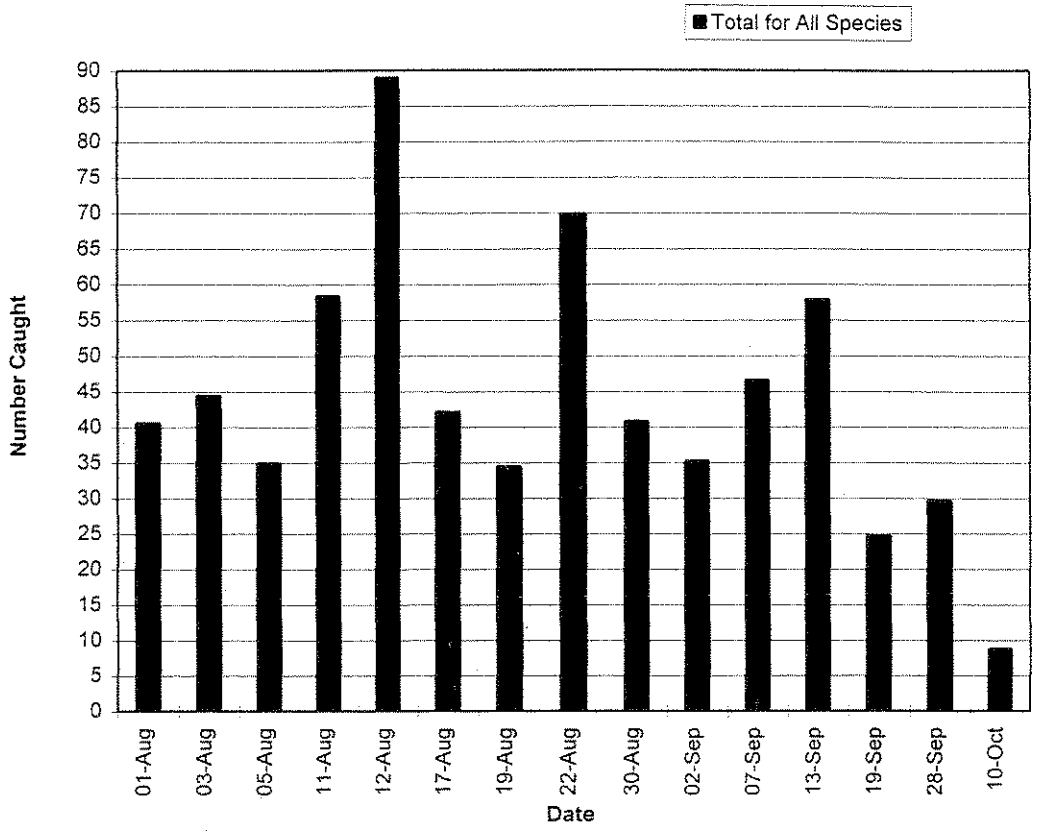


Figure 2b: Adjusted Daily Catches at Rock's Farm, 1994.

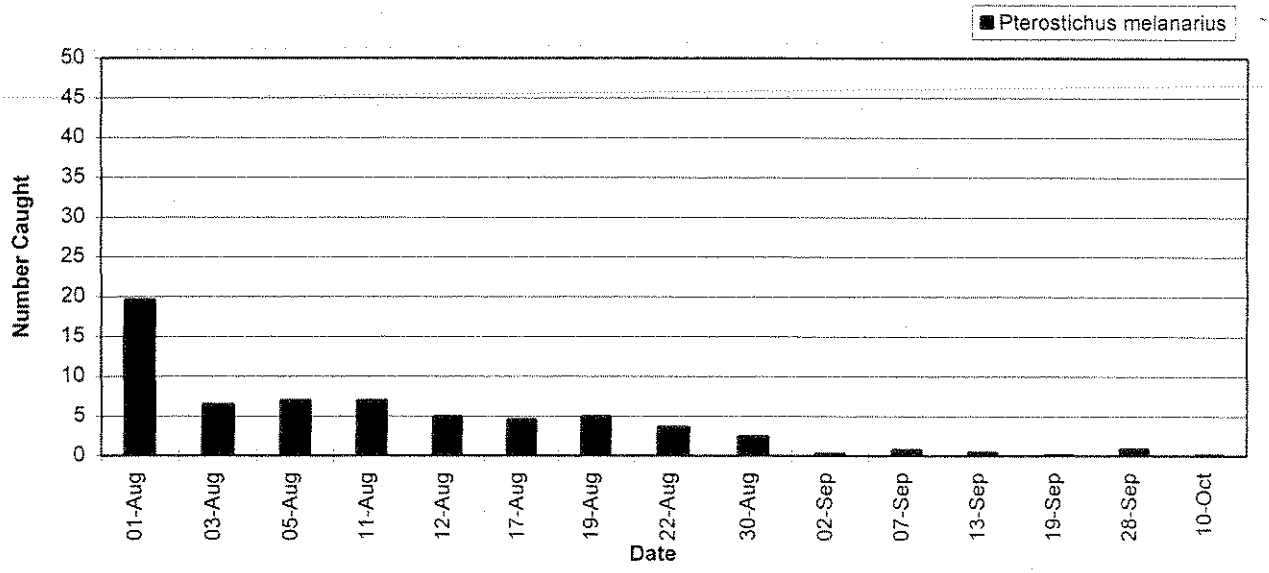


Figure 2c: Adjusted Daily Catches at Rock's Farm, 1994

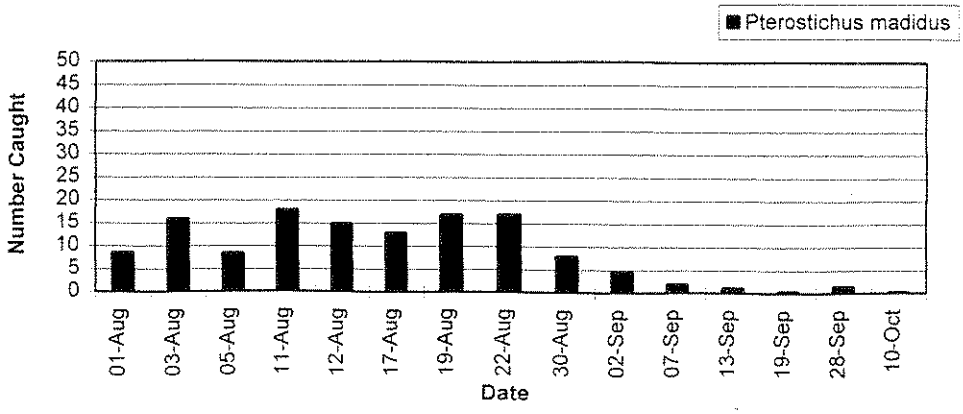


Figure 2d: Adjusted Daily Catches at Rock's Farm, 1994.

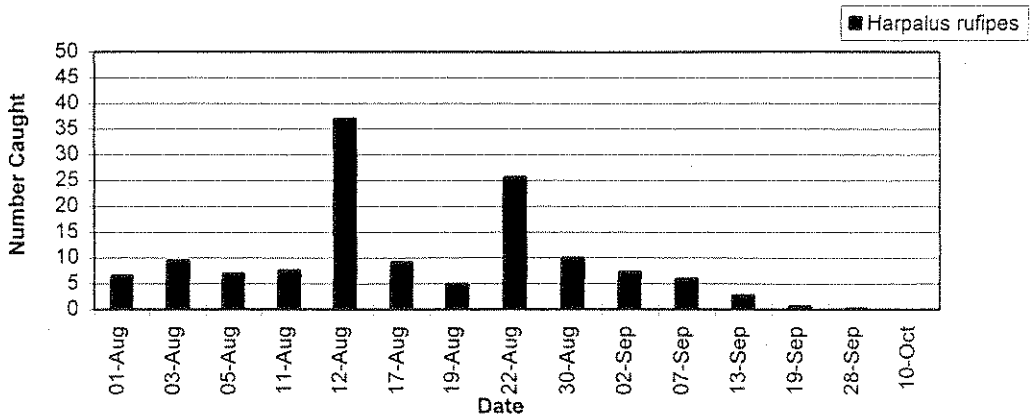


Figure 2e: Adjusted Daily Catches at Rock's Farm, 1994.

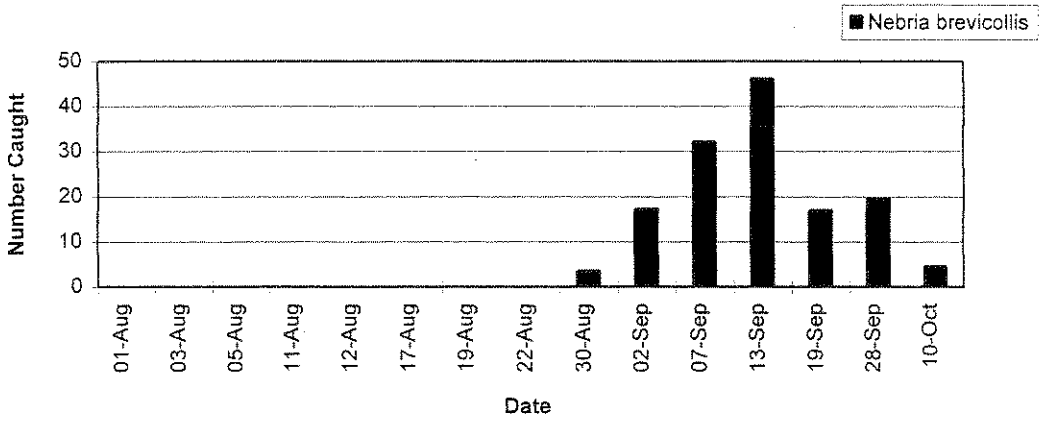


Figure 2f: Adjusted Daily Catches at Rock's Farm, 1994.

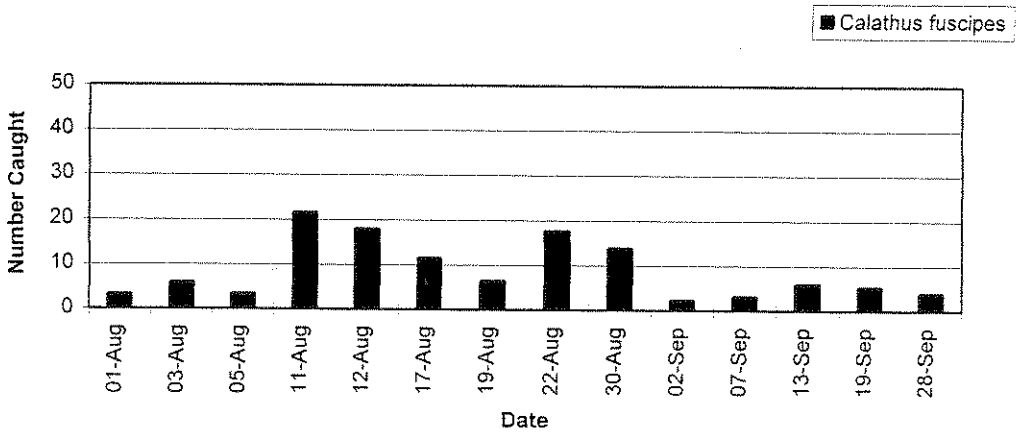
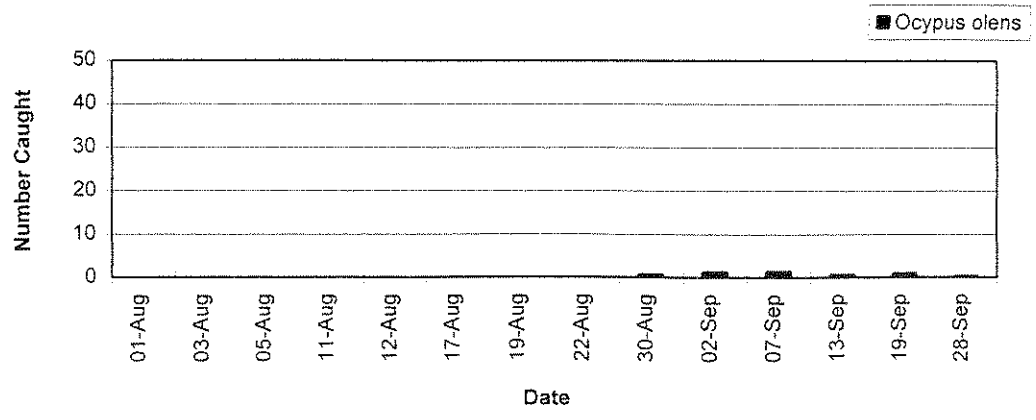


Figure 2g: Adjusted Daily Catches at Rock's Farm, 1994.



level).

Table 4: $\chi 2$ test for significance between number of predators found on 14 plots each with a different individual plant species.

PREDATOR SPECIES	MINIMUM	MEAN	MAXIMUM	χ^2 VALUE	DEGREES OF FREEDOM	p VALUE
Calathus fuscipes	11	43.07	62	60.6186	13	<0.001
Nebria brevicollis	8	60.93	125	265.9332	13	<0.001
Pterostichus madidus	9	34.86	66	105.3361	13	<0.001
Harpalus rufipes	8	32.00	94	287.3125	13	<0.001
Pterostichus melanarius	4	16.36	35	69.0349	13	<0.001
Harpalus aeneus	0	3.93	11	36.3818	13	<0.001
Total of all species	58	204.40	289	225.8071	13	<0.001

Table 5: $\chi 2$ test for significance between number of predators found on 7 plots each with a different individual plant species (cow parsley, selfheal, poppy, corn cockle, bird's foot trefoil, cornflower & corn chamomile).

PREDATOR SPECIES	χ^2 VALUE	DEGREES OF FREEDOM	p VALUE
Calathus fuscipes	12.7159	6	0.0478
Nebria brevicollis	104.4595	6	<0.001
Pterostichus madidus	57.7153	6	<0.001
Harpalus rufipes	43.6795	6	<0.001
Pterostichus melanarius	23.4118	6	<0.001
Harpalus aeneus	13.4839	6	0.0360
Total of all species	3.9775	6	0.6797

Although a single plant species was not expected to influence beetle populations, it was possible that the growth pattern of the plant may have an effect; ie, the density of the plants and the degree of ground-cover they produced. No clear patterns were established between vegetation type and predator numbers. A combination of factors, including soil dampness and prey availability, may have obscured any direct relationship between predator abundance and plant type. However, this experiment identified a range of predators which were active during the period that vine weevil adults are laying eggs, and the larvae are hatching, migrating to the roots and actively feeding.

The Calves Ley Field Trial: Enclosure & Exclusion Plots

This field trial was assessed in April 1995. No leaf-notching was found when the strawberry plants were inspected, confirming that no adult vine weevils had emerged. This was an important check because, unlike carabids, the adult weevils are capable of climbing out over the barriers. Fluon, painted under the external lip of the barrier, prevented any vine weevils present in the field entering the plots, but could not prevent newly emerged adults escaping from the plots.

	Initial Number of Eggs	No. Larvae Recovered
Predators Present	500	6
Predators Absent	500	12

Chi-squared test for difference in proportion of eggs recovered = 1.41 on 1 df (NS)

There was no significant difference between the numbers of surviving larvae in the two treatments. A total of six larvae were collected from the ten plots which contained predators, while twelve were recovered from the controls. Both had initially been inoculated with 500 eggs.

It was found that carabid adults and larvae were able to tunnel under the barriers, which were sunk to a depth of 7-10 cm. This obscured any differences in vine weevil mortality which may have been due to predation. Independent egg and larval mortalities were probably high due to the sudden onset of cool wet weather in September 1994 while the trial was being established. Therefore, a second field trial was established in 1995 to examine predation effects using a different type of enclosure/exclusion plot in the next season.

East Egham Field Trial: Enclosure & Exclusion Plots

This field trial was assessed in March 1997. In this field trial the barriers extended 45 cm below the soil surface, and 60 cm above it. The two infestation rates of 21 and 3 vine weevils per plant, are termed 'high' and 'low' densities, but are in fact both representative of large populations on a field-size scale. Five plants were sampled from each plot, and the number of larvae recovered from the soil was recorded. The means of the log-transformed data per 100 plants are given below, with the untransformed means given in parenthesis.

	High vine weevil density		Low vine weevil density	
Predators present	2.04	(180)	1.14	(26)
Predators absent	2.29	(270)	2.03	(124)

The data have 12 degrees of freedom and the s.e.d. is 0.38.

The analysis shows that at the lower vine weevil density, the effect of the presence or absence of predators was statistically significant, but there was no significant difference in the number of larvae recovered from the two treatments at the higher vine weevil density. The results reflect the high natural mortality, that is expected for all vine weevil eggs laid outdoors. The impact of predators on the low vine weevil density plots indicates their potential to provide a useful contribution to the reduction of vine weevil populations in commercial plantings.

Survey Of Predators Found In Commercial Farms

The results of the pitfall sampling programme are shown in Table 6.

Table 6: Summary of Predators caught in Commercial Farms

Predator Species	Harefield Field, Norham Farm	Dogleg Field, Norham Farm	Baldwins' Field, Upper Horton Farm	Andrew's Field, Upper Horton Farm	Westfield NW Gore Farm	Store Field Wilford Court Farm	Totals
	Blackcurrant 30.5.96-24.10.96	Blackcurrant 14.5.96-24.10.96	Blackcurrant 10.5.96-7.8.96	Blackcurrant 2.9.96-24.10.96	Strawberry 30.5.96-19.8.96	Strawberry 4.6.96-7.8.96	
Harpalus rufipes (c)	866	1939	902	14	233	1	3955
Nebria brevicollis (c)	297	380	392	375	28	17	1489
Ocypus olens (s)	239	147	0	1	0	0	387
Bembidion lampros (c)	228	139	8	1	0	0	376
Notiophilus biguttatus (c)	111	98	26	8	1	3	247
Harpalus aeneus (c)	12	111	97	6	12	1	239
Carabus violaceus (c)	105	130	1	0	0	0	236
Calathus fuscipes (c)	120	61	22	4	7	0	214
Pterostichus melanarius (c)	32	57	62	3	3	0	157
Pterostichus madidus (c)	24	107	22	0	0	0	153
Asaphadion flavipes (c)	38	24	0	0	0	0	62
Carabid larvae	30	23	0	5	0	0	58
Pterostichus niger (c)	15	31	0	0	0	0	46
Trechus quadristriatus (c)	17	27	0	0	0	0	44
Other staphylinids, including *Tachyporus hypnorum*	10	19	10	4	2	0	45
Other carabid species (10) with <20 individuals	24	46	15	15	7	0	107
Totals	2168	3339	1557	436	293	22	7815

(c) = carabid species
(s) = staphylinid species

The period of sampling varied between sites because the strawberry fields were grubbed after harvest; the Baldwin Field of blackcurrants was also grubbed after harvest, and pitfall traps

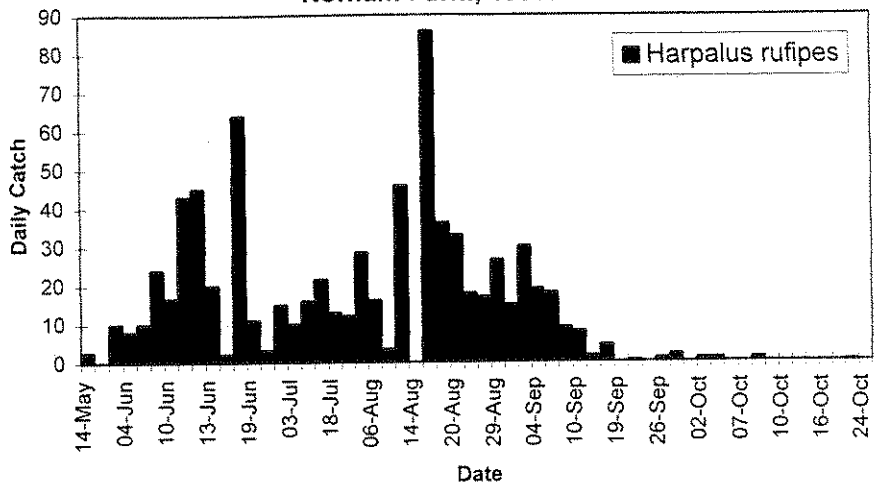
were then established in the adjacent field (Andrews Field) to continue the survey. In Andrew's Field, the absence or low abundance of species other than *Nebria brevicollis* is unsurprising after looking at Figures 3f which show the time of year each species is most active. These indicate that after September, only *Nebria brevicollis* and *Ocypus olens* were at their most active. Although *Calathus fuscipes* and *Notiophilus biguttatus* were caught throughout the survey period, most other species had specific periods of activity when they were caught in large numbers (3a- 3f). There was considerable overlap between species so that there were always a number of different species active at the same time.

Very few predators were found in the two strawberry fields, compared to either the blackcurrant plantations, or to other strawberry fields investigated since. Both these sites had a high usage of pesticides.

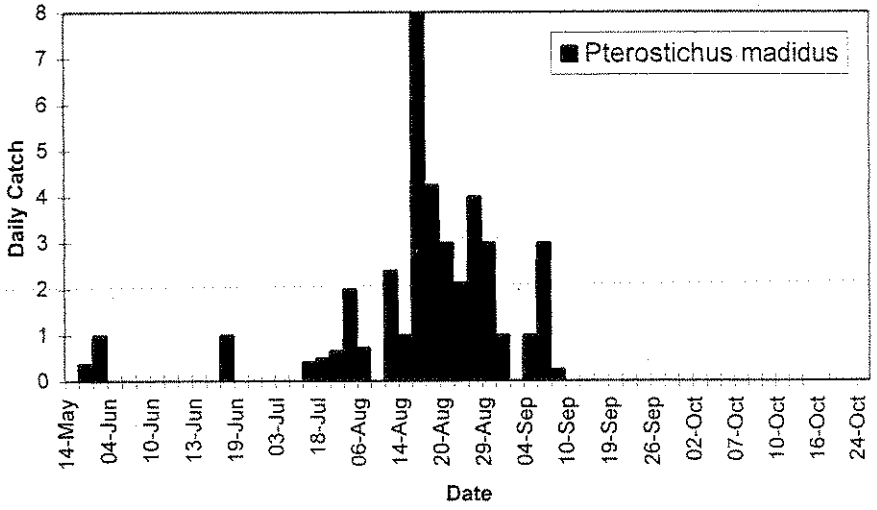
A number of species were found in similar numbers at Norham and Upper Horton Farms, namely *Harpalus rufipes* and *Nebria brevicollis*, which were the most abundant; followed by *Notiophilus biguttatus, Harpalus aeneus, Calathus fuscipes, Pterostichus melanarius* and *Pterostichus madidus*. However, the following were common at Norham Farm but absent from Upper Horton; *Ocypus olens*, *Bembidion lampros* and *Carabus violaceus*. Other species were found in modest numbers at Norham Farm but were again absent from Upper Horton Farm. *Carabus violaceus*, in particular, is a common and widely distributed beetle, so it is unlikely that it had simply failed to colonise the fields. The blackcurrants had been sprayed with chlorpyrifos during the previous winter. The answer to the absence of some species at Upper Horton Farm may lie in differential susceptibilities to insecticides.

Figures 3a- 3f

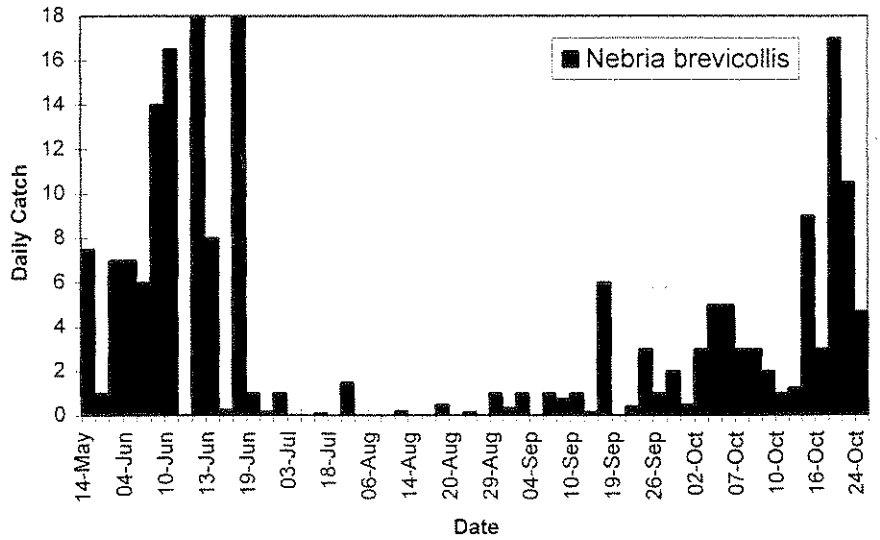
(a) Adjusted Daily Catches from Dogleg Field, Norham Farm, 1996.



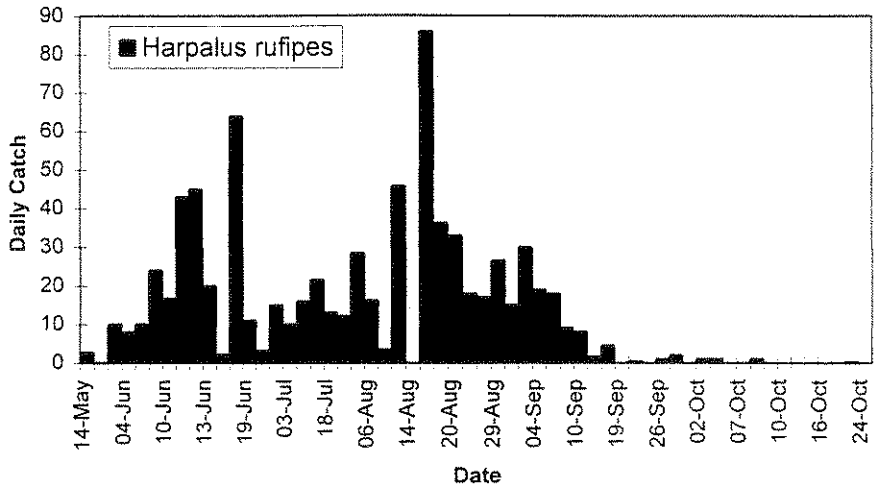
(b) Adjusted Daily Catches from Dogleg Field, Norham Farm, 1996.



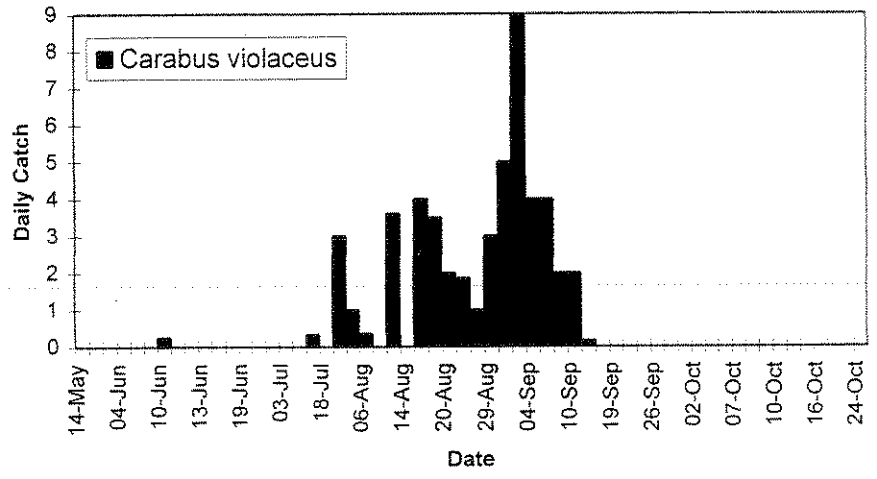
(c) Adjusted Daily Catches from Dogleg Field, Norham Farm, 1996.



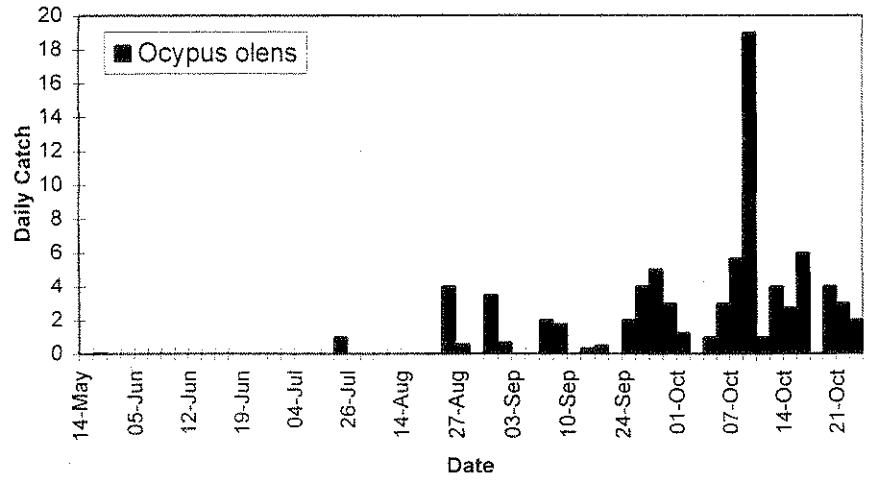
(d) Adjusted Daily Catches from Dogleg Field, Norham Farm, 1996.



(e) Adjusted Daily Catches from Dogleg Field, Norham Farm, 1996.



(f) Adjusted Daily Catches from Dogleg Field, Norham Farm, 1996.



Gut Content Analysis Of Predators Caught In Commercial Farms

The results of the investigation of predation on vine weevils using MAbs are shown in Table 7.

Table 7: Summary of Predator Gut Content Analysis Results

Species	Number of beetles assayed	% positive for vine weevil eggs material	% positive for vine weevil larvae material	% positive for vine weevil adults material
Harpalus rufipes	1050	4.7	18.6	8.2
Nebria brevicollis	455	2.9	8.6	4.6
Ocypus olens	160	5.6	4.4	4.4
Bembidion lampros	80	6.2	10.0	7.5
Notiophilus biguttatus	75	13.3	22.4	6.6
Calathus fuscipes	73	4.1	2.7	9.6
Harpalus aeneus	66	4.5	9.1	7.6
Carabus violaceus	64	3.1	10.9	32.8
Pterostichus madidus	51	0.0	19.6	7.8

Although ELISA is highly sensitive, being able to detect minute quantities of prey material consumed, it is unable to distinguish between predation, secondary predation (the predator has eaten something that had itself fed on vine weevil previously) and scavenging (Sunderland, 1996). In the case of *Bembidion lampros* and *Notiophilus biguttatus*, the adult vine weevil material detected in their gut is likely to have been a result of scavenging, since they are too small to kill an adult vine weevil. Adult predation by other species was greatest during the period just after emergence when their bodies are still soft, and medium sized beetles such as *Harpalus rufipes* and *Calathus fuscipes* are able to kill adult vine weevils more easily. The larger beetles, *Carabus violaceus*, *Pterostichus madidus* and *Ocypus olens* (the Devil's Coach-horse beetle) were observed to be very predatory in the laboratory, and the ELISA results show that this is also true in the field, particularly of *Carabus violaceus*. *Ocypus olens* is a voracious predator, able to kill most arthropod species that it encounters. The relatively low incidence of vine weevil remains in their guts is probably a reflection their very diverse diet.

The majority of the predators under examination are very mobile through the soil, and most have been observed digging tunnels when kept in laboratory cultures. One exception was *Calathus fuscipes*, which may explain why it fed on egg and adult stages, but much less on vine weevil larvae. Both *Bembidion lampros* and *Notiophilus biguttatus* are able to move through the soil following cracks and crevices, and were found to have fed on the youngest larval stages. These two species, which are also the smallest in size, were the most important predators of eggs. Table 8 below summarises the three major predators of each vine weevil stage.

Table 8. Major Predators of each vine weevil stage

Predators of Eggs	Predators of Larvae	Predators of Adults
Notiophilus biguttatus *Bembidion lampros* *Ocypus olens*	*Notiophilus biguttatus* *Pterostichus madidus* *Harpalus rufipes*	*Carabus violaceus* *Calathus fuscipes* *Harpalus rufipes*

Results And Discussion- Laboratory Studies

Development Of The Polyclonal Antisera And Subsequent Testing

Dilution Series Results

The titres of the two antisera, AS_1 and AS_2, were similar. Both gave positive readings at large dilutions of antigen and antiserum. Background levels were very low. The dilution series experiment showed that 1:12,500 dilutions of both antisera could detect a 1:10,000 diluted antigen sample (AS_1 A_{450}=0.283; AS_2 A_{450}=0.267; PBS negative control A_{450}=0.012). A 1:2,500 dilution of the antisera could positively recognise a 1:100,000 of antigen (AS_1 A_{450}=0.390; AS_2 A_{450}=0.365; PSB negative control A_{450}= 0.012). Freshly prepared larval vine weevil antigen was compared with frozen samples to investigate any effect of freezing on the sample. No difference was detected between frozen and fresh antigen material.

Comparison of Cross-absorbed IgG & Non Cross-absorbed IgG

Cross-reactions between the IgG and eleven non-target species (adult *Pterostichus madidus, Amara eurynota, Notiophilus biguttatus, Trechus quadristriatus, Tachyporus hypnorum*, larvae of *Pterostichus melanarius* and *Nebria brevicollis* and the following species from other taxonomic groups; *Coccinella septempunctata, Forficula auricularia,* a salticid spider and a millipede) were largely removed by cross-absorption. Cross-absorbed IgG, at a concentration of 2 µg/ml, was still able to recognise vine weevil, having lost little of its activity. The results are summarised in Figure 4.

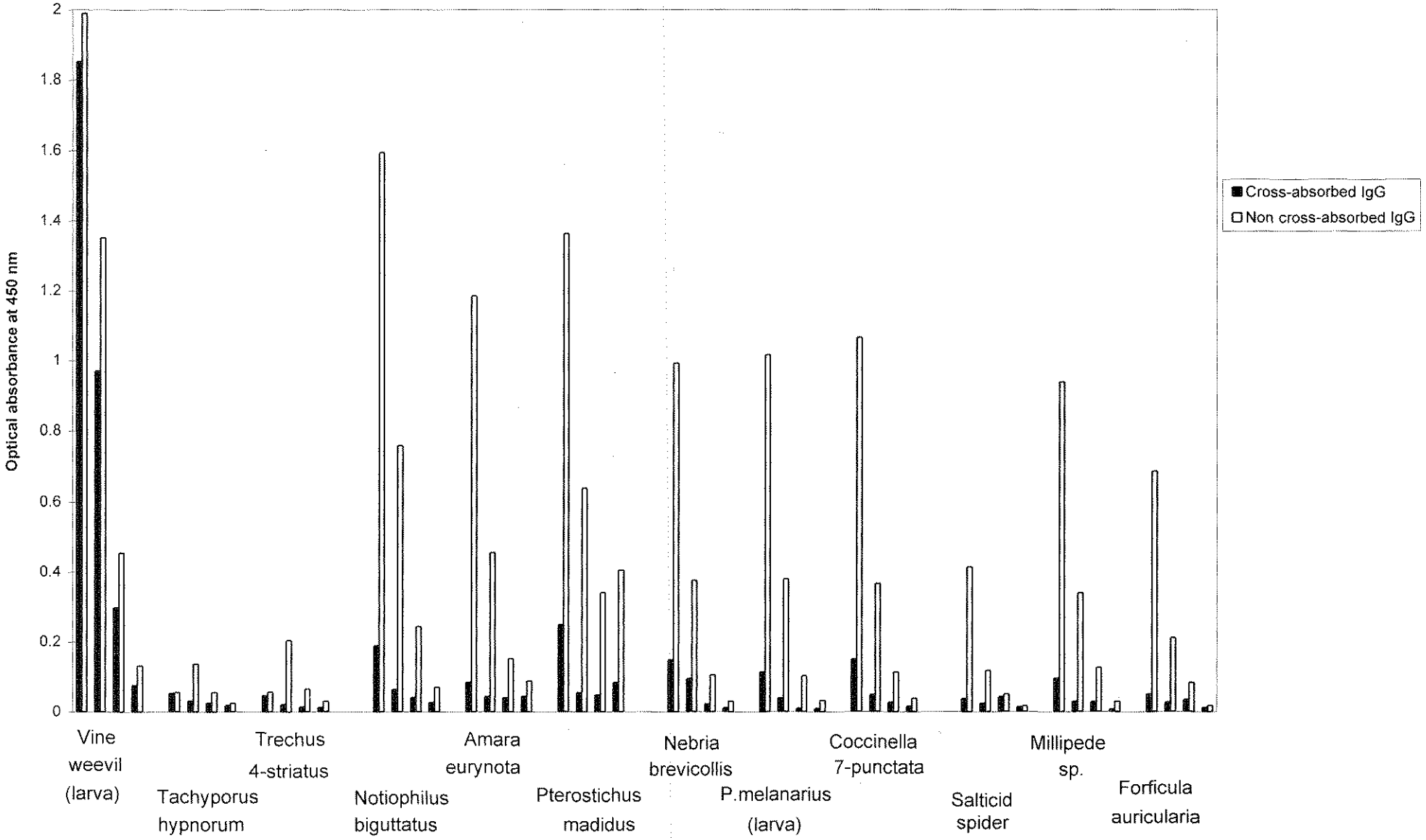
Cross-reactions between Developmental Stages of Vine Weevil

The results are summarised in Figures 5a & 5b, indicating that although the polyclonal antiserum was raised against larval antigens, it recognised all vine weevil developmental stages; however it was unable to distinguish between them. Interestingly, the highest antigen concentration (1:100) did not give the highest reading, possibly due to inhibition.

Comparison of Results given by Different Batches of Cross-absorbed IgG and the Effects of Storage

The results illustrated in Figure 6 indicate that there is no deterioration of IgG over a six month period, as both freshly prepared and stored IgG produced similar readings. Secondly, Figure 7b shows that at a concentration of 10 µg/ml, the freshly-prepared IgG recognised all vine weevil stages, even when they were diluted to 1:50,000. After examining the results given in Figures 5a, 5b, and 7, it was concluded that the IgG would be used in future at 10 µl/ml.

Figure 4: Cross-reactivity of Cross- & Non Cross-absorbed IgG with non-target Antigens



Antigen: Each species has IgG applied in a 5-fold dilution series (10, 2, 0.4 & 0.08 µg/ml)

Figure 5a: Reactivity of Vine Weevil Stages to IgG at 10 µl/ml

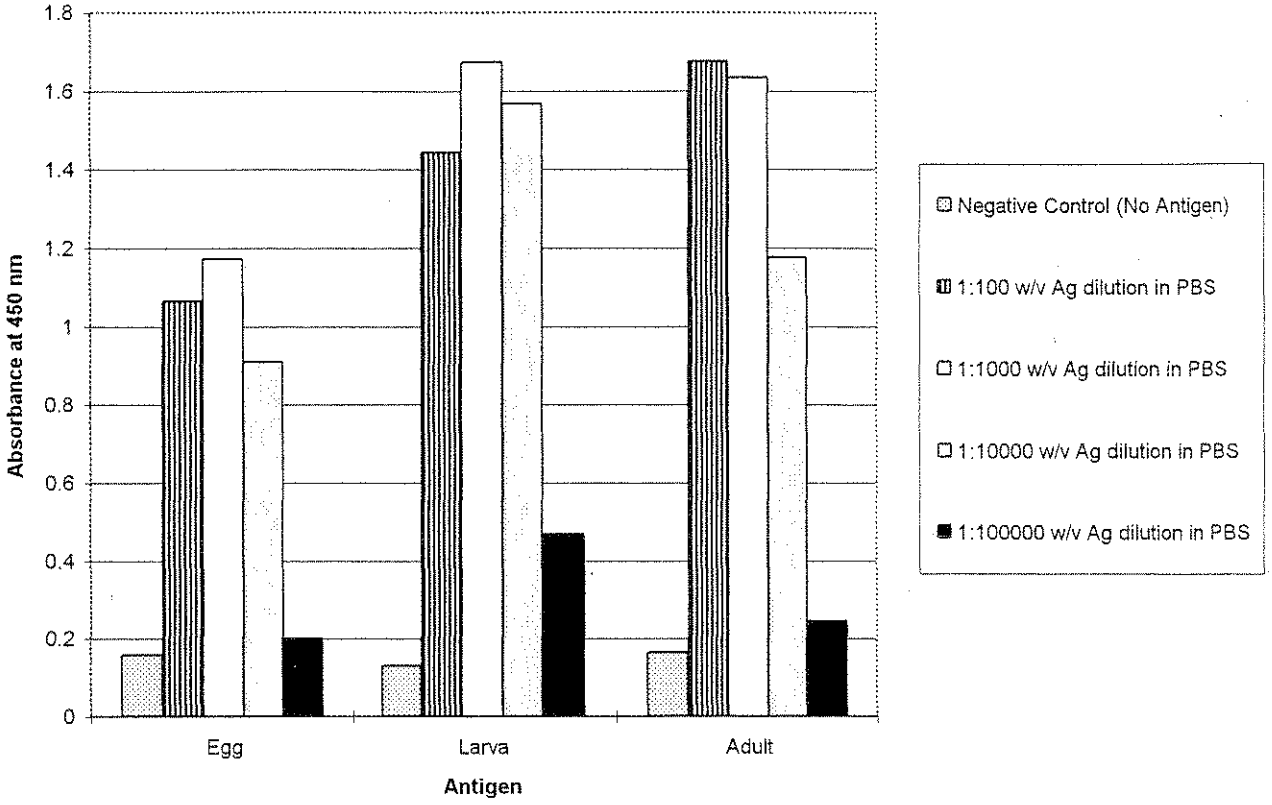


Figure 5b: Reactivity of Vine Weevil Stages to IgG at 2 µl/ml

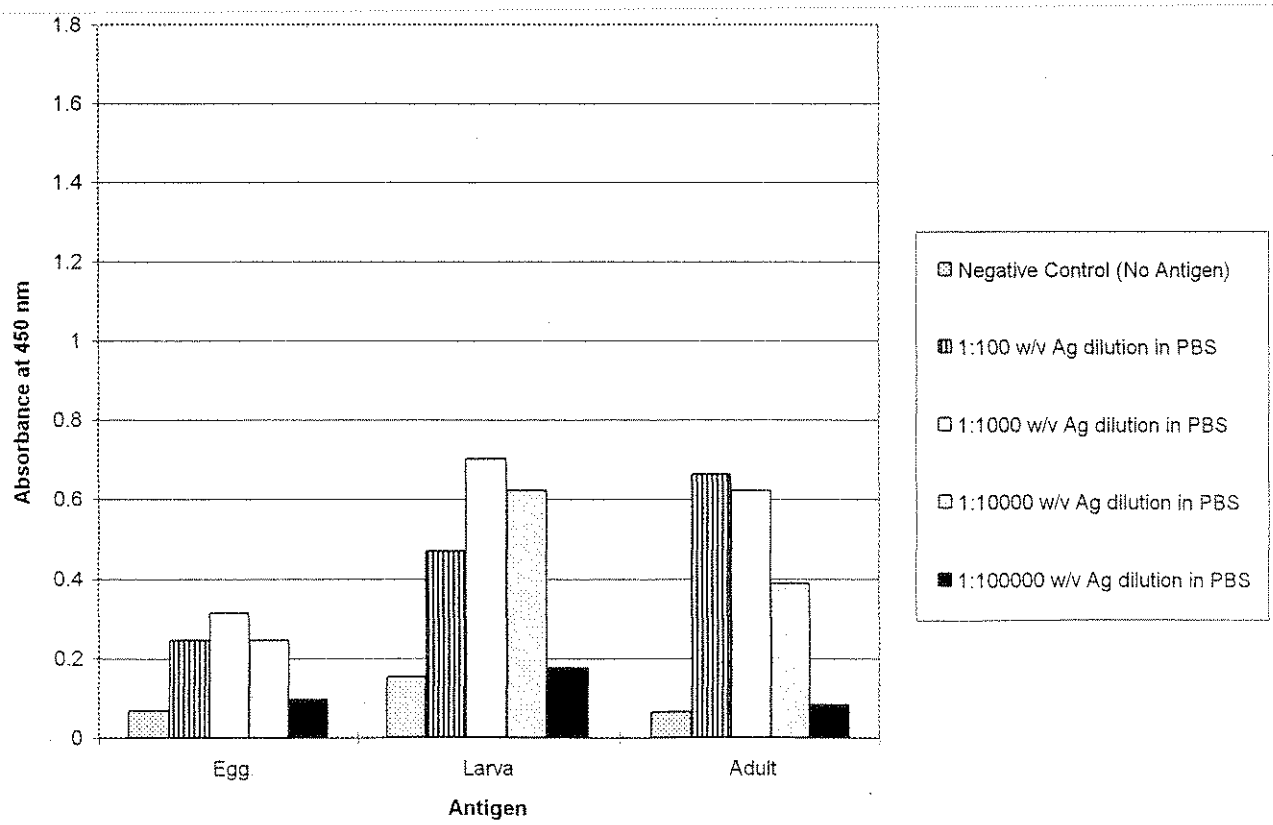


Figure 6: Comparison of Stored and Fresh IgG (applied at 10µg/ml)

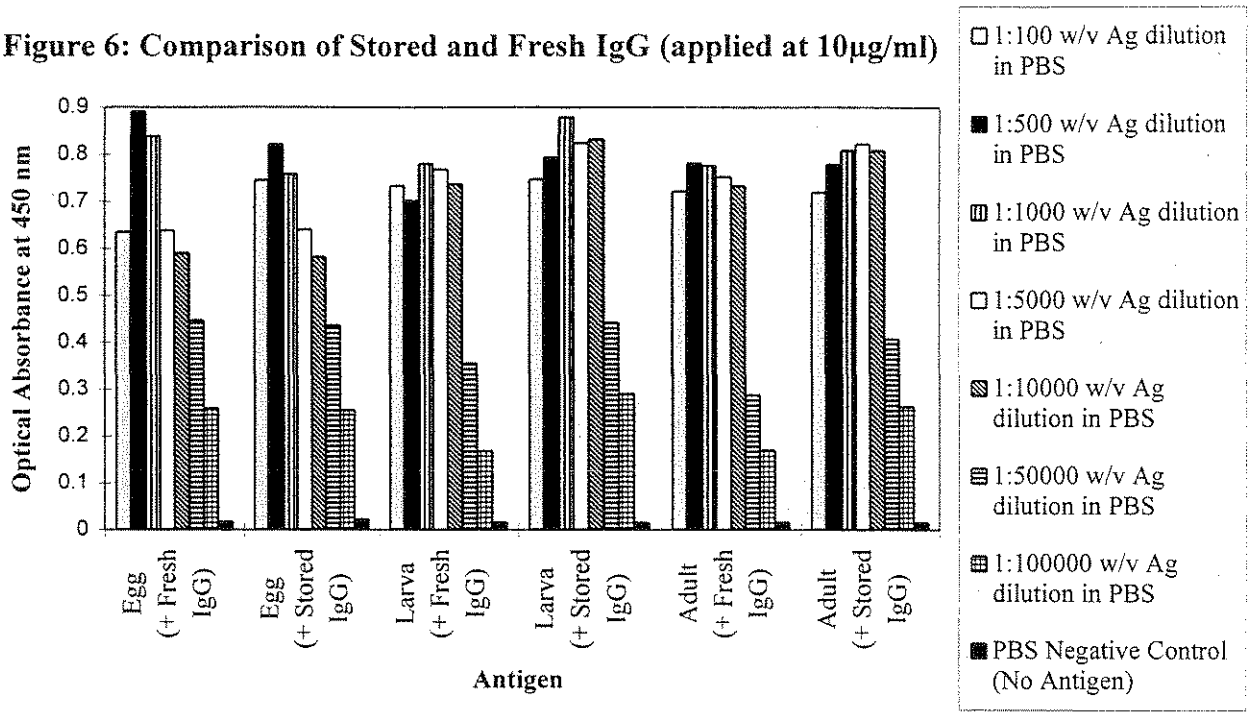
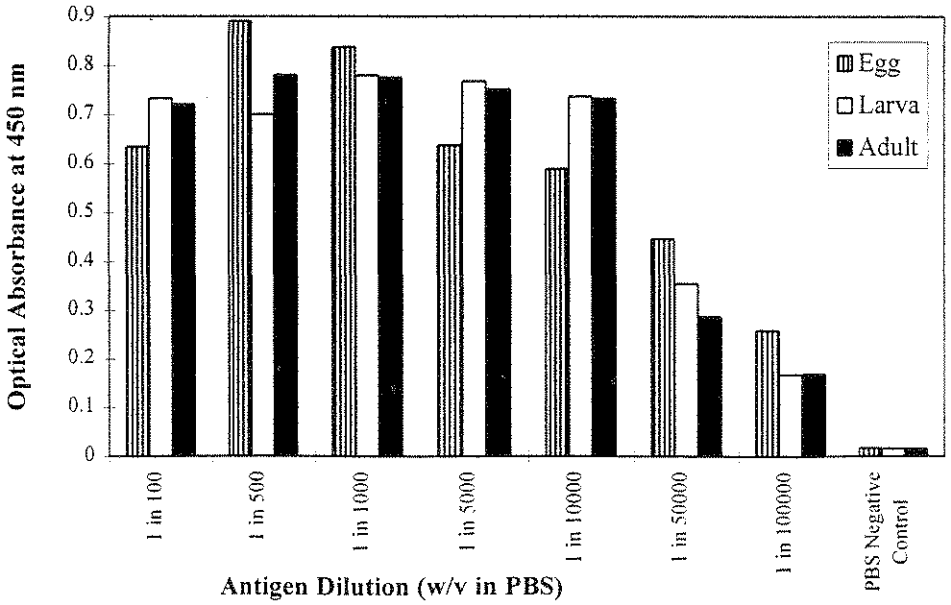


Figure 7: Reactivity of Vine Weevil Stages to Fresh IgG at 10 µg/ml



Analysis Of The Composition Of Vine Weevil Proteins By Electrophoresis And Western Blotting

Electrophoretic Comparison of Total Protein Content of Egg, Larva & Adult Vine Weevil

After staining with Coomassie Blue, the protein bands were most easily visible in the 1:150 and 1:300 dilutions. Egg-specific bands were observed, two with very similar high molecular weights (approximately 110,000 KDa) which appeared as a single band at 1:150 and 1:300 dilutions but were clearly resolved at 1:600, and a third protein with a very low molecular weight (approximately 10,000 KDa), close to the travelling front.

Western Blotting & Probing the Gel with AS_1 IgG

After probing with the polyclonal antiserum, a number of proteins, common to all three developmental stages, were recognised. In addition, two proteins of approximately 100,000 KDa and a third of 28,000 KDa, which were present in both larvae and adults, but absent from the eggs, were identified from the blot.

The egg-specific proteins which were found in the electrophoretic gel stained with Coomassie Blue were not identified by the polyclonal antiserum. This was expected, as the polyclonal antiserum was originally raised against larval vine weevil material which did not contain this protein, and therefore antibodies would not have been raised against it.

These techniques revealed the protein composition of the different vine weevil stages. Some proteins were specific to a particular stage, others were common to all stages. The polyclonal antiserum recognised some of the common proteins, and therefore responded to all the stages.

The fact that each stage had a number of specific proteins led to the decision to develop a panel of monoclonal antibodies with the expectation of them constituting a stage specific detection technique.

Production And Selection Of Monoclonal Antibodies For Use In Detecting Predation On Vine Weevil

Three separate fusions were carried out, one against each of the three vine weevil stages. Of the 239 positive hybridomas screened against predator antigens, 89 were vine weevil specific. Table 9 lists the final panel of fifteen monoclonal antibodies (MAbs), which were selected for their specificity and sensitivity. Stage-specific MAbs were raised against adult and egg antigens, and seven other MAbs were produced with specificity to either two stages or all three. EMA 133 recognised all vine weevil stages and also cross-reacted strongly with all predator species examined, and was therefore developed for use as a positive control.

Table 9: Specificity and Isotype of MAbs Produced

Monoclonal Antibodies	Specificity	Isotype
EMA 122, EMA 150	E,L,A	IgG1
EMA 133	E,L,A + all predators	IgM
EMA 134	L,A	IgG1
EMA 149	L,A	IgG2b
EMA 154	L,A	IgM
EMA 159	A,E	IgG1
EMA 160	A,E	IgG2b
EMA 131, EMA 151, EMA 152	A	IgG1
EMA 130, EMA 135	A	IgM
EMA 162	E	IgG1
EMA 161	E	IgM

Pest-specific MAbs provide a valuable tool for the analysis of predator gut contents. Their use in sensitive techniques such as ELISA provides a rapid method for large-scale screening of predators. This was the first study to produce a panel of MAbs as a diagnostic probe to determine which developmental stages are subject to predation. They were used in the 1996 field trial to screen field caught predators from commercial strawberry and blackcurrant plantations in Kent.

Using an egg-specific, an adult specific, and a larva-plus-adult specific MAb, it was possible to identify predators that had fed on eggs, larvae or adults, as well as those that had eaten eggs-plus-larvae. A predator that had fed on an adult vine weevil would elicit the same response in the multiple-ELISA as one that had fed on an adult and larva; it was possible to differentiate these two possibilities on the basis of which vine weevil stages were present in the field at the time of sampling. Likewise, a predator that had fed on both eggs and adults could not be distinguished from one that had fed on all three stages, but the latter is sufficiently improbable that it could be discounted.

Few other workers have used monoclonal antibodies in field predation studies. Symondson and Liddell (1993) have developed a MAb capable of distinguishing arionid slugs from those of other genera. Hagler and co-workers have successfully produced MAbs to egg antigens of three different pest species. In each case the MAbs recognised adult females of the same species (Hagler *et al.* 1991, 1993, 1994). The highest degree of MAb specificity has been achieved by Greenstone and Morgan (1989), who produced an ELISA system which was capable of identifying predation on the fifth instar of *Heliothis zea*.

Concluding Comments

The difficulties in making direct observations of predation on the different life cycle stages of vine weevil led to a situation where very little information existed on the subject. It was not known which, if any, of the many polyphagous predators found in soft fruit plantations consumed vine weevils.

The extreme sensitivity and specificity of the monoclonal-antibody-based detection system developed in this research programme has led to analysis of vine weevil consumption by predators in soft fruit plantations.

Two small species, *Notiophilus biguttatus* and *Bembidion lampros*, were found to be key consummers of vine weevil eggs and young larvae. Adult beetles of a number of species were found to be highly active within the soil. Several species consumed vine weevil larvae in large numbers, particularly in addition to *Notiophilus biguttatus*, the larger carabid species, *Pterostichus madidus* and *Harpalus rufipes*. The staphylinid beetle, *Ocypus olens*, was found to feed on all stages in the laboratory, but did not consume as many vine weevils as expected in the wild. This is probably due to the presence of many alternative food sources available to it in the field. The largest of the carabid species found, *Carabus violaceus* was evidently the greatest consumer of the adult weevils.

The East Egham field experiment with strawberry plants grown within barriers indicated that predators are capable of reducing vine weevil numbers, and that this might be sufficient as a control technique if vine weevil population density is not too high. It is clear therefore that these polyphagous predatory beetles should be encouraged in soft fruit plantations. Further work is required to examine the effectiveness of the various ways that plantation management policy might be modified to favour predators, but it is clear that in general any techniques that provide daytime sheltering sites for predators are likely to be beneficial.

Although no systematic survey of predator abundance in relation to pesticide use was conducted in this project, it was apparent that there were big differences in predator numbers between sites. For instance, numbers were very low in the strawberry plantation at Wilford Court Farm, and Gore Farm, but observations at other sites revealed much higher numbers in some strawberry plantations. An important contributory, if not the major, factor likely to be responsible is pesticide use. Some information on the toxicity of particular pesticides to some predatory beetles exists; in general the broad-spectrum organophosphorus compounds and pyrethroids are likely to be damaging.

Now that a panel of monoclonal antibodies has been prepared, future research work on predation on vine weevil in soft fruit, or indeed in any other growing systems, is greatly facilitated. The MAbs are self perpetuating, so that with a little periodic culture maintenance this valuable resource can be maintained. This will make possible the detection of predation on the various life cycle stages by predators collected from a range of different sites at different times of year. Thus predation on vine weevil could be examined on a wider scale, to check that the pattern of consumption revealed in this study is a general one.

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Glossary

Antibodies:

Host proteins produced in response to the presence of foreign molecules in the body They circulate through the blood and lymph where they bind to foreign antigens.

Antigen

A protein molecule recognised by an antibody

Carabid beetles:

Members of the Carabidae family of beetles, they are also known as ground beetles.

Electrophoresis (SDS-PAGE):

'Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis' - A method of separating proteins of different sizes.

ELISA (enzyme-linked immunosorbant assay)

A system used for the detection of antigens.

Fluon:

A PTFE suspension, similar in its properties to non-stick Teflon.

Hybridoma cells:

Formed by the fusion of spleen cells and myeloma cells, they are self-replicating antibody-producing cells.

IgG :

One of the five classes that antibodies are divided into based on their structure. IgG antibodies are the most abundant in serum. When purified from antiserum, they constitute the most concentrated antibody fraction.

Immunoglobulin:

A term interchangeable with 'antibody'. Technically, an antibody binds to a known antigen, while an immunoglobulin is a protein of this type irrespective of whether or not their binding target is known.

Isotype:

The sub-class of an antibody.

Monoclonal antibody (MAb):

An antibody originating from a single hybridoma cell line.

Parthenogenesis:

A form of asexual reproduction which does not require the existence of males.

Pitfall trap:

A trap based on a plastic beaker submerged in the ground, used to collect beetles as they fall into it.

Polyclonal Antiserum:

A mixture of antibodies present in plasma.

Staphylinid beetles:

Also known as rove beetles, they are members of the taxonomic group the Staphylinidae

Appendix

Stock Reagents Used During The Indirect Elisa Protocol

(A) PBS (Phosphate buffered saline)
8g Sodium chloride
0.2g Potassium chloride
0.2g Potassium hydrogen orthophosphate
Made up to one litre with distilled water

(B) PBS-TPO
0.5g Tween-20
20g Polyvinypyrrolidone
2g Ovalbumin
Made up to one litre with PBS
The powder was emulsified in a small volume of liquid, then the remainder added, stirring until dissolved.

(C1) Enzyme conjugate for use with polyclonal antisera:
10 µl Protein A/Horseradish peroxidase
10ml PBSTPO

(C2) Enzyme conjugate for use with monoclonal antibodies:
10 µl Antimouse IgG (Sigma)
10 ml PBSTPO

(D) Substrate
9ml distilled water
1ml 10X 1M Sodium acetate, pH 5.8
100 µl Tetramethylbenzidine (TMB) (10mg/ml in Dimethyl Sulphoxide (DMSO))
20 µl hydrogen peroxide

Preparation Of IgG By Ammonium Sulphate Precipatation Protocol

1ml of antiserum was mixed with 9ml of distilled water. An equal volume (10ml) of saturated ammonium sulphate solution was added dropwise to the antiserum, while the antiserum was slowly stirred. Slow addition of the ammonium sulphate was important as high local concentrations would precipitate out other proteins, such as albumin. The solution turned cloudy when three-quarters of the volume had been added as the proteins started to precipitate out. The mixture was then stored at 4 °C for 3 hours before being spun at 4 °C, 12,000 rpm for 15 minutes. The supernatant was then discarded, the pellet resuspended in 5 ml of saturated ammonium sulphate and respun as before. The pellet was washed to reduce contamination without significantly reducing the yield. The supernatant was again discarded and the pellet redissolved in 1ml half-strength PBS.

Ammonium ions interfere with subsequent reactions the IgG would undergo during an

ELISA, so the ammonium sulphate was removed by dialysis against PBS. The resuspended pellet was placed in dialysis tubing and dialysed against 750 ml of half-strength PBS at 4 °C. The buffer was changed after 1 hour and again at the end of the day and left overnight. The dialysed IgG was then passed through an ion-exchange cellulose column (Whatman DE52). For 1ml of antiserum, a bed volume of 5 ml was used. When the surface of the DE52 bed was just dry, it was pre-run with at least 8 volumes of half-strength PBS and then drained. The antiserum was transferred from the dialysis tubing to the column. A series of 2 ml tubes was placed under the column. Half-strength PBS was slowly added, allowing the column to drain between additions, until 8-10 tubes were filled. Samples from each tube were tested in a spectrophotometer at a wavelength of 280 nm to determine which tube or tubes contained most of the IgG. As the extinction coefficient of a 1cm vial at 280 nm is 1.4, it was possible to calculate the concentration of IgG solution collected. Thus, 2 mg of cross-absorbed IgG was collected in a volume of 1.7 ml, and 3.65 mg of non cross-absorbed IgG in 5 ml. Sodium azide was added to each (0.05 %) , and they were stored at 4 °C.

Electrophoresis Protocol

Stock Reagents

(A) Acrylamide/Bis (30% T, 2.67% C)
87.6 g acrylamide (29.2 g/100 ml)
2.4g N'N'-Bis-Methylene-Acrylamide (0.84 g/100 ml)
Made up to 300 ml with distilled water. Filtered and stored at 4 °C in the dark for a maximum period of one month.

(B) 1.5 M Tris-HCl buffer, pH 8.8
27.23 g Tris base (18.15g/100 ml)
Approximately 80 ml distilled water
Adjusted to pH 8.8 with 1N HCl, then made up to 150 ml with distilled water and stored at 4 °C.

(C) 0.5M Tris-HCl buffer, pH 6.8
6g Tris base
Approximately 60 ml distilled water
Adjusted to pH 6.8 with 1N HCl, then made up to 100 ml with distilled water and stored at 4 °C

(D) 10% SDS (Sodium Lauryl Sulphate)
10 g SDS was dissolved in a little distilled water with gentle stirring and then made up to 100 ml.

(E) Sample Buffer (SDS Reducing Buffer)
4 ml distilled water
1.0 ml 0.5m Tris-HCl buffer, pH 6.8
0.8 ml Glycerol
1.6 ml 10% (w/v) SDS
0.4 ml 2beta-mercaptoethanol

0.2 ml 0.05% (w/v) bromophenol blue
Samples were diluted a minimum of 1:4 with sample buffer and heated to 95 °C for 4 minutes.

(F) 5X Electrode (Running) Buffer, pH 8.3
9 g Tris base (15 g/l)
43.2 g Glycine (72 g/l)
3 g SDS (5 g/l)
The solution was made up to 600 ml with distilled water and stored at 4 °C. If a precipitate formed the buffer was warmed to 37 °C before use. 60 ml of 5X stock solution was diluted with 240 ml distilled water for each electrophoretic run.

(G) Reagents for Coomassie Blue Staining
Fixative = 40% methanol/ 10% acetic acid
Stainer = 40% methanol/ 10% acetic acid/ 0.1% Coomassie Blue

Protocol: Gel Preparation

Discontinuous gels consist of an upper stacking gel and a lower separating gel. The stacking gel concentrates the sample in a narrow band at the interface between the gels, regardless of the volume in which the sample was applied. The resolving gel then separates the proteins. This system, developed by Laemmli (1970), is capable of very high resolution.

The 12% monomer was prepared as above by combining all reagents, except the TEMED and APS, and degassing for 15 minutes under vacuum. Polymerisation was initiated by adding the APS and TEMED and swirling gently to mix. A glass pipette was then used to pour the solution smoothly into the 0.75 mm space between the glass plates to prevent it from mixing with air until two-thirds of the volume was filled. The monomer was then immediately overlaid with water-saturated isobutanol. The gel was allowed to polymerise for 45 minutes and then the overlay completely rinsed off with distilled water. The alcohol must not remain on the gel for longer periods as it will cause dehydration of the gel.

If the rest of the gel was to be cast the next day, the separating gel could be stored overnight if the gel was covered with 5 ml of 1:4 diluted stock solution B to prevent dehydration.

Table A1: Production of Separating and Stacking Gels.

	Separating Gel 12% gel, 0.375M Tris, pH 8.8	Stacking Gel 4.0% gel, 0.125M Tris, pH 6.8
Distilled Water	3.35 ml	6.1 ml
0.5M Tris-HCl, pH 6.8	-	2.5 ml
1.5% Tris-HCl, pH 8.8	2.5 ml	-
10% (w/v) SDS (stored at room temperature)	100 µl	100 µl
Acrylamide/Bis (30% stock) (Degassed for 15 minutes at room temperature)	4.0 ml	1.3 ml
10% ammonium persulfate (APS) (made fresh daily)	50 µl	50 µl
TEMED (NNNN'N'-Tetramethylethylene-diamine)	5 µl	10 µl
TOTAL STOCK MONOMER	10 ml	10 ml

The stacking gel monomer solution reagents were then combined together except for the APS and TEMED, and degassed for 15 minutes. The area above the separating gel was dried with filter paper. After addition of the APS and TEMED to the monomer solution, it was laid over the separating gel. The well-forming comb was then placed in the top of the stacking gel so that the 'T' of the comb rested on the spacers. The gel was allowed to polymerise for 30-45 minutes before the comb was removed and the wells covered with distilled water.

Loading sample wells

300 ml of electrode buffer was prepared as above. The inner cooling core was lowered into the lower buffer chamber of a BioRad Mini-PROTEAN II cell. The upper chamber was filled to within 2 mm of the top of the outer longer glass plate. The remainder of the buffer was poured into the lower chamber, ensuring that at least the bottom 1 cm of the gel was covered.

The sample buffer contained 10% sucrose in order to underlay the sample in the well without mixing. The samples were loaded using a Hamilton syringe which was inserted to within 1-2 mm of the bottom of the well before delivery.

Alternatively, a single sample could be loaded across the top of the gel. In this procedure, the stacking gel was cast without a comb, forming a continuous flat surface.

Running and fixing the gel

The gel was run at 180 volts for 45 minutes. If the gel was to be stained by either Coomassie Blue or Silver Staining methods, it was then transferred into fixative solution. If the proteins on the gel were to be electrophoretically blotted onto a membrane, the gel was placed instead into BEB equilibration buffer (see below).

Coomassie Blue Protein Staining

The gel was placed in the fixative for approximately 1 hour. The fixative was then replaced with stainer for approximately 30 minutes and then destained with fixative until the background blue had been removed so only the protein bands were visible.

Western Blotting An Sds-Page Gel Onto Nitrocellulose Paper Protocol

Stock Reagents

(A) BEB (Blot Electrode Buffer)
25mM Tris (3g/l)
192mM Glycine (14.4g/l)
20% methanol (200ml/l)

Preparation

The buffer was prepared in advance and refrigerated so that it was at 4 °C at the start of transfer. The nitrocellulose paper was cut to the dimensions of the gel and the top right-hand corner removed to identify the orientation of the membrane subsequently. The membrane was soaked for 15-30 minutes in transfer (BEB) buffer. The pre-cut filter paper and fibre pads were also saturated in the transfer buffer.

The buffer tank was half-filled with transfer buffer and the frozen cooling unit installed next to the electrode a few minutes prior to the start of transfer. The Bio ice cooling unit was prepared in advance by filling it with de-ionized water and storing it in the freezer. The ice in the cooling unit serves as a heat sink for the heat generated during the electrophoretic transfer.

Electroblotting of the SDS-PAGE gel onto a nitrocellulose membrane

NB. Gloves were worn throughout this procedure to prevent contamination of the membrane.

The gel was first blocked for 30 minutes in 10% dried milk powder. The gel holder consists of two panels, the clear panel being the anode and the black one being the cathode. Correct orientation of these panels is very important. The cassette was opened so that the cathode lay flat in a plastic tray, and a pre-soaked fibre pad placed on top followed by a piece of saturated filter paper and then the gel. The surface of the gel was flooded with transfer buffer and the pre-wetted nitrocellulose laid on top ensuring total contact and no air bubbles. The

sandwich was then completed with another piece of saturated filter paper and a fibre pad, and the cassette was closed. The cassette was placed in the buffer tank so that the black panel correlated with the cathode. The cell was then connected to a power supply and run at 30 volts for 14 hours.

Immunoperoxidase Staining Of Nitrocellulose Blots

Stock Reagents

(A) TBS (Tris Buffered Saline)
10mM Tris-HCl, pH 7.4 (1.21g/l for 1X stock)
140mM NaCl (8.18g/l for 1X stock)
Made up as 10X stock.

(B) TBS-Tw
TBS containing 0.1% Tween 20

(C) HST buffer
10mM Tris-HCl, pH 7.4 (1.21g/l)
1M NaCl (58.4g/l)
0.5% Tween 20 (5.0ml/l)

(D) Substrate
(D1) If Protein A-Alkaline Phosphatase was used, it was made up using 0.05M Tris-HCl + 10mM $MgCl_2$, pH 8.6.
(i) 5mg Na_1-Naphthyl phosphate was dissolved in 25 ml buffer.
(ii) 3 mg/ml (ie. 15mg in 5ml) of Fast Red TR was dissolved in distilled water.
(iii) 1 volume FR-TR was added to 5 volumes 1-NP and was used to develop bands within 1 hour.
(D2) If Protein A-Horseradish peroxidase was used, it was made up as shown below.
(i) 18mg 4-Chloro-1-naphthol was dissolved in 6 ml methanol.
(ii) 94 ml of TBS and 0.075 ml 30% hydrogen peroxide (ie. 125 µl of 6% H2O2) were added.
(iii) The reagents were mixed well and used immediately.

Protocol

The nitrocellulose was removed from the blotting apparatus and washed twice for 5 minutes with gentle agitation in TBS-Tw. The membrane was then blocked for 15 minutes in 10% dried milk powder before being washed again in TBS-Tw. The antibodies were diluted in HST buffer and the nitrocellulose paper incubated with the antibodies for 45-90 minutes at room temperature. Unbound immunoglobulins were removed by a series of 5 minute washes at room temperature: twice with TBS-Tw, a single wash with HST buffer and then twice again with TBS-Tw.

The nitrocellulose membrane was then incubated for 45-90 minutes in the protein A-ALP conjugate which had been diluted in HST buffer. The unbound conjugate was removed by

another series of 5 minute washes: three times with TBS-Tw, once with HST, another three times with TBS-Tw and a final wash with TBS.

During this time, the protein A-ALP based substrate was prepared, as it must be prepared immediately before use. The nitrocellulose sheet was placed in the substrate and agitated. Purple bands developed and darkened over 30 minutes. The reaction was stopped by washing the blot for a minimum of 10 minutes in several changes of distilled water.

Blots could be stored, protected from light after drying between sheets of filter paper.

Monoclonal Antibody Production

Three fusions were carried out; the immunisation protocol detailed below describes the production of antibodies to vine weevil larvae, with details of adult and egg preparation in parentheses.

Six mice of the strain Balb C, aged 6-8 weeks, were immunised by intraperitoneal injection of 200 µl of a 1:2 emulsion of Hunter's Titermax™ and 6 mg crude larva extract (6 mg crude egg extract; 8.8 mg crude adult extract) in phosphate buffered saline (PBS).

Four weeks later, the mice received a second booster intraperitoneal injection of 200 µl each, containing the same quantity of vine weevil extract in PBS without adjuvant. Tail bleeds were carried out 10-14 days afterwards and the concentration of anti-vine weevil larva (or egg/adult) antibodies was estimated by ELISA. The serum with the highest titre determined which mouse was selected for fusion. A pre-fusion boost, identical to the second immunisation, was administered four days prior to fusion with SP2/O-Ag14 myeloma cells.

Splenocytes from the pre-immunised mouse were fused with SP2/O-Ag14 myeloma cells in the ratio of 5:1 using polyethylene glycol (PEG 1500) as described by Galfre & Milstein (1981). The fused cells were resuspended in 80 % DMEM (Dulbecco's modified Eagle medium)/20% FCS (foetal calf serum), containing hypoxanthine, aminopterin and thymidine (HAT), and plated out on a splenocyte feeder layer in eight 96-well tissue culture plates, which were incubated at 37 °C in a water-saturated atmosphere of 5% CO2.

Three to four days later, the hybridomas were fed by adding 100 µl per well of 80 % DMEM/ 20 % FCS medium containing HAT. Half of the medium was replaced with fresh medium every 4-5 days until day 14, when HT medium replaced HAT medium. The supernatants were then screened by ELISA (see below) against the homologous and heterologous vine weevil antigens, and against predator antigens. Positive cell lines were selected and twice cloned by limiting dilution.

Hybridoma supernatant screening

Crude soluble protein extracts of each vine weevil stage were made at the following concentrations in PBS: 100 µg/ml egg; 66 µg/ml larva, and; 125 µg/ml adult. The extracts were homogenised, centrifuged at 13,000 rpm for 5 minutes at room temperature, and the supernatant

retained. For long term storage, 100 mg/ml stock solutions were made and stored at -80 °C.

Microwell plates were coated with 100 µl of vine weevil extract, and incubated at 4°C overnight. The plates were briefly rinsed three times in PBS-Tween 20. Culture supernatants were diluted 1:3 in PBSTPO (PBS/ Tween 20/ Polyvinylpyrrolidone/ Ovalbumin) and 100 µl was placed in each well, and incubated for 2 hours at 37 °C. Positive and negative controls were always included on each ELISA plate.

Bound antibodies were detected with anti-mouse horseradish peroxidase (HRP) conjugate, and tetramethylbenzidine (TMB) as substrate. Reactions were terminated with 15% sulphuric acid and the optical absorbance measured at 450 nm.

Monoclonal antibody cross-reactivity screening

Positive hybridomas were screened against all three vine weevil stages to examine their specificity. Cell lines of interest were then tested by ELISA as previously described, to examine cross-reactivity with starved adults and larvae of predator species. Although not screened against predator eggs, the supernatants were screened against individual female adults which were gravid. Extracts of 100 µg/ml were made from predatory insects of interest (Table 2), and 100 µl used to coat individual wells of a 96-well plate. Only cell lines that demonstrated no cross-reactivity with predator species were cloned.

Table A2: Total number of each species caught on each plant species

FLORAL SPECIES:	CORIANDER	BUCKWHEAT	BELLFLOWER	PHACELIA	PARSLEY	SELFHEAL	POPPY	COCKLE	BFTREFOIL	CORNFLOWER	CAMOMILE	DAISY	MARIGOLD	CARROT	TOTALS	
Nebria brevicollis	93	125	81	80	32	55	110	49	44	16	77	61	22	8	853	Nebria brevicollis
Calathus fuscipes	48	38	48	50	51	62	33	59	58	44	45	27	29	11	603	Calathus fuscipes
Pterostichus madidus	24	25	31	47	60	66	22	31	56	24	22	43	28	9	488	Pterostichus madidus
Harpalus rufipes	94	79	49	34	32	3	19	26	10	44	17	14	13	9	448	Harpalus rufipes
Pterostichus melanarius	10	4	16	26	25	13	16	7	19	29	8	9	35	10	229	Pterostichus melanarius
Harpalus aeneus	3	6	10	0	5	4	3	11	3	4	1	1	4	0	55	Harpalus aeneus
Amara simulata	3	1	6	3	3	0	0	4	6	1	11	1	2	1	42	Amara simulata
Amara bifrons	4	4	5	1	0	0	6	4	0	5	2	1	0	3	35	Amara bifrons
Ocypus olens	1	0	0	4	2	2	0	3	2	8	0	5	3	2	32	Ocypus olens
Bembidion lampros	3	0	5	0	1	1	1	1	0	2	3	0	3	0	20	Bembidion lampros
Amara familiaris	1	2	4	0	0	1	0	0	0	4	0	0	0	0	12	Amara familiaris
Carabus violaceus	0	0	0	0	1	0	0	2	0	2	1	2	0	2	10	Carabus violaceus
Agonum dorsale	0	1	1	0	2	0	0	2	0	0	0	1	0	0	7	Agonum dorsale
Notiophilus biguttatus	0	0	0	0	0	1	0	0	1	5	0	0	0	0	7	Notiophilus biguttatus
Bembidion genei	1	0	1	0	0	0	0	1	0	0	0	1	0	2	6	Bembidion genei
C.melanocephalus	0	0	1	0	0	0	1	1	0	1	0	0	0	1	5	C.melanocephalus
Harpalus latus	2	0	1	0	1	0	0	0	0	0	0	0	0	0	4	Harpalus latus
Abax parallelopipedus	1	0	0	0	0	0	0	0	0	0	1	0	0	0	2	Abax parallelopipedus
Amara convexicolor	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	Amara convexicolor
Cychrus caraboides	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	Cychrus caraboides
Loricera pilicornis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Loricera pilicornis
Trechus 4-striatus	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	Trechus 4-striatus
TOTAL	289	285	259	245	215	215	212	202	199	189	188	166	140	58	2862	TOTAL