Comparing Predatory Insects of *Helicoverpa spp.* in Australian Cotton: Approaches to Measuring Prey Consumption.

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Declaration

I certify that the substance of this thesis has not already been submitted for any degree and is not currently being submitted for any other degree or qualification.

I certify that any help received in preparing this thesis, and all sources used, have been acknowledged in this thesis.

Marie-Louise Johnson

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Dedicated to Maui

Abstract

The Australian cotton industry relies heavily on pesticides to control key pests. These pests include the immature stages of moths of the genera *Helicoverpa* (formerly *Heliothis*). As a result of heavy pesticide use the cotton industry has been facing continual problems of *Helicoverpa* developing resistance to pesticides and has come under increasing pressure to reduce the contribution of high pesticide usage to environmental pollution. As a result, the Australian cotton industry has put considerable effort into finding alternative ways to reduce the impact of *Helicoverpa* on yield of cotton and reduce the use of pesticides.

One of the alternative ways to control *Helicoverpa* is the use of natural enemies. There is scant information available on whether natural enemies contribute to *Helicoverpa* mortality and even less on the impact that individual natural enemy species have in reducing *Helicoverpa* populations. This thesis investigates the impact of several natural enemy species (predators) on *Helicoverpa armigera* (Hübner) and *Helicoverpa punctigera* (Wallengren) eggs from the Namoi valley in New South Wales, Australia.

The predators *Nabis kinbergii* Reuter, *Dicranolaius bellulus* (Guerin Meneville) and *Coccinella transversalis* (Fabricius) occur in reasonably high numbers throughout the cotton growing season. Adults of these species were selected for experiments that were conducted at the University of New England, NSW, Australia. These species all consume immature stages of *Helicoverpa* but little is known of how many prey they consume or under what circumstances they will feed on *Helicoverpa*.

Using a series of trials in an environment cabinet it was determined that the factors that influenced predation, by the three predator species, were temperature, the position of prey on cotton plants and the hunger status of the predators. The optimal temperature for studying predation rates under artificial conditions was 30.2 °C. At the two extremes of 20°C and 36.8°C consumption of *Helicoverpa* was generally reduced. These temperatures also affected the proportion of individuals within a species finding prey. Placing eggs on the upper surface of leaves of a cotton plant was suitable for studying predation rates. Starvation of predators for different lengths of time prior to their use in experiments caused differences in both the number of prey consumed and the number of predators that found and consumed prey. This was of consequence only when an experimental time of 8 hours as opposed to 24 hours was

used. The optimal conditions determined by these experiments enabled studies on predation rates of each predator species to be done.

Studies on predation rates showed that the predator species *N. kinbergii*, *D. bellulus* and *C. transversalis* fed on an average of 51, 34 and 30 *H. armigera* eggs a day in petri dishes, if no restrictions to accessing prey were provided. However, the number of *Helicoverpa* prey provided on more realistic arenas, such as cotton plants, was found to affect the number of prey consumed in some predator species. In spite of this, at both realistic (low) and high numbers of *Helicoverpa* eggs, all predators exhibited a linear response to increases in prey density. That is, their hunger was not saturated by the number of prey provided. The predators' ability to find prey was hampered in larger search arenas (larger plants). *Nabis kinbergii* consistently consumed more *Helicoverpa* eggs than the other two species.

When alternative prey were included with *Helicoverpa* eggs, *N. kinbergii* preferred *Helicoverpa* neonates to eggs, *D. bellulus* showed an initial preference for neonates but consumed more eggs as neonate numbers declined and *C. transversalis* showed no preference at all. Including aphids of different densities did not reduce the number of *Helicoverpa* eggs consumed by *N. kinbergii* and *C. transversalis*. Only *D. bellulus* appeared to be affected by the presence of aphids, consuming fewer eggs at higher densities of aphids. All the predators tested consumed *Helicoverpa* eggs and early larval stages as well as the aphid *Aphis gossypii* Koch.

The difficulty in the extrapolation of laboratory results to field conditions led to the use of immunodot assay (a serological technique) to determine if predators feed on *Helicoverpa* directly in the field. A comparison of a monoclonal and polyclonal antibody showed that only the monoclonal antibody was suitable for field trials. The polyclonal antibody cross reacted with predator haemolymph. Immunodot assay using chemiluminescence as a detection substrate provided the best method for determining whether predators had fed on *H. armigera* eggs. The method was tested on *Diomus notescens* (Blackburn), *D. bellulus, N. kinbergii, C. transversalis, Micraspis frenata* (Erichson) and *Harmonia* spp. Smaller predators provided the most consistant results. Trials in the field provided the first direct measurement of predation in cotton in Australia using this technique. A high proportion of the two predator species *D. notescens* (up to 86%) and *Harmonia conformis* (Boisduval) (up to 54%) were found to consume *H. armigera* eggs in the field.

The data presented in this thesis suggest that there are important differences in the factors that affect the consumption of *Helicoverpa* eggs by each predator species. These differences occur as a result of the number of *Helicoverpa* eggs available, the presence of other prey types and environmental parameters, such as temperature. These results verify that it is important to assess predator species individually if they are to be used in future control of *Helicoverpa* in Australian cotton.

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Chapter 1

1. Introduction – Why study natural enemies of *Helicoverpa* species ?

1.1 The pest and the industry

Species of the genera *Helicoverpa* (formerly *Heliothis*) and *Heliothis* (Lepidoptera: Noctuidae) include some of the world's major pests of crops and ornamentals (Fitt 1989). In Australia there are two species of *Helicoverpa*: both are major pests of cotton. One, *Helicoverpa armigera* Hübner, is a cosmopolitan pest of the former USSR, Europe, Asia, India, Africa, and Australia (Romeis and Shanower 1996, Van den Berg and Cock 1995, Sugonyaev 1994, Fitt 1989). The other, *Helicoverpa punctigera* Wallengren, is endemic to Australia (Fitt 1989). In Australia both species are found on horticultural crops such as tomatoes, corn, beans and roses. They are also found in summer crops such as tobacco, sunflowers, sorghum, maize, soybean and cotton. Whilst this thesis is restricted to *Helicoverpa* in cotton, both species are known to feed on a wide variety of crop and non crop plants (Zalucki *et al.* 1994, Zalucki *et al.* 1986). There is potential for confusion as a result of the change in nomenclature from *Heliothis* to *Helicoverpa* (Matthews 1991). To avoid this, unless specified by a species name, the genera *Helicoverpa* and *Heliothis* will be referred to by the common name heliothis (not in italics).

In order to understand the ecology and assess the impact of heliothis on a local and regional level a great deal of work has been done both overseas and in Australia over the past two decades. The majority of this work has been reviewed in Fitt (1989) and Zalucki *et al.* (1986). Similarly, studies on the migration and ecology of *Helicoverpa* spp. from inland Australia has been extensively covered by Gregg *et al.* (1993), Fitt *et al.* (1990), Gregg *et al.* (1989) and Farrow and Daly (1987). Both *H. punctigera* and *H. armigera* are migratory. *Helicoverpa armigera* are found predominantly along the east coast and eastern inland of Australia as well as in the Northern Territory, and occasionally in South Australia and Western Australia (Fitt *et al.* 1995, Zalucki *et al.* 1994, Zalucki *et al.* 1986) whereas *H. punctigera* are found all over Australia (Zalucki *et al.* 1994). Spring migrations of both species can occur but *H. armigera*

is considered a facultative migrant and a large proportion of the population overwinter in cropping areas. Evidence suggests that *H. punctigera* depend on migration to initiate a first spring generation in cropping areas because of the low numbers found in the cropping region of the Namoi valley (Fitt and Daly 1990). *Helicoverpa punctigera* is believed to migrate from inland Australia towards the east coast when the right temperature, rain and food conditions (flowering plants) occur. Both species are capable of migration of up to 2000 km. However, both species also have a resident overwintering population of diapausing pupae (although thess are predominantly *H. armigera*) within the crop growing areas in eastern Australia that begin to emerge in October (Fitt and Daly 1990). Although *H. punctigera* is generally the dominant species early in the crop growing season and *H. armigera* in the mid to late season, both species may occur at any time during this period. The generation time is variable depending on temperature and plant hosts but in the temperate regions there are usually 3-5 generations per year. Generations are not discrete (Fitt 1989). In summer, both *H. armigera* and *H. punctigera* took about 34 days from egg to adult at temperatures around 28 °C (Zalucki *et al.* 1986). Migration, short generation time and high fecundity contribute to heliothis being pests that are difficult to control.

Due to high fecundity and the need for control measures of the pest, it is necessary to understand the natural history of heliothis in the field. Both species usually lay eggs singly on cotton plants. The oviposition sites include the leaves, buds, flowers, fruit and stems (Zalucki *et al.*1986). Eggs are of uniform quality, age and condition but their distribution is clumped in cotton crops (Stanley 1997). Adults mate at day one or two and continue mating throughout their life. In the laboratory females live for two weeks and produce eggs for the entire time. The number of eggs laid varies with temperature. Female *H. punctigera* kept at 24 °C laid a mean of 1437 (± 229) eggs per lifetime. This equates to about 112 eggs laid per day per female over 12 days (Zalucki *et al.* 1986). This information is not available for *H. armigera* in Australia although females have been cited as laying up to 3000 eggs in a life time (Fitt 1989). These laboratory-based estimates of longevity and fecundity probably represent maximum intrinsic values. The extent to which they are realised under field conditions remains unknown. In both species most activity occurs 3-4 hours after dusk. Egg laying in *H. armigera* is bi-modal with most eggs laid early in the night. Maximum egg laying is thought to occur when plants are flowering and producing nectar but in cotton peak egg laying may also occur at the pre-flowering stage. Hairy or rough textures are the most likely cue for oviposition but

chemical cues such as secondary chemicals and nutrients may also be important. The presence of nectar in extra floral nectaries may also influence oviposition (Adjei-Maafo and Wilson 1983).

The number of eggs found per metre is highly variable regionally, within regions and seasonally. Murray *et al.* (1996) showed egg numbers per metre reached on average five to ten but sometimes numbers reached up to 55 per metre in unsprayed cotton in Queensland. According to Mensah *et al.* (1996) in New South Wales conventionally grown cotton, the number of eggs averaged two per metre and reached 4.4 per metre. In another growing season data from Mensah and Singleton (1998) indicate that there were no more than six eggs per metre throughout the cotton growing season at 'Yarral' Station in the Namoi valley. In Kunanurra (Western Australia) egg numbers reached 500 per metre on one occasion but generally were below 50 (Strickland *et al.* 1996). The implications of numbers of eggs per metre are discussed in Chapter 4. In general the eggs hatch 3-4 days after being laid and five instar stages occur. Egg stages are usually referred to as white, brown and black as they develop. The development of larvae is also dependent on temperature, diet and possibly compounds in the host plant (Zalucki *et al.* 1986). It is the larval stages that cause damage to cotton plants.

Damage and losses to the industry

The damage to cotton by heliothis generally occurs as a result of larvae feeding on meristematic tissue, causing delayed growth of the cotton plants. Depending on the time of feeding cotton plants can recover from this type of damage (Ring and Benedict 1993, Brook *et al.* 1992). Direct damage also occurs by feeding on squares (flower buds) and bolls later in the season. This causes reduced yield from cotton bolls. Yield is generally referred to as bales of cotton produced per hectare. Yields from irrigated cotton in the Namoi valley in the 1996/7 season averaged 6.4 bales per hectare but reached up to 9.9 bales per hectare in some parts. The amount of damage caused (or loss of yield) is dependent on the number of moths in the region and the numbers of larvae that survive from the eggs that are laid. This can be highly variable due to seasonal conditions such as rainfall and availability of plant food sources prior to oviposition.

The total annual cost of control and damage losses to production as a result of heliothis in Australia are around $226 million. Without control the loss of income would equate to $800 million annually. In cotton, the cost to the industry is $124.5 million (Adamson *et al.* 1997 a and b cited in Zalucki *et al.* 1998). The main control method for these pests is the use of pesticides (usually broad spectrum). For example, in cotton 11 to 12 pesticide applications were carried out in the Darling Downs (Queensland) during the 1996/7 season for control of heliothis. Cost of insect pest control is one of the largest production costs to the industry. In the Darling Downs the cost per hectare for control of heliothis reached $800 in the 1997/98 season (Bligh 1998). Costs are also derived from scouting for the pests although this is considered a saving to some growers (McIntyre and Fresser 1998). Apart from the economic loss, two problems arise from consistent pesticide usage. One is the development of resistance to chemicals by the insects and the other is environmental damage.

Resistance to pesticides

Put briefly, resistance occurs as a result of consistent use of a chemical or group of chemicals with the same mode of action. The insects that are not susceptible to the insecticide are selected through x generations as a result of constant use of a particular chemical. Under such selection there is the potential for 100% resistance to a chemical to occur. Cross resistance can occur if two chemical groups have similar modes of action. There is no evidence of resistance by *H. punctigera* to DDT, endosulfan, deltamethrin and fenvalerate or any other chemical group used in cotton (Zalucki *et al.* 1998, Gunning and Easton 1994a). However, resistance to pesticides does occur in *H. armigera*. In *H. armigera* resistance has occurred to most of the major groups of chemicals including organochlorines, cyclodienes, synthetic pyrethroids, endosulfan, carbamates and organophosphates (Gunning and Easton 1994b). Resistance to pesticides by *H. armigera* occurred firstly to DDT in the 1970s and more recently to synthetic pyrethroids in the early 1980s (Daly and Murray 1988). Since then resistance has occurred to most of the major chemical groups. There is now high resistance to pyrethroids, moderate resistance to endosulfan, low but increasing resistance to profenofos and thiocarb. As yet there is no resistance to the bacterium *Bacillus thuringiensis* (Bt) as a spray. In order to prolong the use of pesticides an Insecticide Resistance Management (IRM) strategy was formulated in Australia and has been running since the 1983/84 cotton growing season (Forrestor 1994).

It is well known that resistance to pesticides by heliothis in the Ord river scheme (Western Australia) in the 1970s devastated the cotton industry (Bottrell and Adkisson 1977). How far have we come since then? Unfortunately transgenic cotton, expressing the Cry1Ac insecticidal crystal protein of *Bacillus thuringiensis* (the latest control tactic), is potentially no less subject to resistance than pesticides are (Roush 1996). Consequently, another type of resistance management is a requirement of using transgenic cotton (Fitt 1998). It involves the use of conventional cotton to dilute moth populations that may develop resistance to transgenic cotton. Although resistant management strategies have bought time for some pesticides and will aid in diluting the resistance of moths to transgenic cotton, resistance still remains a major problem in the future of the industry.

Public image

The other problem with consistent pesticide usage is that whilst IRM of pesticides has been worthwhile, the industry still remains heavily reliant on pesticides. This puts the cotton industry in the spotlight as potentially causing environmental pollution and health problems. This is to such an extent that "due to public pressure the level of pesticides used must decrease in the next ten years" (Edge 1996). The industry has, therefore, put a great deal of effort into finding more environmentally friendly ways of controlling heliothis.

1.2 Controlling heliothis in Australian cotton

The main type of control of heliothis in cotton has been with the use of pesticides either as the only form of control or, integrated with other management strategies. IRM has, for the most part, been used as part of conventional or 'soft options' control programs. In cotton, conventional spray management is based on application of pesticides when heliothis numbers reach a pre-determined threshold (currently two small larvae per metre of cotton in the early season) (Fitt 1994). Under conventional management up to 11 spray applications per season may be used (Pyke and Fitt 1998). 'Soft option' management was designed to preserve beneficial insects for as long as possible by monitoring for pest levels, thus using pesticides only when required. Also, by using 'soft option' pesticides that are more specific to certain pests and pesticides that have short residual times, there is a decrease in mortality of beneficial insects (Brook *et al.* 1992). Up to seven spray applications are used in 'soft options' programs

although this is variable and sometimes soft and conventional programs do not provide a difference in the number of sprays applied.

Also available for better pesticide usage are decision making aids such as EntomoLOGIC (Deutscher and Plummer 1998), its predecessor SIRATAC (Room 1979a) and recently the use of the LEPton kit® to determine when *H. armigera* are present (Trowell *et al.* 1993). All these tools enable the grower to optimise pesticide usage thus reducing the use of some pesticides and slowing resistance. Other aids for reducing the impact of heliothis include the development of 'Okra' leaf shapes to deter oviposition of heliothis (Brook *et al.* 1992), the pupae busting program (Murray *et al.* 1995a), viral pathogens such as nuclear polyhedrosis virus (NPV) and *Bacillus thuringensis* (Bt) insecticides as sprays. Also used and still being developed are the growing of lucerne strips in cotton fields as refuges for beneficial insects and sinks for other pests (Mensah and Khan 1997), and the application of food spray Envirofeast® to encourage beneficials to be in the crop before heliothis arrive (Mensah and Harris 1996a). Envirofeast is used as a food supplement for natural enemies and may deter oviposition of heliothis (Mensah 1996). Most recent is the establishment of commecially available transgenic (Bt) cotton expressing insecticidal protein genes from *Bacillus thuringiensis* (INGARD®) in the 1996, 1997 and 1998 seasons (Pyke and Fitt 1998). All these aids can be incorporated as part of integrated pest management (IPM) programs.

The definition of IPM is reviewed by Kogan (1998). He concludes that "IPM is a decision support system for the selection and use of pest control tactics, singly or harmoniously co-ordinated into a management strategy, based on cost/benefit analyses that take into account the interests and impacts on producers, society and the environment". IPM is relatively new in the Australian cotton industry (Edge 1994). It aims to reduce the reliance of pesticides as the main tool for insect control through the use of alternatives such as those mentioned above. These tools are incorporated into a management program that is usually dynamic. The majority of current entomological research in cotton aims to develop tools and strategies that will enable the implementation of IPM programs. Fitt (1994) has reviewed research in some of the areas, that relate to implementation of IPM.

The main tools currently available for use in IPM programs are those described above including pupae busting, soft options pesticides and transgenic cotton. Future challenges in

gaining grower acceptance of IPM will be to provide cost effective alternatives to chemicals and implementing IPM on a regional level (Wilson *et al.* 1998b, Zalucki *et al.* 1998). Also under investigation are furthering the development of technologies that are less likely to harm beneficial insects. Beneficial insects are seen as part of the development of IPM (Fitt 1996). The technology includes development of viral insecticides (NVP) for control of heliothis (Christian and Richards 1996), heliothis stunt virus (HaSV) and plant based attractants that aid in luring and killing heliothis (Gregg *et al.* 1998). Other future alternatives include improved transgenic cotton based on other insecticidal genes, further conventional plant breeding (Fitt 1994), and rearing and releasing the parasitoid *Trichogramma.* Continued research into the use of food sprays will also be of value to the industry as their effect on individual natural enemy species is not yet known. Other potential tools are the use of biological pesticides, mating disruption with pheromones and new synthetic pesticides such as insect growth regulators. All these strategies are designed to be used as part of IPM programs. However, in the absence of a commercially tested IPM program, there is still an ongoing reliance on pesticides as the major control measure for heliothis.

Transgenic cotton

Transgenic cotton (Bt) (sold commercially in Australia under the trade name INGARD) warrants a special mention as it is relatively new to the industry and seen by some as a final solution to the pesticide problem or alternatively as a means to advance the adoption of IPM (Pyke and Fitt 1998). Transgenic cotton is currently in its third commercial year with some 60,000 ha planted across all cotton growing regions (about 15% of the Australian cotton area) and has so far reduced pesticide usage by 50-60% (Fitt 1998, Fitt *et al.* 1998). However, this still equated to an average of 4.2 pesticide applications in its second season (Pyke and Fitt 1998). Just as resistance occurs to pesticides it may also occur to transgenic cotton. An important requirement of transgenic cotton is the inclusion of refuge crops of conventional cotton which aid in diluting resistant populations of *H. armigera.* Refuge crops are not sprayed with the same group of chemicals as the transgenic crop. Without adequate refuges, resistance to transgenic cotton has the potential to occur within three or four years (Fitt 1996). Refuges will not prevent resistance occurring: they will delay it. Consequently, *H. armigera* is most likely to develop resistance to transgenic cotton (Fitt 1996). Resistance is seen as a serious threat to the longevity of transgenic cotton (Tabashnik 1998, Roush *et al.* 1998, Roush

1996). As resistance increases the number of pesticides used will also increase. Therefore, a heavy reliance on pesticides is still likely to be a problem.

All the control measures mentioned above are aimed to control heliothis but many also aim to conserve natural enemies (Fitt 1989). Many researchers suggest that it is important to conserve beneficial insects and consider them in management programs (Abbott and Fitt 1998, Alexander 1998, McIntyre and Fresser 1998, Murray *et al.* 1998, Deutscher and McKewen 1996, Mensah and Harris 1996b). However, with few exceptions (Mensah and Singleton 1998, Titmarsh 1992, Room 1979b) the research to date in Australia has not provided evidence as to why it is important to conserve natural enemies and, in many cases being generalist feeders, whether they actually contribute to mortality of heliothis.

1.3 Natural mortality of heliothis

In Australia natural mortality of heliothis eggs and young larvae has been attributed to weather, competition, host patch size, host plant phenology, predation, cannibalism, feeding habits, disease and parasitism (Dillon *et al.* 1992, Titmarsh 1992, Kyi *et al.* 1991, Fitt 1989, Twine 1973). Few studies are able to quantify how each of these contributes to mortality. Weather, as well as the direct effects of temperature on development and survival, can also include indirect effects such as rain induced drowning and soil splash (Titmarsh 1992). Titmarsh (1992), Dillon *et al.* (1992) and Kyi *et al.* (1991) all conclude that a large proportion of egg and neonate mortality can be apportioned to abiotic factors. Similarly overseas studies attribute mortality of *Helicoverpa zea* (Boddie) (formally *Heliothis zea*) to abiotic factors such as wind, rain and abrasion but also from natural enemies (Nuessly and Sterling 1994).

Mortality in the field due to natural enemies

Studies both in Australia and overseas have shown that natural enemies can impact on heliothis populations in the field. The information available is difficult to compare due to varying methodologies and heliothis species. It should be noted that there are also numerous studies on predation tested in laboratories and glasshouse situations. These are referred to in Chapters 4 and 5 of this thesis. In Australia, studies have shown that natural enemies including pathogens, predators and parasites (Seymour and Jones 1991) contribute to heliothis mortality in the field. The use and effectiveness of pathogens are tabled in Teakle (1977).

Most studies that report the impact of natural enemies generally include both predators and parasitoids as a group. However, there has also been ongoing research in the sole use of parasitoids as biological control agents. Several species have been studied. The native *Microplitis demolitor* (Wilkinson) is thought to contribute to mortality of heliothis larvae, when included as part of IPM (Annetts *et al.* 1998). Two larval parasitoids (*Hyposoter didymator* Thunberg and *Cotesia kazak* Telenga) were also introduced into Western Australia in 1983 and in eastern Australia in 1991, but their impact has been minimal (Murray *et al.* 1995b). Twine (1973) found the parasite *Telenomus* sp. nr. *triptus* Nixon accounted for 97% of sampled heliothis eggs found parasitized in the cotton growing areas of New South Wales and southern Queensland. He also found *Trichogramma sp.* accounted for some of the parasitism. Research into *Trichogramma* species has been explored for some 60 years (Scholz and Zalucki 1998). Through the application of inundative releases some *Trichogramma* species are known to be effective biological control agents of heliothis (Oatman *et al.* 1983) but within Australia their performance has been variable (Scholz and Zalucki 1998). With the exception of these trials, most research to date on effective control in Australian cotton has been based on using a parasitoid agent to reduce heliothis to below current economic thresholds. Evaluation of parasitoids as a tool to aid in reduction of the target pest under IPM situations has not been widely considered. However, there are a few examples of their efficiency in conjunction with other control methods such as predators and alternative spray systems.

More often studies include the use of both parasitoids and predators together. In comparisons of unsprayed, biological systems (Bt spray, natural enemy conservation and release of parasitoids), reduced pesticide systems and conventional cotton systems, Murray *et al.* (1994) showed natural enemies can be used to manage heliothis in some seasons in rain grown cotton. Yield was used to infer that natural enemies reduced heliothis damage. Natural enemy activity was considered higher on unsprayed and biological systems. Furthermore, Scholz *et al.* (1996) showed that use of the naturally occurring parasitoids *Trichogramma sp.* and beneficials, as well as NPV were sufficient to prevent numbers of heliothis exceeding economic thresholds in dryland cotton. This level of control was largely attributed to parasitoids.

The majority of studies do not single out specific natural enemy species as efficient control agents but show that the natural enemy complex helps to control heliothis. Mensah and Singleton (1998) showed that beneficial insect numbers are higher when Envirofeast or IPM plots are used, and the number of pesticide sprays are reduced in Envirofeast food spray and lucerne strip crops. Although no direct evidence was provided on the relationship between beneficial insects and heliothis numbers, the number of eggs and larvae was consistently lower in plots where IPM was practised (Mensah and Singleton 1998). In another study heliothis egg losses of 31-88% were attributed in part to predation (Kyi *et al.* 1991). Likewise loss of small larvae was partly related to the presence of beneficial insects including nabids, coccinellids, lygaeids and spiders on plants (Kyi *et al.* 1991). In a comparison between open and caged cotton plants Titmarsh (1992) showed that predation on heliothis eggs was between 14 and 44% and on first instar larvae between 15 and 44%. This contributed in part to an explanation of unknown disappearance of heliothis eggs and instar stages from life tables on mortality of heliothis. In the case of brown eggs, predation accounted for 83% of unknown losses in the field (Titmarsh 1992). Titmarsh and McColl (1992) suggest natural enemies provide a valuable (although variable) resource.

In some cases, presence of beneficials is thought to imply predation. Pyke (1980) estimated predator numbers per metre of cotton in IPM and conventional crops. Predators were ranked according to persistent presence and abundance. Peak abundance appeared mid December to late January (Pyke 1980). Nabids (*Nabis kinbergii* Reuter) were considered the most consistent predator even in conventionally sprayed cotton. *Dicranolauis bellulus* (Guerin Meneville), *Coccinella transversalis* (Fabricius), *Micraspis frenata* (Erichson), *Deraeocoris signatus* (Distant) were also considered to be consistent predators. Pyke (1980) stated work on *N. kinbergii* should be given priority. Stanley (1997) showed a correlation between abundance of *C. transversalis* and heliothis eggs but found the majority of predators were correlated with the jassids *Austroasca viridgrisea* (Paoli) and *Orosius argentatus* (Evans). This perhaps was because they are present in high numbers consistently throughout the season. Bishop and Blood (1981) compared four spider species in cotton and found a numerical relationship between two of the species (*Chiracanthium diversum* Koch and *Oxyopes mundulus* Koch) and heliothis abundance. Titmarsh (1992) suggested abundance of populations alone does not measure predator effects, as mortality due to abiotic factors, has not been considered. He concluded that little mortality of heliothis can be attributed to natural enemies. However, as

mentioned above he did attribute a large proportion of "unknown disappearance" of brown eggs and early instars to predation. It remains important to verify that if a predator species is present, it is contributing to mortality of the prey present. It is acknowledged that this is very difficult to do (Kyi *et al.* 1991, Seymour and Jones 1991).

Overseas studies

Studies on the impact of natural enemies on heliothis in cotton are also highly variable. In his review of the effectiveness of various predators, Ives (1980) found that a search of the literature over ten years revealed only about 20 studies that gave data on predation rates, search efficiency, prey preference and prey size. In spite of this, predators are considered important control agents of heliothis (Greenstone and Morgan 1989). More detailed studies overseas have shown that predators do contribute to heliothis mortality in the field. Sugonyaev (1994) in his review of cotton pest management, in the former USSR, documents that natural enemies control 60-70% of pests in cotton including *H. armigera*. These natural enemies form the basic component of IPM programs. Likewise, Mamedov (1989) showed predation was the main cause of mortality to *H. armigera* eggs and early instars in cotton. In the United States, Nuessly and Sterling (1994) showed mortality of heliothis eggs reached over 80% in the field when eggs were placed artificially on plants. Hogg and Nordheim (1983) showed that part of heliothis mortality was attributed to the action of natural enemies. McDaniel and Sterling (1979, 1982) showed predation of *Heliothis virescens* (F.) eggs was on average 73% and 78% throughout the season in each study in cotton. Earlier studies on *H. virescens* and *H. zea* have shown the potential of predators to reduce populations of heliothis by up to 96% (Ridgeway and Jones 1969, Van den Bosch *et al.* 1969, Lingren *et al.* 1968, Ridgeway and Jones 1968, Whitcomb and Bell 1964, Fletcher and Thomas 1943). In India, Romeis and Shanower (1996) in their review of natural enemies of *H. armigera* provide detailed information on many studies that show the impact of natural enemies in several crops. Also in India, work by Dhandapani *et al.* (1992) showed that two *Chrysopa* spp. contributed to 40% larval mortality in field grown cotton. In Africa, Van den Berg and Cock (1993) showed anthocorids and ants were important predators of *H. armigera* in cotton. A later study showed anthocorids accounted for 65% loss of *H. armigera* eggs in the field (Van den Berg and Cock 1995). There remain few examples of quantitative data on predation in cotton.

Collectively these reports are highly variable (perhaps due to differing methods) and as Stanley (1997) pointed out due to them being studies in different climatic regions, with varying numbers of prey initially available to the predators. Given many of the predators are generalist feeders these studies contain information that must be considered from a point of view that, there is a danger in assessing predators in the absence of alternative prey (Ables *et al.* 1978, Ridgeway and Jones 1968) and with laboratory or artificial studies only (Seymour and Jones 1991). These issues are further addressed in Chapters 4 and 5 of this thesis.

The impact of individual species in Australia

There are differences between heliothis species and predators in Australia to those from overseas. The main differences are the number of prey available to predators and, although the predator genera are similar, the species are different. However, given the results from overseas and the similarity in predator complex (but not heliothis numbers) it seems likely that in Australia predators will feed on heliothis. This has been confirmed by Room (1979b) in laboratory and field studies. There are about 10-15 main families and at least 24 species of predacious insects on heliothis in cotton (Room 1979b). Zalucki *et al.* (1986) lists 30 species of potential predators. These include the parasitoids, ground dwelling carabids and generalists such as coccinellids and hemipterans. Other important predators include *Chrysopa* spp. (which have been commercially reared) and *Orius* spp. (Broadly 1980). Stanley (1997) provides a table of insects he found in cotton and their feeding status. He lists 32 predators and 34 parasites but does not determine which attack heliothis. Room (1979b) lists *Diomus notescens* (Blackburn), *Campylomma livida* (Koch) (synonymous with *C. liebknechtii* Girault) *Oxycarenus luctuosis* (Montrouzier and Signoret) and *C. diversum* as the predators that were most regularly detected as having predated on heliothis eggs in the field. Using a field cage study Stanley (1997) found that when five *D. bellulus* adults were in one metre of cotton mortality of *H. punctigera* was 7% on eggs and 22% on larvae. This increased to 31% on eggs with 30 beetles per metre. The lacewing, *Mallada signata* (Scheider) larvae were found to have a similar impact. Some predators such as the smudge bug, *D. signatus* are considered pests but are also known predators (Chinajariyawong and Harris 1987). Many of the species listed above are generalist predators known to feed on other pests within cotton (Wilson *et al.* 1998a, Stanley 1997).

Use of natural enemies in Australian cotton pest management programs

There are three basic ways to use natural enemies; these are via conservation, augmentation and inundation. They can also be used independently or integrated as part of decision making management on spray programs in the form of IPM (Dent 1991). To date, the main thrust in cotton has been to conserve natural enemies, especially early in the season. Examples of these strategies are limited in Australia but do exist. As the use of specific parasites has been mentioned above this section only refers to predators. Predator conservation was part of a management strategy in sprayed cotton on a property in south-east Queensland in the late 1970s (Pyke 1980). Here, predator numbers influenced the type of spray used or the timing of the spray. Decisions were based on abundance of predators in total. In another program the reason for not spraying in the early season was attributed to predator presence and using a threshold of two heliothis larvae per metre (Deutscher and McKewen 1996). The most work on the use of natural enemies to date is by Mensah (1997) and Mensah and Harris (1995, 1996a and 1996b) and Mensah *et al.* (1996). They incorporated predators into pest management decisions through the use of predator:prey ratios and, as mentioned earlier, through conservation of natural enemies by using lucerne as refugia and Envirofeast sprays to encourage beneficial insects. This research is ongoing and to date has contributed considerably towards preparing growers in the adoption of IPM in Australia cotton. However, the predator/prey ratios are not yet based on a knowledge on the effects of individual predator species (Mensah *et al.* 1996). As yet there appears to be little direct and systematic consideration of natural enemies in current cotton management programs.

1.4 Conclusion

To date the literature suggests that natural enemies can impact on heliothis populations and most researchers agree that it is important to conserve natural enemies in order to aid control of heliothis. However, in light of the research already done the fundamental limitation to their incorporation into IPM programs at present seems to be a lack of understanding of the management and role of natural enemies in predation. Many researchers suggest that until we know more about the impact of natural enemies they cannot be effectively incorporated into

management programs. Broadly (1980) suggested manipulation of natural enemy populations can minimise or eliminate pesticide application but an understanding of the predator complexes must be clear before they can be used in management. Murray and Mensah (1996) state that there is little known about the role of individual species of natural enemies in Australian cotton. This was earlier suggested by Murray (1992) who stated that "we don't have confidence to rely on natural enemies because we don't know enough about them". Strickland *et al.* (1996) suggests that one of the key focuses on the future of IPM should be maximising the use of beneficials. Even more recently Alexander (1998) wrote "farmers should be looking for beneficials when making heliothis decisions" and Wilson *et al.* (1998b) states that "clarifying the role of predation ... is essential for developing integrated pest management systems". Fitt (1989) also says that "until the efficacy of beneficials is quantified, their potential is unlikely to be utilised efficiently". These are just a few examples of many references that argue for the need to conserve beneficial insects. However, as Seymour and Jones (1991) point out there remains very little evidence on the direct impact of beneficials on heliothis in Australia. If they are to be incorporated into management programs as the above authors suggest then we need to know considerably more than the current literature provides on their role in mortality of heliothis including the role of individual natural enemy species within Australian cotton.

Limitations to our understanding of predator impact

As mentioned earlier the lack of detailed information on the direct impact of natural enemies of heliothis can be partly attributed to the fact that it is very difficult to do such studies. One reason for this has been the negative impact of spray drift (associated with pesticides) onto experimental plots of unsprayed cotton (Gibb 1998, Mensah and Harris 1996a, Murray and Mensah 1996, Wilson *et al.* 1996). Also, until recently, the ecology of most natural enemy species has not been studied outside the cotton field (Walker *et al.* 1996, Yee 1998). This is important when considering the potential of a natural enemy (Metcalf and Luckmann 1994) and their impact on a regional level. As Matthews (1997) states, research into IPM requires interdisciplinary action and co-operation on a regional basis. This view is shared by Zalucki *et al.* (1998). There remains a need for both growers and researchers to develop methods to allow the impact of natural enemies to be studied without these limitations.

1.5 Purpose of this study

In Australian cotton very little is known of the efficiency of individual predators as control agents on heliothis. The aim of this study is to compare and evaluate individual predator species in order to add to our understanding of their potential use for control of heliothis. There are obvious gaps in our understanding of the impact predators have. These include: how many prey each species consumes, where and when they consume and even which pest or prey species is consumed. The way in which predator species have been assessed also requires further investigation as methods such as exclusion cage studies often do not address the needs of predators that are generalist species. Some of these issues are addressed in the following chapters.

It is important to determine which method should be used for assessing the impact of generalist predators, based on the practicality of each method and the optimal way of studying the predators. These questions are discussed in Chapters 2 and 3. Chapter 2 describes the general methods used to collect and study predators throughout this study. Chapter 3 investigates parameters such as temperature, prey location and predator preparation that may influence results of predation studies and interpreting results when evaluating the efficiency of each species. Chapters 4 and 5 discuss the way in which evaluations of natural enemies can be studied and evaluate three species of predators taking into consideration the need to evaluate predation under realistic conditions and conditions applicable to generalist rather than host specific natural enemies. This includes the numbers of prey consumed, the size of the search arena and the provision of alternative prey.

Given the degree of difficulty of assessing predation in the field it is always important to explore new methods for assessing predation. Chapter 6 and 7 provide an insight into using more recent technology to study predation directly in the field with the use of serological techniques never before used in Australian cotton. Chapter 8 is the first attempt to put the serological technique into practice and provides some useful initial information on direct predation of heliothis in Australian cotton. Chapter 9 collates the information of this thesis and puts it into the context of both current management practices and future practices that involve conservation and IPM.

Chapter 2

2. General Methods.

In order to compare the ability of different predator species to consume heliothis under various conditions such as searching environments and prey availability, laboratory and serological trials were conducted at the University of New England (UNE), Armidale, New South Wales. Since trials required the use of large numbers of predators, and rearing facilities and methods were not readily available at the commencement of this study, adults of each species were collected from the field. The three main species collected were *N. kinbergii, D. bellulus* and *C. transversalis*. These were used for both laboratory and serological studies. When available, other species (*D. notescens, Harmonia octomaculata* (Fabricius), *H. conformis* (Boisduval), *M. frenata* and *D. signatus*) were used for serological trials.

2.1 Collection of predators

Collections were made on a regular basis throughout the cotton growing season so that newly captured predators were always used in trials. Collecting trips were made every 4-5 weeks from early October to late February each growing season. Collection sites were located in the Narrabri district around the Australian Cotton Research Institute (ACRI) at Myall Vale west of Narrabri (30°13'15"S, 149°37'33"E) and along Bullawa creek (30°17'15"S, 150°1'30"E), NE of Narrabri towards Mt Kaputar National Park (see Figure 2.4 in section 2.4). Due to high mortality *N. kinbergii* could not be kept in captivity for long periods so it was collected at the UNE farm, "Lauredale" (30°29'15"S, 151°39'33"E) in Armidale, NSW.

Generally the best sites found for collection of mass numbers of predator species (100+) were patches of weeds and grasses growing in damp areas along creeks, or soaks in fields. The species of weeds and grasses on which predators were most common were; medics *Medicago* spp. (Fabaceae), variegated thistle *Silybum marianum* L. (Asteraceae), purple clover *Trifolium incarnatum* L. (Fabaceae), wire weed *Polygonum aviculare* L. (Polygonaceae), and the grasses *Urochloa* spp. and *Lolium rigidum* Gaudin (Poaceae). When *D. bellulus* numbers were high they were also found on *Allocasurina cunninghamiana* (Casurinaceae) growing along creeks. *Dicranolauis bellulus* were most abundant on river rocks (possibly feeding on lichen and moss) and easily collected using an aspirator. The coccinellids (*C. transversalis, M. frenata* and *Harmonia* spp.) and *D. bellulus* were particularly common on purple top verbena *Verbena bonariensis* L. (Verbenaceae). *Nabis kinbergii* were more common to grass sites. Another predator species, *D. notescens* was found most commonly on *S. marianum*, wild oats *Avena fatua* L. and *Avena ludoviciana* Durieu (Poaceae) and *V. bonariensis*. Crops that were good

for collecting most predator species included lucerne *Medicago sativa* L. (Fabaceae), and chickpea *Cicer arietinum* L. (Fabaceae) mixed with weed grasses. Occasionally large numbers of coccinellids were found on saffron thistles *Carthamus lanatus* L. (Asteraceae) and sunflowers *Helianthus annuus* L. (Asteraceae).

Field collections

As predators collected from the field were for use in laboratory trials only, a formal sampling strategy was not required. Predators were collected using a sweepnet in most locations with the exception of thistles and creek-side weeds where a beating tray or aspirator was used. Sampling methods used to determine the abundance of predators in cotton for serological assay are outlined in Chapter 8. Identification of predator species was confirmed with type specimens from the Australian National Insect Collection (ANIC) by Tom Weir, Ian Neumann, Mary Carver and myself.

Storage of predators

As predators were collected from the field, each species was placed in Chinese food containers (17 x 12 x 6 cm) using an aspirator. A moist dental wick and some vegetation from the collecting site were placed in each container. The vegetation was particularly important for *N. kinbergii* as they are known to attack each other. Fine gauze (curtain material) was placed over the container and a lid (into which a 5 cm square had been cut) was sealed onto the container. This allowed for adequate aeration and the dental wicks to be kept moist with distilled water from a squirt bottle. All containers were placed in a large esky (cooler) containing ice bricks and kept cool (c.17 ^{o}C) until transported to the laboratory in Armidale.

2.2 Laboratory trials

Once at the laboratory, all predators were kept at room temperature (25 ^{o}C) and provided with moist dental wicks each day. Predators were pre-fed cultured heliothis eggs, pollen (Flower Power Bee Pollen® granules) or cotton aphids (*Aphis gossypii* Glover) until used in experiments. The type of prey each species was fed, depended on the experiments to be conducted. These are discussed in detail in the relevant chapters. Female and male predators were not separated until they were used as this may affect feeding (R. Mensah pers. comm.).

Cages

Single plant experimental cages were made from anti-static (0.0178 cm thick) plastic with gauze tops and two side ventilation panels covered with gauze (Figure 2.1). Each cage was approximately 20 cm in diameter and 75 cm in height. The cage base was pushed into the soil to avoid predators escaping. Petri dishes were 8.5 cm in diameter and contained only a moist dental wick. Four large multi-plant cages were used in searching experiments (Chapter 4). Each was made from the same material as the smaller cages. Multi-plant large cages were 48 x 100 x 80 cm in size with gauze tops for adequate aeration and aluminium frames for stability. Once plants were placed in a large cage, a final panel was fixed onto the aluminium frame with velcro strips.

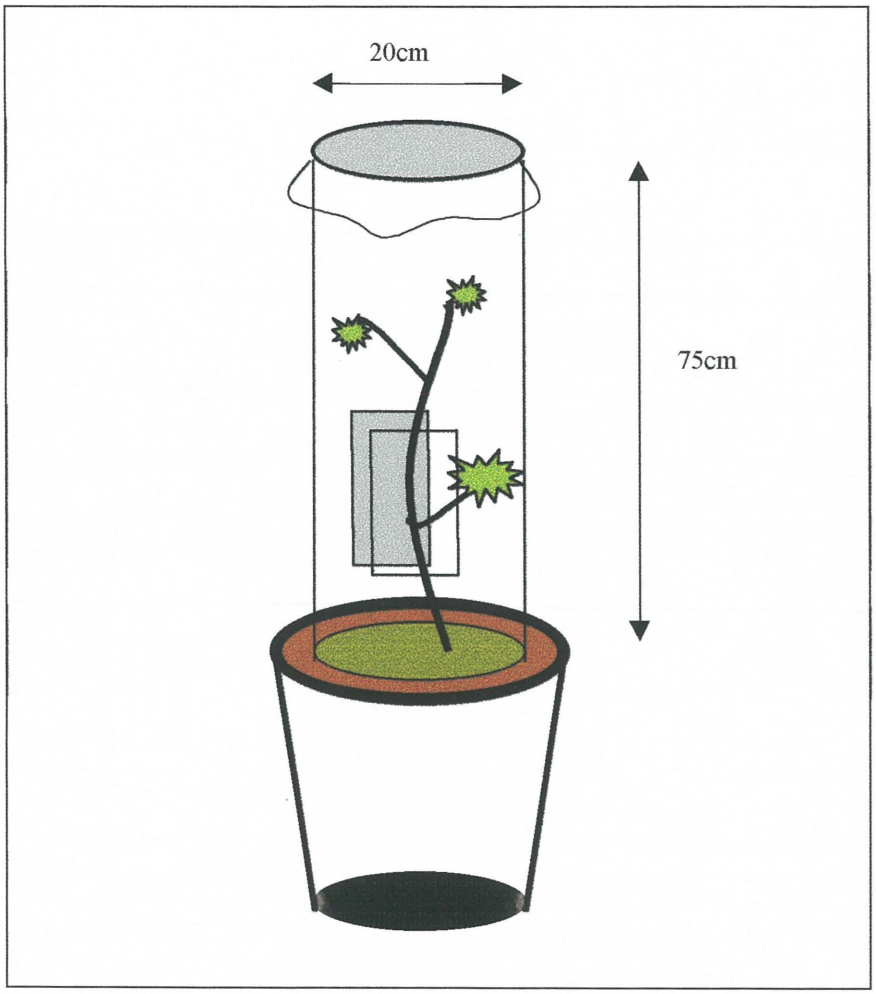

Figure 2.1 Schematic diagram showing a single plant cage used for evaluation of predators in an environment cabinet.

Environment cabinet conditions

Most experiments were conducted on caged potted plants or in Petri dishes in a Honeywell Thermoline T7300 environment cabinet. Unless specified the temperature inside the cabinet was set at 25 °C. Temperatures within the cabinet were recorded using a Tain® micropower data logger. In each experiment the wet/dry temperatures were recorded near both a plant without a cage and a caged plant to check whether caging affected temperature and, if differences occurred between experimental days. Figure 2.2 shows typical wet and dry temperatures inside and outside a small cage when the environment cabinet was set at 25 °C. The average day/night dry temperatures inside the cages were 30.7 °C and 24.9 °C respectively showing the cages increased temperature. Figure 2.3 shows a typical reading of wet and dry temperature inside and outside a large cage. In larger cages the average temperature during the day was 29 °C and 23 °C at night. Photoperiod was set at 14:10 hours day/night. Light was increased gradually over the first (6:00 am) and last (20:00 pm) hour to simulate dawn and dusk.

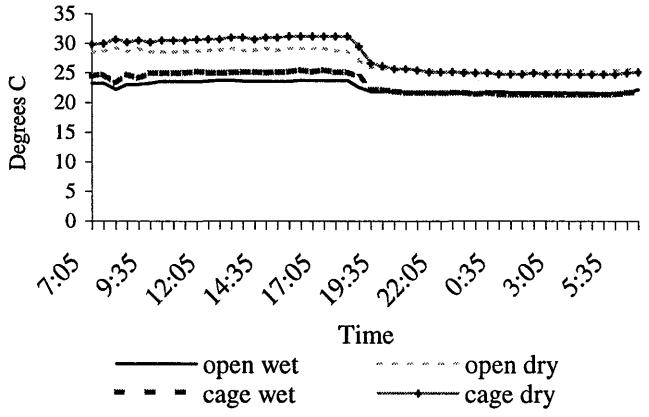

Figure 2.2 Typical wet and dry temperatures recorded over 24 hours in small cages compared with the surrounding environment cabinet. "Open wet" and "open dry" refer to wet and dry temperatures recorded inside the cabinet near an uncaged plant. "Cage wet" and "cage dry" refer to wet and dry temperatures recorded near a plant inside a cage within the cabinet.

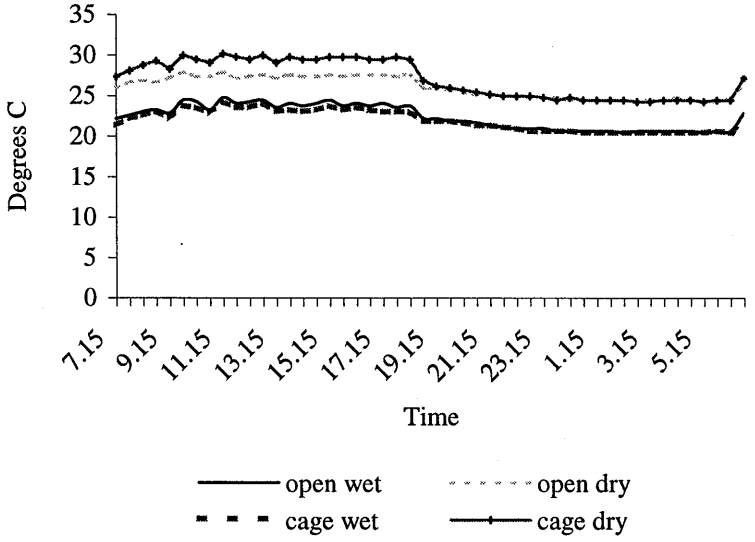

Figure 2.3 Typical wet and dry temperatures recorded over 24 hours within a large cage compared with the surrounding environment cabinet. "Open wet" and "open dry" refer to wet and dry temperatures recorded inside the cabinet near an uncaged plant. "Cage wet" and "cage dry" refer to wet and dry temperatures recorded near a plant inside a cage within the cabinet.

Plant cultivation

Sicala V2 cotton seeds were germinated directly in black clay soil obtained from the ACRI in Narrabri. Fifty grams of Starter 15® fertiliser were added to each 25 cm pot. At week four, plants were fertilised using a rate equivalent to 60 kg/ha nitrogen, 40 kg/ha phosphorous, 50 kg/ha sulphur, and 4 kg/ha zinc and added together at 400 ml/pot. Plants were checked each day for signs of mite, aphid or thrips infestation. Plants that were heavily infested with pests were removed immediately or, if infestations were low, the pests were removed with a small paint-brush. Unless specified in each chapter, all plants were used at the pre-squaring stage, from four to six weeks after emergence.

2.3 Prey

Helicoverpa armigera and *H. punctigera* were supplied from a stock culture at UNE or ACRI. The diet used for culturing is described in Teakle and Jensen (1985).

In the first season, the UNE culture did not allow for a continuous supply of heliothis eggs. Eggs of each species (*H. armigera* and *H. punctigera*) were only available for one week on a six week cycle. Therefore, the number of experiments that could be done was limited. In order

to increase the number of experiments *H. punctigera* eggs were used for some experiments in the first season, particularly when predator numbers were low and experiments had to be completed whilst predators were available. In order to test whether the use of *H. punctigera* eggs changed results, a comparison of consumption of *Helicoverpa* species eggs by each predator species was conducted. The methods and results of this experiment are provided in Chapter 3.

In the second season a source of heliothis eggs from ACRI (Narrabri) was obtained. As a result only *H. armigera* eggs were used. These were delivered weekly from the ACRI culture. In the third season a semi-continuous culture of *H. armigera* was set up at UNE to allow for daily supply of eggs. This provided eggs for approximately three out of every five weeks for the season.

2.3.1 Prey handling

In culture, moths are allowed to lay fresh eggs each night on paper towelling. The towelling was collected each morning and on the morning of each experiment, eggs were brushed off the paper towelling with water and painted on to plants with a camel hair brush. The eggs are able to adhere to plants due to the protein substance for forming egg white (egg albumen) on the egg creating a natural glue. Placement of eggs on the plants is discussed in detail in Chapters 3 and 4. As plants were re-used, all excess eggs were removed at the end of each experiment and the plants were washed down with water to remove traces of egg albumen in case this influenced predator feeding in the next experiment.

2.4 Serological trials

Serological trials explored the establishment and use of immunodot assays to assess predation by identifying prey remains in the guts of predators. The methods are discussed in detail in Chapters 6 and 7. However, the majority of trials required similar collection and feeding of predators, which are discussed below.

Feeding trials

In order to establish an immunodot assay method predators were collected using the techniques described earlier in this chapter (2.1). Approximately 30 individuals of each predator species were required for each feeding trial. For the serological tests predators that

had been starved of any previously consumed prey and then allowed to feed on one heliothis egg were required.

Feeding trials were done during the day at 27 ^{o}C and 14:10 hours light/dark in a small culture room at UNE. Each individual was starved of heliothis and any other prey for 24 -72 hours prior to feeding it one heliothis egg. Initial trials comparing the clarity of seeing an individual feed were done using eppendorf 1 ml tubes, potted cotton plants and Chinese food containers. Chinese containers were found to be best for observing if a predator had fed on prey. Continuous observation during the day was found to be the only way to ensure, without doubt, that the predators had fed. Each individual predator was therefore placed in a Chinese container into which 50 or more fresh eggs had been painted on to the inner surface of the container.

Two main experiments were required for the serological work. First, in order to test whether an insect had fed (a positive result), predators were allowed to feed on a single prey. Each predator was observed until it had fed on one heliothis egg. After it had moved away from the egg the predator was removed and placed into a small 1 ml eppendorf tube, labelled and stored in liquid nitrogen until required for assaying. Another predator was then placed in the feeding container and so on until enough replicates were obtained. For each predator that had fed, another that was starved of heliothis prey for 48 hours (a negative result) was also frozen as a control.

Secondly, predators were required for digestion studies, which determine the length of time prey antigen can be detected in the predator gut after feeding. These were fed in the same way as the positive and negative trials but after feeding they were placed in a small container with an alternative food source (bee pollen) and water, allowed to digest for pre-determined times (outlined in Chapter 6), and then frozen in liquid nitrogen. Details of each trial are outlined in Chapters 6 and 7. Feeding trials took anywhere from eight hours to one week to complete. This depended on predator availability and how readily they ate. Predators collected fresh from the field were found to be the most active feeders. This accelerated the feeding trials. For each digestion trial, controls of predators, which were starved of any heliothis for 48 hours, were frozen and stored in liquid nitrogen as well.

2.4.1 Field study

Once established, the serological method was tested on field collected predators. Two sites were chosen. In the 1996/7 season plots of Sicala V2 conventionally sprayed cotton (using conventional management practices) and unsprayed (no pesticides) cotton were sampled. The sprayed site was located at "Yarral" property on the Wee Waa road near the ACRI and the

unsprayed site at the Wheat Research Institute (WRS) just north of Narrabri. As there was a shortage of unsprayed cotton at the WRS in the 1997/8 season a new site was utilised at the ACRI. Conventionally sprayed cotton did not yield enough predators to assay in the first season so it was not included in the final season. Figure 2.4 shows approximate locations of each sampling site and sites where the majority of predators were collected.

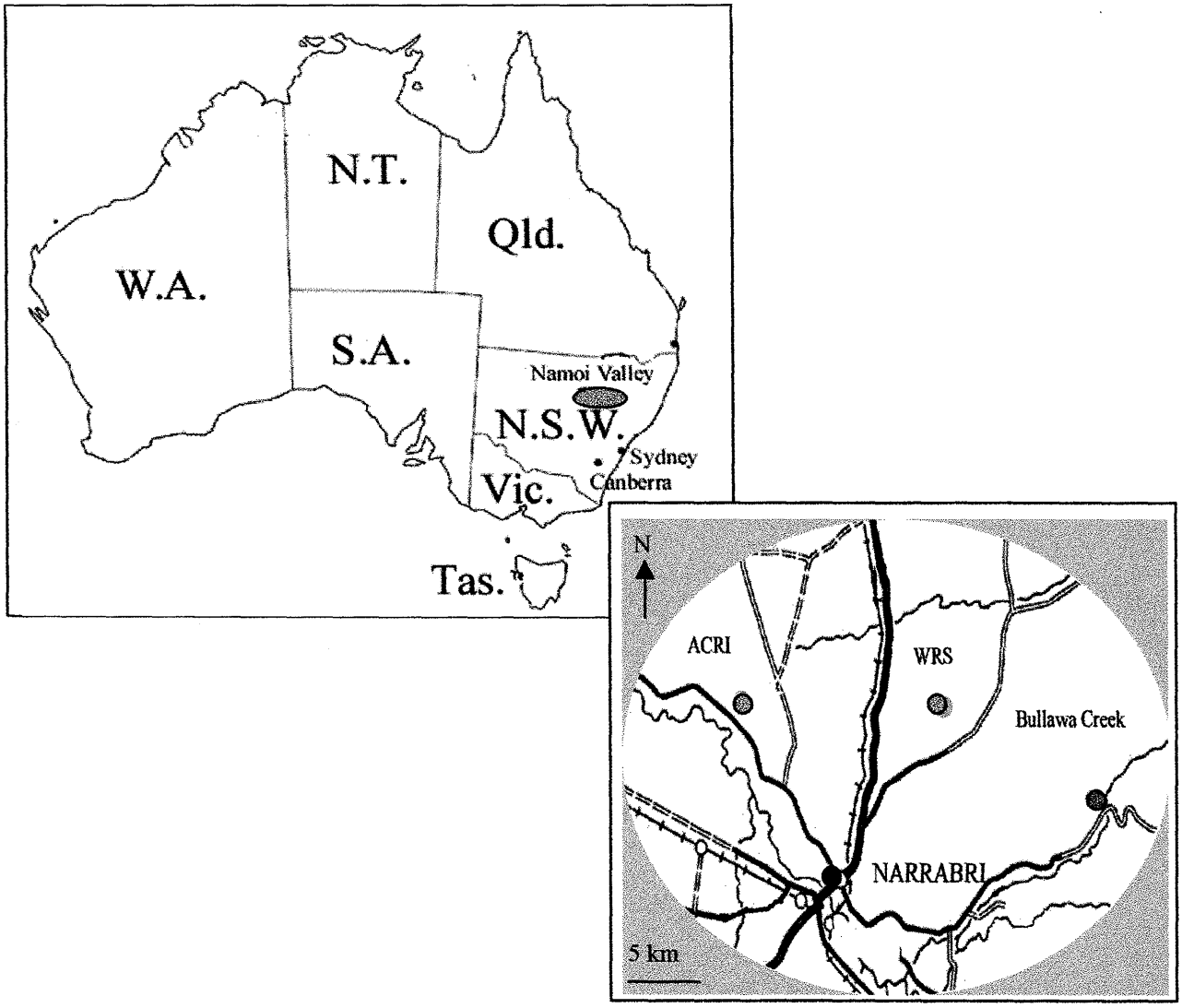

Figure 2.4 Map showing the location of the Namoi valley where field work was conducted. Inset shows the ACRI and WRS sites where serological field trials were done (green dots) and sites where most insects were collected for laboratory studies (green and red dots).

Sampling

In the 1996/7 season one of the above sites was sampled per day on each of four field trips. In both seasons a check was done at each site early in the morning to obtain heliothis egg

numbers. This was done following the protocol outlined in the entomoLOGIC '96 manual for cotton management in Australia (Anon 1996a). Thirty whole plants were checked, one metre at a time (about 5 plants per metre). As predator numbers were on occasion low, in the following season one site was sampled over one week to obtain sufficient predators for assaying.

After checking heliothis numbers, each site was suction sampled using a Homelite® HB 180V Blower/Vacuum (D-vac) for collection of predators. Although suction samplers have limitations when used for quantitative estimation of populations (Stanley 1997) in this thesis they were used solely for obtaining insects for laboratory experiments. Nets made of knitted voile were fitted over the opening of the vacuum nozzle. Twenty metres of cotton plants were sampled by suctioning one metre diagonally up and one metre diagonally down the cotton plants whilst walking along the row. The diagonal ran from the tip of a plant to a base so that *D. bellulus* would be included (Stanley 1997). The samples were immediately frozen in a car fridge and taken back to the laboratory for sorting. Predator species that were required for assaying were stored in individual small tubes, labelled and placed in liquid nitrogen for future assay. The abundance of each species of predator and prey type was also recorded. Details of sampling strategies and abundance are outlined in Chapter 8. Once collections were made material was assayed. The final protocol used for these assays is discussed in detail in Chapter 8. Details of the establishment of the assay method are discussed in Chapters 6 and 7.

2.5 Analysis

For all experiments treatment means and standard errors were calculated for each data set. These were plotted and where it was obvious that no significant differences occurred the data were not further analysed. Analysis was carried out when plots did not clearly show non-significant results. Data were analysed using Genstat 5.3.2. (Anon 1995). Unless specified in each chapter all analysis used Generalised Linear Models (GLM). For data with repeated measures Generalised Linear Mixed Models (GLMM) were used.

Generalised Linear Models

It was assumed that the data collected from experiments followed a binomial error structure as prey were either eaten or not. The EXTRABINOMIAL procedure of Genstat 5.3.2 was used as well as a logistic link function in calculations for fitting each model. The EXTRABINOMIAL procedure allows individual terms to be dropped from a saturated model thus it allows an examination of their contribution to the deviance explained by the model. This facilitates obtaining an amended model that fits the data. The amended model is run to determine if the terms are significant. Plots of deviance residuals showed the data to be consistently over

dispersed, therefore the over dispersion model III (Williams 1982) was included in each analysis. The final output of each analysis did not provide an ANOVA table. Consequently, p values were calculated from t values and the differences between estimate values divided by the standard errors of pairs. Where there was more than one term in the model this provided multiple t tests. As a result significance levels were adjusted using the Bonferoni method (Hannah 1996) to guard against over optimistic error rates. This provides a conservative estimate of significance. The significance level assigned to each experiment is referred to in the text as the 'bf' value. The terms included in each amended model and the significant results from the amended model are given with each experiment within each chapter. These degrees of freedom used for each analyses are shown in the text as 'df'. An example of obtaining an amended model, analysis of the amended model and how p values were obtained is provided in Appendices 1 and 2. Non significant results within an analysis are referred to as $p > 0.05$.

Repeated measures

Data recorded over time were analysed with Generalised Linear Mixed Models (GLMM) using repeated measures. In all tests the marginal methods of Breslow and Clayton (1993) were used. Unlike the EXTRABINOMIAL procedure the GLMM did not allow terms from the saturated model to be dropped to test for the contribution of each term to the deviance in the model. Main treatment effects were determined using the Wald statistic (Anon 1995). The significant terms from the Wald statistic were used as the amended models. These were tested for how well they fit by plotting each model to the data means. Once a good fit was obtained the amended model was run. Results from the analysis allowed a comparison of slopes and the point at which 50 % of heliothis eggs were consumed, to be calculated. Where slopes did not differ y intercepts and predicted means were calculated and each compared. As some of the analyses required multiple t tests the significance level was adjusted using the Bonferoni method as above (Hannah 1996). The adjusted significance value is shown in each results section for each experiment by the term 'bf'. An example of this type of analysis using an amended model is provided in Appendix 3.

2.6 Conclusion

The collection and feeding of predators and the experimental arenas used in the following chapters are shown above. However, each chapter (Chapters 3-8) also provides further details of the methods used in each experiment done in this thesis. As the effects of the environment cabinet, using starved predators, prey location on plants and prey species were unknown, the next chapter (Chapter 3) looked at the effects of these parameters on the number of prey consumed by predators.

Chapter 3

3. Determining experimental conditions.

3.1 Introduction

There are many parameters that affect feeding by generalist predators both in the field and the laboratory. These include temperature, hunger status and prior feeding history, searching ability and search area, prey type and prey density (DeClercq and Degheele 1994, Donahoe and Pitre 1977, Holling 1959). The type and size of a study arena used to assess predation can influence the results obtained from experiments. This is also due to the influence of parameters such as temperature. The source of predator and prey can also affect the level of predation that occurs. It is, therefore, important to optimise the conditions of the study arena so that predation is not hampered and variation from sources other than inherent predator efficiency is accounted for. This chapter determines the optimal conditions for studying predation in a laboratory situation.

Temperature

Temperature may affect the searching ability of insects by slowing them down or forcing them to escape desiccation. Crocker *et al.* (1975) showed the potential rate of predation by *Geocoris punctipes* (Say) was altered by temperature. Glen (1975) found searching by the mirid *Blepharidopterus angulatus* Fall decreased with night temperatures. Beisner *et al.* (1997) also found that temperature influenced predation rates. They showed that an increase in temperature led to stronger functional and numerical responses by *Mesostoma ehrenbergii* (Focke) on *Daphnia pulex* Leydig. Temperature may slow searching down but, it may also speed up movement thereby hampering intensive searching. This was observed in *C. transversalis* where temperatures above 30 °C in an enclosed cage caused an increase in frantic behavioural patterns such as frequent flying (personal observation). There may also be variation in optimal foraging temperatures between species of predators. DeClercq and Degheele (1994) suggested that the predatory stink bug *Podisus sagitta* (F.) was likely to perform better at higher temperatures than *Podisus maculiventris* (Say). Just as temperature may influence predation rates the gender of the predator may also be of consequence.

Gender of predators

Female insects require more nutrients in order to sustain reproductive capacity and egg production and may therefore feed on more prey (Hemptinne *et al.* 1996, Chapman 1971). Lingren *et al.* (1968) found *Nabis alternatus* Parshley females consumed more *H. zea* than males. Maximum consumption by hemipteran females is reported during peak egg production and laying (Perkins and Watson 1972). Male *P. sagitta* consumed less than females when offered eggs of *Spodoptera exigua* (Hübner) (DeClercq and Degheele 1994). Conversely, female predators may sometimes be more interested in searching for oviposition sites than feeding. Adult male *Adalia bipunctata* L. ate fewer aphids than females and exhibited a different functional response than females (Hemptinne *et al.* 1996); the males did not increase consumption with aphid density whereas females did. Males also did not change to an area-concentrated search upon an encounter with prey and were less active than females. It is likely that the males spend more time looking for females than feeding (Honek 1985 cited in Hemptinne *et al.* 1996).

Hunger

Holling (1959) considered hunger to be one of the main factors affecting the functional response of a predator. Food deprivation can influence predatory behaviour by increasing rates of attacks and, in ambush predators, the attack distance is increased with hunger (Taylor and Schmidt 1996, Wallin and Ekbom 1994). Hunger can also increase prey species selection and type (size and stage). Starvation can result in death so there is a point where attack overrides security or energy expenditure. This point is reached at 96 hours in *Sinea diadema* (F.) nymphs (Taylor and Schmidt 1996). Hungry insects may also be more responsive to prey than satiated individuals; on the other hand it may decrease predation efficiency for some time. Ambrose *et al.* (1985) noted this in reduvids. In addition most laboratory studies tend to starve predators for 24 to 48 hours prior to use in order to start feeding on an even basis thus providing a uniform predator population (Hemptinne *et al.* 1996, Heimpel and Hough-Goldstein 1994a, Horn 1991, Nakamuta 1984). As well as starvation, obtaining a uniform predator population can be affected by the source of predators.

Source of predators

Hutchinson and Pitre (1983) concluded that field collected predators provide a more realistic look at predation. They used field collected *G. punctipes* to study predation on *H. virescens*. Using field collected adult predators means that the feeding history and age of the predators are unknown. This may affect the uniformity of the predators' feeding. Variation in consumption with field collected predators can occur as older adults feed on significantly less prey than young adults (Quayogode and Davis 1981, Donahoe and Pitre 1977). DeClercq and Degheele (1994) also found great variation in consumption rates by adult hemipterans *P. maculiventris* and *P. sagitta*.

Prey and predator location

In their experiments on cotton Hutchison and Pitre (1983) placed eggs (prey) in the upper third of the canopy on young terminal leaves as this was the preferred oviposition site of the prey. They placed predators on terminal leaves (their preferred activity site) in the upper canopy of the plant. Wilson and Gutierrez (1980) found that predators' preferred locations were on the fruits and underneath leaves close to the main stem and terminal of cotton plants. Coll *et al.* (1997) found that the preferred location of *Orius insidious* Say was different between plant species. In tomatoes the majority were found on the upper leaf surface as opposed to beans and corn where the majority were on the under surface of leaves. Braman and Yeargan (1989) noted that there was a difference in the position of different *Nabis* spp. on the same species on plants. The placement of predator and prey can influence predation results in artificial arenas. Therefore, it is important to know the preferred feeding sites of the predators and (if eggs are used) the oviposition sites of the prey.

In Australia heliothis eggs are laid singly on plant leaves, buds, flowers, fruits and stems (Zalucki *et al.* 1986). In the United States Wilson *et al.* (1980) showed *H. zea* laid the majority of eggs on both leaf surfaces and mostly on main stem leaves. The stage of the prey may also affect consumption (McDaniel and Sterling 1979). As the target prey in Australian cotton are two species of heliothis it is important to know if there are any differences in location between them that may affect consumption by predators. The preys' food plant can also have a significant effect on the predator's functional and numerical response and

searching strategy (Coll and Ridgway 1995, McCall *et al.* 1994). The placement of prey is also important as predators may alter their searching behaviour after encountering and consuming a prey (Coll *et al.* 1997).

It may be of advantage for a predator to feed on prey that has consumed fresh plant material as this may contain greater protein content than prey feeding on mature feed (Horan-Strohmeyer *et al.* 1998). Therefore, it is likely the predators will be found where prey can feed on fresh plant material. There is unlikely to be a difference between male and female predator location on the plant. The position of male and female nabids on plants was similar when studied by Braman and Yeargan (1989).

3.1.1 Purpose of this study

The aim of this study was to provide as near to natural field conditions as possible by testing which parameters may affect the predator's ability to consume heliothis eggs. This included determining the optimal temperature in the environment cabinet, whether differences occurred in consumption of each species of heliothis egg, starvation times for predators, gender comparisons and the position of eggs on cotton plants. These preliminary results are important for further experiments reported in the following chapters.

3.2 Methods

Unless specified, all trials that tested predation parameters were carried out on potted cotton plants cv. Sicala V2. The preparation and treatment of these plants are described in Chapter 2 - General Methods. Predators were collected, contained and provided with H_2O and pollen or alternative prey prior to their use. All predators were placed in the environment cabinet without prey for 24 hours prior to use, to allow adaptation to the light and temperature conditions. Unless specified the environment cabinet temperature was set at 25 ^{o}C and 14:10 day/night photoperiod.

3.2.1 Species of heliothis egg and egg placement

In order to test if eggs of one species of heliothis were preferred, a pilot trial was done using petri dishes in the environment cabinet. Newly laid eggs of both *H. punctigera* and *H. armigera* were obtained early in the morning from the UNE culture. Ten eggs of each species were placed alternatively in two rows, 1 cm apart, across the centre in a petri dish (8 cm diameter) containing a moist dental wick. A single predator was placed in each dish and, as the time of day that feeding occurred was not known, each was allowed to feed for 24 hours. The predator species tested were *N. kinbergii*, *D. bellulus* and *C. transversalis*. Six replicates of male and female predator species were tested in the environment cabinet. The number of eggs consumed from each row were recorded hourly from 10:00 am to 18:00 pm for a total of eight hours and a final count was done at 24 hours.

Egg placement experiments - plant site and egg position

The majority of experiments in this thesis required egg consumption to be counted on cotton plants without disturbance to the predators. Removing cages from the pots to count eggs could disturb the predators. Therefore, in order to see the eggs clearly (without removing the cage from the potted cotton plant), they needed to be placed on the upper surface of open leaves. As this may not be where the predators feed, their preferred feeding sites were tested. Observations in the field on early season plants showed heliothis eggs could be found on the growing tips, and both upper and lower leaf surfaces (personal observation). Consequently, these three sites were chosen as egg position sites for this experiment. Noting where and when eggs were consumed would determine if predators prefer to feed at a particular site and whether the number of eggs consumed differed between sites.

Eight replicates of *N. kinbergii*, *D. bellulus* and *C. transversalis* were prepared for this experiment. Ten eggs were placed on the upper and lower surface of the first fully opened leaf on cotton plants and on the growing tip or first unopened leaf; a total of 30 eggs per plant. Three control plants containing ten eggs at each egg position site were included to make sure no eggs were lost due to factors other than predation. The number of eggs consumed on either leaf surface and the tip were recorded every two hours from 9:00 am to 17:00 pm and a final count was done at 24 hours.

Egg position

The placement of eggs on the plant was also important for data analysis. Firstly, 20 *H. armigera* eggs were placed on the upper surface of one newly opened leaf of a cotton plant. This was referred to as the 'fixed' egg treatment. Secondly, 20 *H. armigera* eggs were placed semi-randomly on cotton plants to reflect more natural heliothis oviposition sites (one egg on or near the edge of a leaf). This was done by painting one egg at a time on the first, second and third leaf of the plant until the required egg density was reached. This was referred to as 'random' egg placement. In both fixed and random egg positions the effect of density was also tested. Five, ten, 15 and 20 egg densities were used to reflect normal to high populations of eggs normally found in the field. Each density was replicated five times for each of the predator species (*N. kinbergii*, *D. bellulus* and *C. transversalis*). The number of eggs consumed were recorded after 24 hours.

3.2.2 Preparation of predators

As predators were field collected from natural populations the age and prior feeding history was unknown. As most studies on laboratory reared predators use a 24 - 48 hour starvation time to standardise hunger levels this was tested for the predator species used in this study. The gender of each species could also be determined so the difference in consumption between the two was tested. The optimal temperature under which feeding occurred was not known for these species. To ensure that the temperature of the environment cabinet did not inhibit prey consumption a range of temperatures were tested that reflected temperatures normally found in the cotton field during the growing season.

Starvation times

Twenty five female *D. bellulus* were individually fed ten eggs in petri dishes on the first day. The following day five beetles that were observed to feed were removed and given only water. The remainder were fed another ten eggs. This continued until there were five beetles that had been prefed and starved for 96, 72, 48, 24, and 0 hours. On the fifth day all beetles were provided with 20 heliothis eggs in individual petri dishes (8 cm diameter) and the number

consumed were recorded hourly from 9:00 am to 17:00 pm and after 24 hours. This experiment was repeated for *N. kinbergii* and *C. transversalis* females. Again there were five replicates for each time starvation time. As the *D. bellulus* were tending to consume all the eggs, the next two predator species were given 30 eggs (instead of 20) in the petri dishes.

Gender

Due to the limited numbers of predators available from field collecting, it would be of advantage to use mixed genders. However, the difference in consumption between the two was not known, and needed to be investigated. Gender for each species was distinguished in the following way. Male *D. bellulus* have an enlarged club segment in their antennae. The ovipositor in female *N. kinbergii* is clearly visible and *C. transversalis* males have a slight notch on their last visible abdominal segment which is not present in females (Pope 1988). The difference in prey consumption between genders of each species was tested at five egg densities (one, five, ten, 15 and 20 eggs per plant) and with five replicates for each density, species and gender. The number of eggs consumed over 24 hours were recorded.

Temperature effects

Throughout the cotton growing season there are usually relatively high temperatures in the field. The daily average temperatures in Narrabri vary with the time of year but they rarely go below an average of 15 °C at night and 32 °C in the day when the plants are at pre-squaring stage (ACRI meteorological data). Several temperatures were compared in the environment cabinet to see if consumption of prey was affected for each predator species. To reflect field conditions the temperatures chosen for this study were 15, 20, 25, 30 °C. As shown in Chapter 2 the temperature measured within caged plants in the environment cabinet was slightly higher than the cabinet temperature. Therefore, the temperatures inside the cages are slightly higher than the ones chosen here. The differences between the temperatures inside the cages and inside the environment cabinet were measured in the same way as those described in Chapter 2. Exact temperatures recorded inside the cages at the time of these experiments are shown in the results section.

Data for temperature comparisons were obtained from a series of experiments over time using the same experimental method. Each predator species and gender were used on separate occasions to test egg consumption at different egg densities over four temperatures. In each experiment consumption of eggs was tested at five densities for both male and female *C. transversalis* and *D. bellulus*. *Nabis kinbergii* were not available in sufficient numbers at the time of these experiments so they were not tested. The reproductive status of the females was not known. However, no egg laying was observed during the experiments. The experiments were done on separate days as there was only one environmental cabinet available. Where possible the same predator cohorts were used and eggs from the same heliothis species were used. The experiments were done systematically to reduce any variation between days for plants and predators. The number of eggs consumed were recorded for each species, gender and prey density after 24 hours.

Analysis

The types of analysis and procedures used for all experiments are outlined in Chapter 2 - section 2.5. However, as the final terms for each analysis were obtained from saturated models, the terms used for each experiment are given in individual sections below. In some cases treatments that were not significant were included in the amended model as the model did not appear to fit without them. An overall test for treatments was determined using Chi-square tests from the saturated model (a conservative estimate). For tests that included the repeated measures the Wald statistic (Anon 1995) was used to determine statistical significance of treatments. The final analysis provided a series of t-tests for each term in the model. As a result the Bonferoni method (Hannah 1996) was applied as a multiple comparison method to guard against over optimistic error rates. The adjusted significance levels (bf), the p values (p =) and the degrees of freedom (df =) are provided in each section. When non significant slopes are referred to no result is given. In these cases $p > 0.05$. The estimated time it takes for 50% of heliothis eggs to be consumed were predicted from the models for data from the starvation and site preference experiments. These results are provided within the relevant sections. As specified in Chapter 2 data were not analysed where it was obvious no differences between treatment means occurred.

3.3 Results

The results showed that the placement of eggs on the plant affected each predator species in different ways. This was also true for starvation times of the predators, gender and the temperature in the environment cabinet. The species of heliothis egg did not affect consumption by any of the predator species tested.

3.3.1 Species of heliothis eggs and egg placement

Data obtained from petri-dish trials on the difference in consumption between *H. armigera* and *H. punctigera* eggs were plotted as means and standard errors. As no obvious differences occurred between the cumulative numbers of eggs consumed by any of the predator species over eight hours data were not analysed (Figures 3.1 - Female predators and 3.2 - Male predators). The figures show one prey species was not fed on first in preference to the other. That is, the predators ate similar numbers of eggs of both species at each time period.

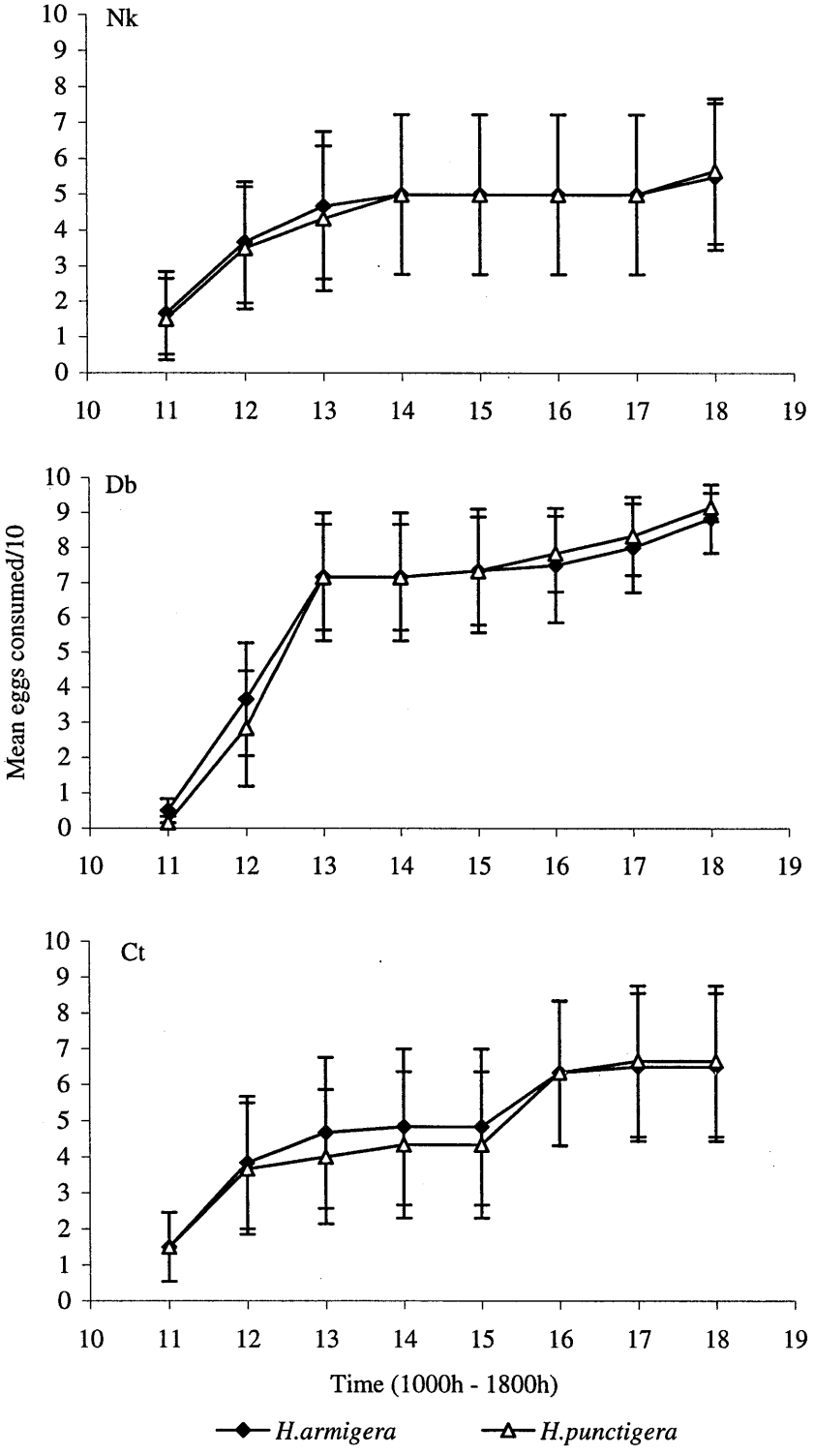

Figure 3.1 The mean cumulative numbers of two different species of heliothis eggs consumed by female predators. Nk = *N. kinbergii*, Db = *D. bellulus* and Ct = *C. transversalis*. Bars indicate standard error of the means.

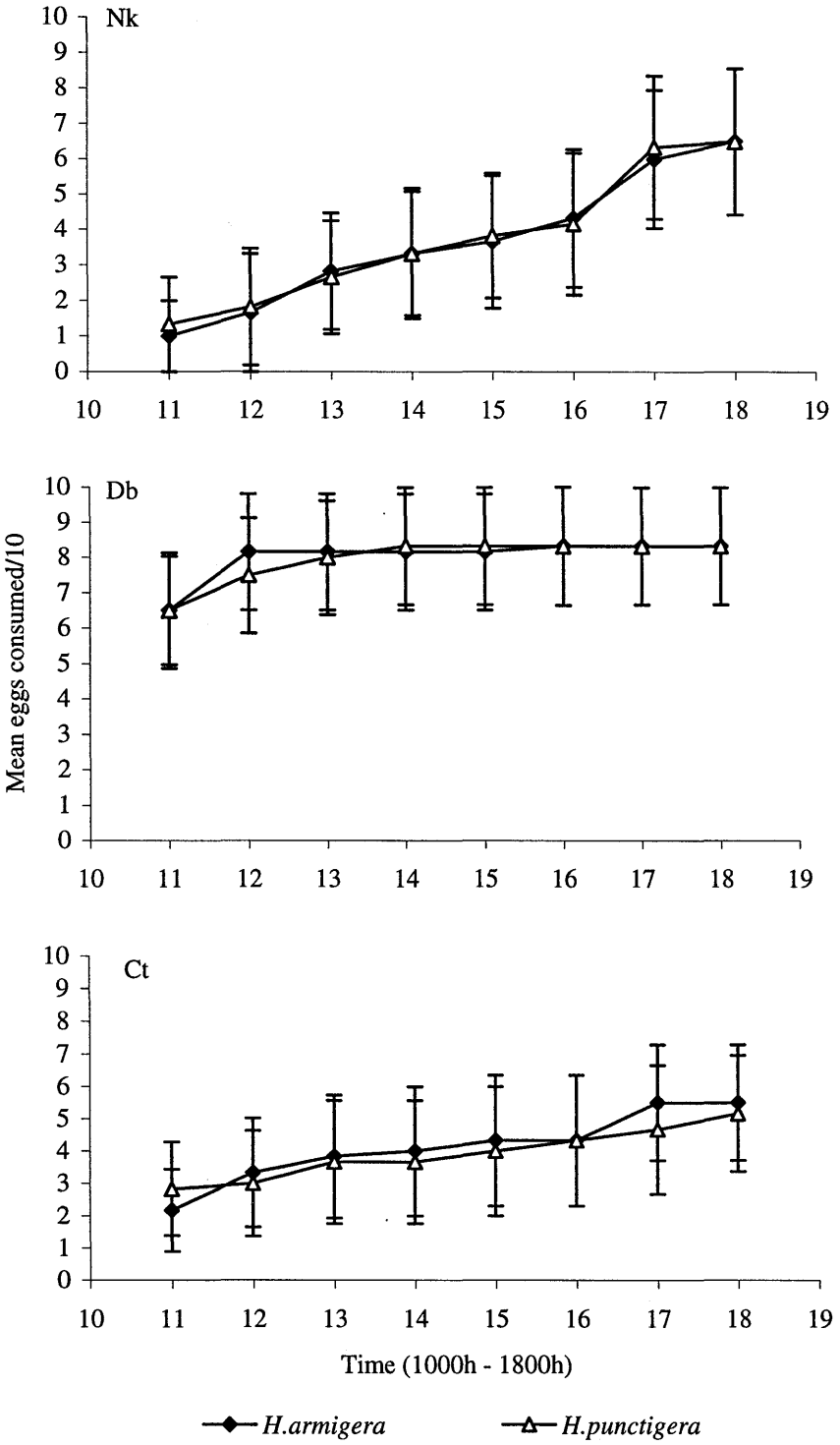

Figure 3.2 The mean cumulative numbers of two different species of heliothis eggs consumed by male predators. Nk = *N. kinbergii*, Db = *D. bellulus* and Ct = *C. transversalis*. Bars indicate standard errors of the mean.

No differences occurred in the numbers of heliothis species eggs consumed over the 24 hours in any of the predator species (Figure 3.3). However, female *N. kinbergii* and *D. bellulus* ate all the prey offered so differences may occur beyond the prey density and experimental time tested

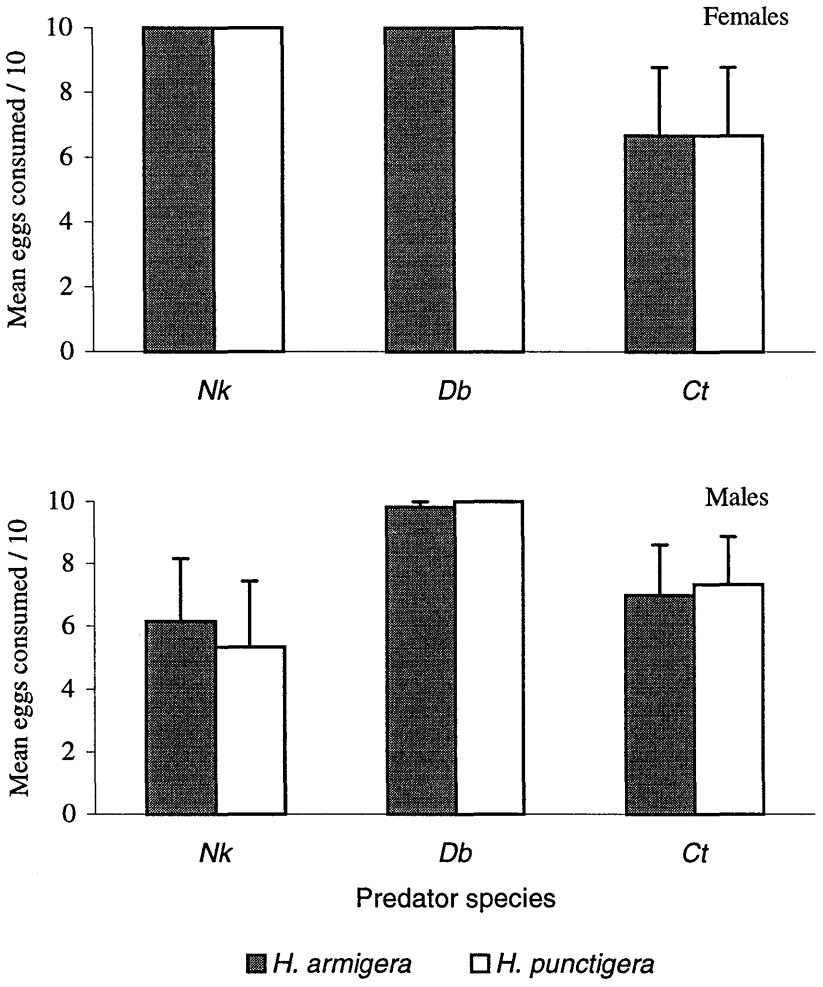

Figure 3.3 The mean consumption of eggs of both heliothis species by female and male predators over 24 hours. Nk = *N. kinbergii*, Db = *D. bellulus* and Ct = *C. transversalis*. Bars indicate standard error of the means.

Plant site

Data from egg consumption between 0900h and 1700h on different plant sites were analysed using a generalised linear mixed model (GLMM) fitted using the method of Breslow and Clayton (1993). Data were analysed as polynomial regression over time. The final terms used in each model for *N. kinbergii* and *D. bellulus* were 'site, time and time^2'. For *C. transversalis*

the terms used in the final model were 'site and time'. To determine the difference between sites within species over 24 hours a regression analysis was used. The final term used for the models was the treatment 'site'.

Results from the analysis of site preference for predator feeding showed that there were no differences between the number of eggs consumed at each site over the first eight hours of feeding. *N. kinbergii* (Figure 3.4) fed at all sites on the plants and slopes did not differ significantly between sites. Results from the graphs indicate that there were fewer eggs consumed on the under side of the leaves than the tips and upper leaf surface. In *D. bellulus* (Figure 3.4) no preference was shown for any of the feeding sites over the eight hours. There were no significant differences between the slopes, expected means or y-intercepts for the number of eggs consumed at each site.

Coccinellids were different to the other two species (Figure 3.4). There was a significant site effect ($p < 0.01$, $df = 3$). Significantly more eggs were consumed on the upper leaf than the under leaf surface ($bf < 0.016$, $p = 0.0052$, $df = 20$) and the tips ($bf < 0.016$, $p = 0.0001$, $df = 20$). The rate at which *C. transversalis* fed at all sites did not differ significantly (slopes were not different). Y-intercepts were significantly different indicating *C. transversalis* initially fed on more eggs at the upper leaf ($bf < 0.016$, $p = 0.0014$, $df = 20$) and the least on the tips ($bf < 0.016$, $p = 0.0001$, $df = 20$). There was no significant difference between the number of eggs consumed initially on the tips and under leaves ($bf < 0.016$, $p = 0.0488$, $df = 20$). Analysis of the expected means showed there were significantly more eggs consumed on the upper leaf surface that the under ($bf < 0.016$, $p = 0.0052$, $df = 20$) and tips ($bf < 0.016$, $p = 0.0001$, $df = 20$). There was no significant difference in the mean number of eggs consumed on the under leaf surface and the tips ($bf < 0.016$, $p = 0.0488$, $df = 20$).

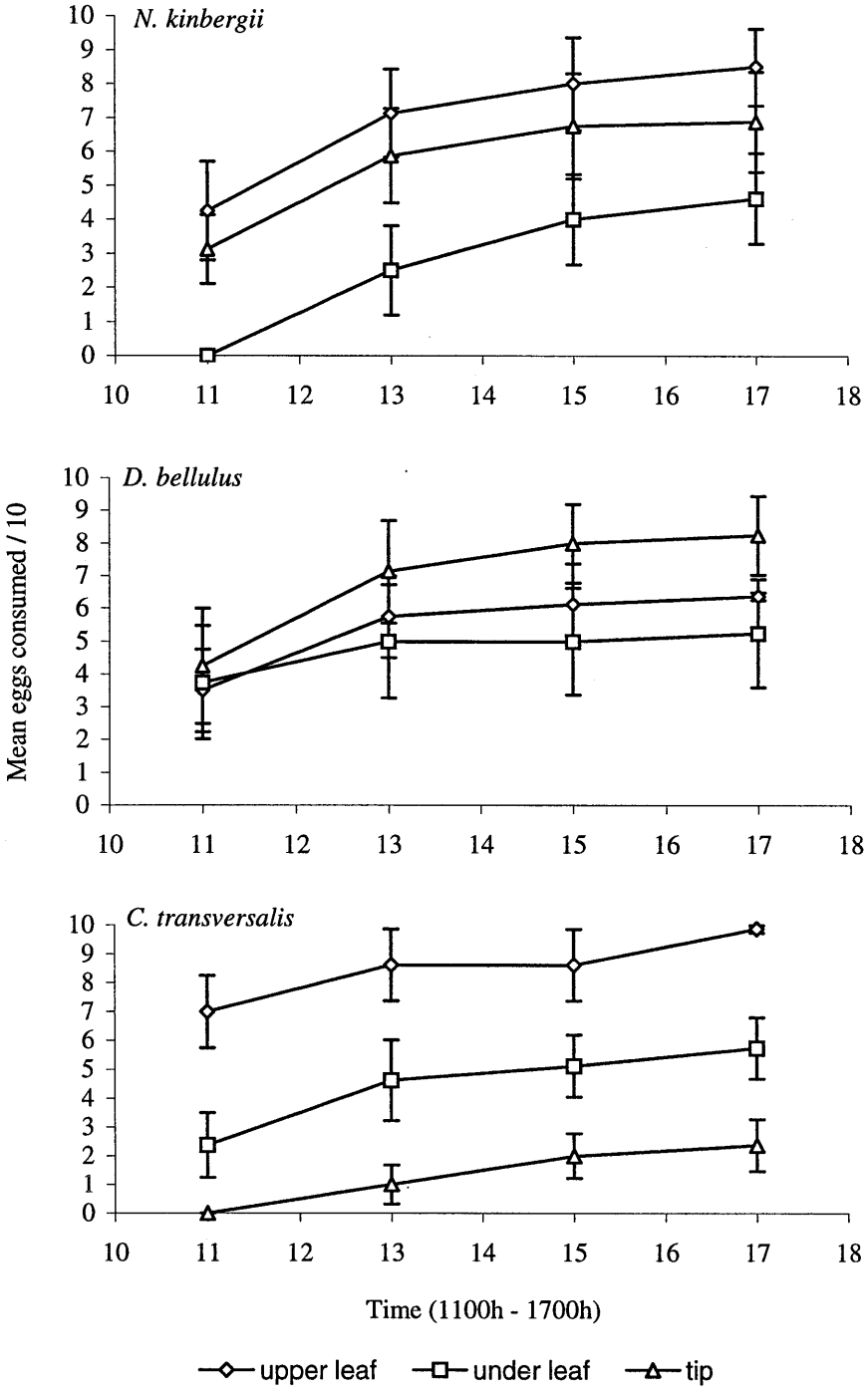

Figure 3.4 The mean cumulative number of heliothis eggs consumed at each site on a cotton plant by each predator species over eight hours. Bars indicate standard errors of the means.

The time taken for a predator species to consume 50% of heliothis eggs differed between species and sites (Table 3.1). *N. kinbergii* did not appear to take longer to feed on eggs at any particular site. *D. bellulus* took longer to feed on 50% of the eggs on the undersides of the leaves than the other two sites. *C. transversalis* were different again. The eggs on the upper leaf were eaten almost straight away and they took longer to feed on eggs on the tips than the other two sites.

Table 3.1 The estimated time taken by three predator species to consume 50% of *H. armigera* eggs at three sites on a cotton plant.

Species	Treatment	Hours taken to consume 50% of prey.
N. kinbergii	Upper leaf	1.25
	Lower leaf	N/A
	Tip	1.78
D. bellulus	Upper leaf	1.7
	Lower leaf	2.19
	Tip	0.92
C. transversalis	Upper leaf	0.3039
	Lower leaf	1.179
	Tip	3.9364

At 24 hours there were no significant differences between the number of eggs consumed at each site by *N. kinbergii* and *D. bellulus* (Figure 3.5). However, at 24 hours *C. transversalis* ate significantly more on the upper leaf surface than the under leaf surface ($bf < 0.016$, $p = 0.0028$, $df = 21$) and the tip ($bf < 0.016$, $p = 0.0002$, $df = 21$) (Figure 3.5).

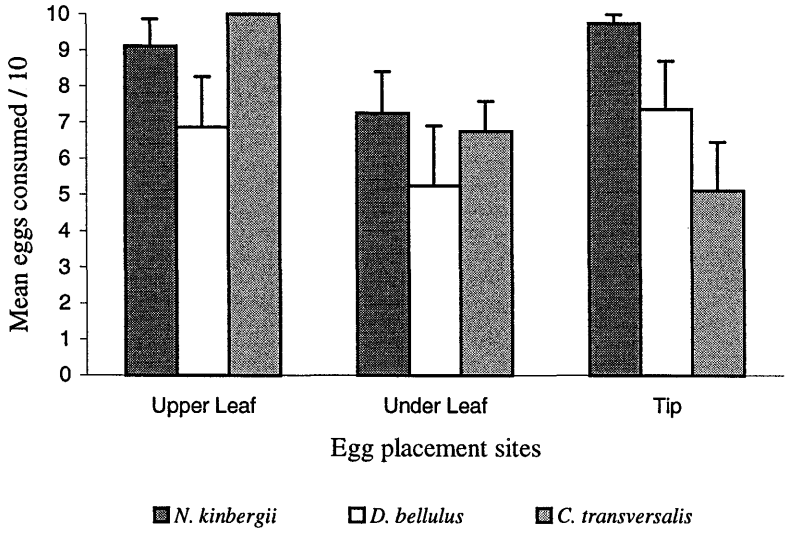

Figure 3.5 The mean number of heliothis eggs consumed at each site by each predator species over 24 hours. Bars indicate the standard errors of the means.

Egg position

The number of eggs consumed at each density was plotted for each species and experiment. Data for each species were analysed using regression analysis and the term used for the final models was 'treatment' where treatment = the type of egg placement (fixed or random). In all species there was no effect of density on the proportion of eggs consumed. In *N. kinbergii* and *D. bellulus* the difference between the number of eggs consumed between fixed and random prey placement was significant ($bf < 0.025$, $p = 0.0124$, $df = 42$ and $bf < 0.025$, $p = 0.0058$, $df = 42$ respectively). The number of eggs consumed on plants with the random egg placement was lower for both species. The number of eggs consumed on fixed and random egg placement treatments did not differ in *C. transversalis* ($p > 0.05$). Figure 3.6 shows that when eggs were all placed on one leaf (fixed eggs) the numbers consumed by individuals tended to be nil or close to the maximum offered, whereas in the random placement of eggs there was a more even spread of data.

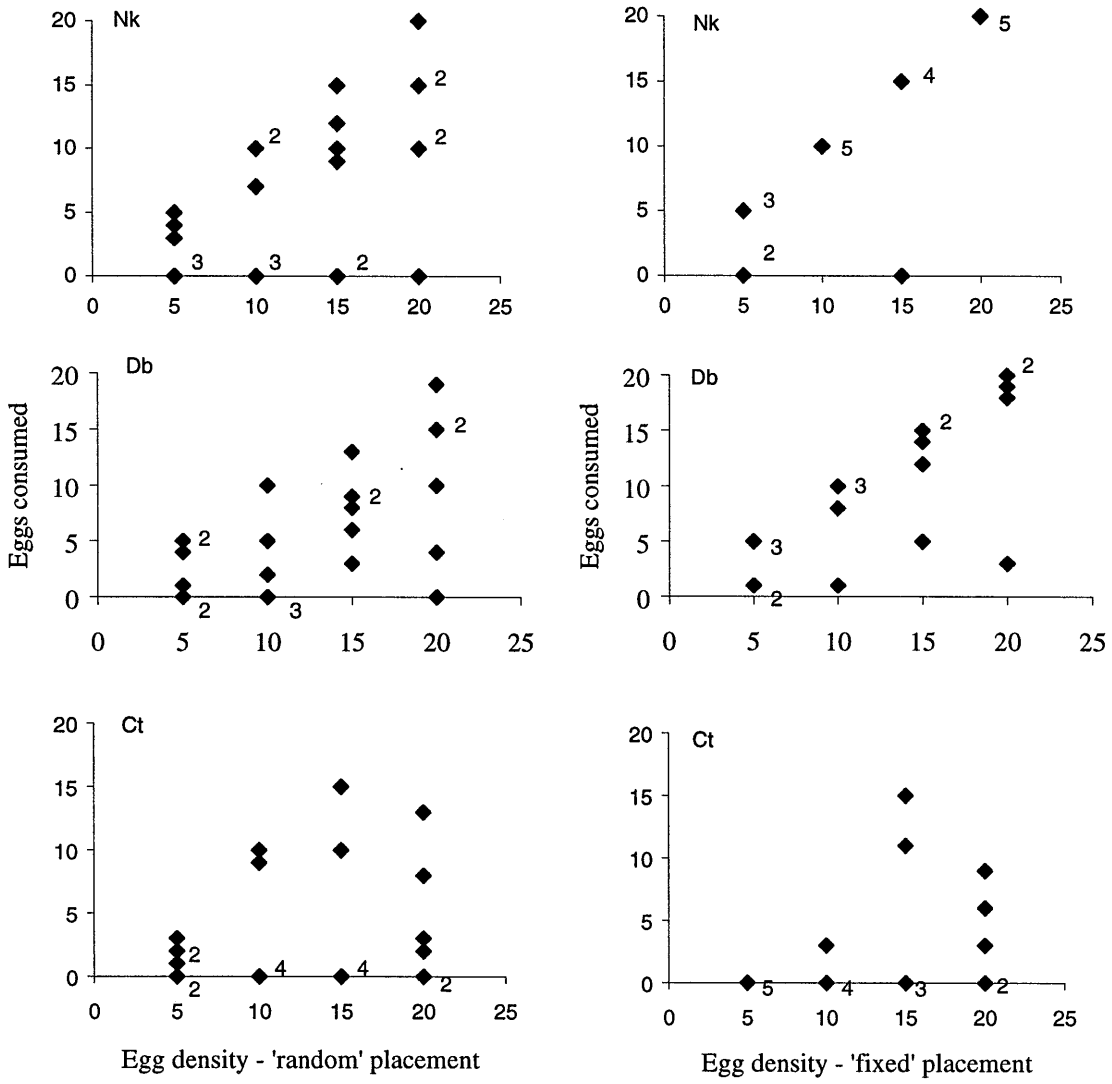

Figure 3.6 The spread of data points when heliothis eggs are placed on one leaf (fixed) or scattered on the upper third of the plant (random). Nk = *N. kinbergii*, Db = *D. bellulus* and Ct = *C. transversalis.*

3.3.2 Preparation of predators

Starvation times

Data from consumption of prey between 0900h and 1700h were analysed using GLMM. Data were analysed as polynomial regression and the amended model terms used for *N. kinbergii* were 'starvation, starvation*time, time^2 and time$^{3'}$ where, starvation = the time that

individuals were starved of prey. The amended model terms for *D. bellulus* were 'starvation and starvation*time'. The amended model terms for *C. transversalis* were 'starvation, starvation*time and time^2'. The final term used in the regression analysis for the 24 hour data was 'starvation' for each species.

There were significant effects of starvation treatment on the number of eggs consumed over the first eight hours in one of the predator species (Figure 3.7). In *D. bellulus* there was a significant difference between the slopes when insects were starved for 0 and 48 hours (bf < 0.005, p = 0.002, df = 16) and between 0 and 96 hours (bf < 0.005, p = 0.0008, df =16). In all data sets the variation was high. Consequently the differences between other starvation times that appear to occur in the data were unable to be shown statistically.

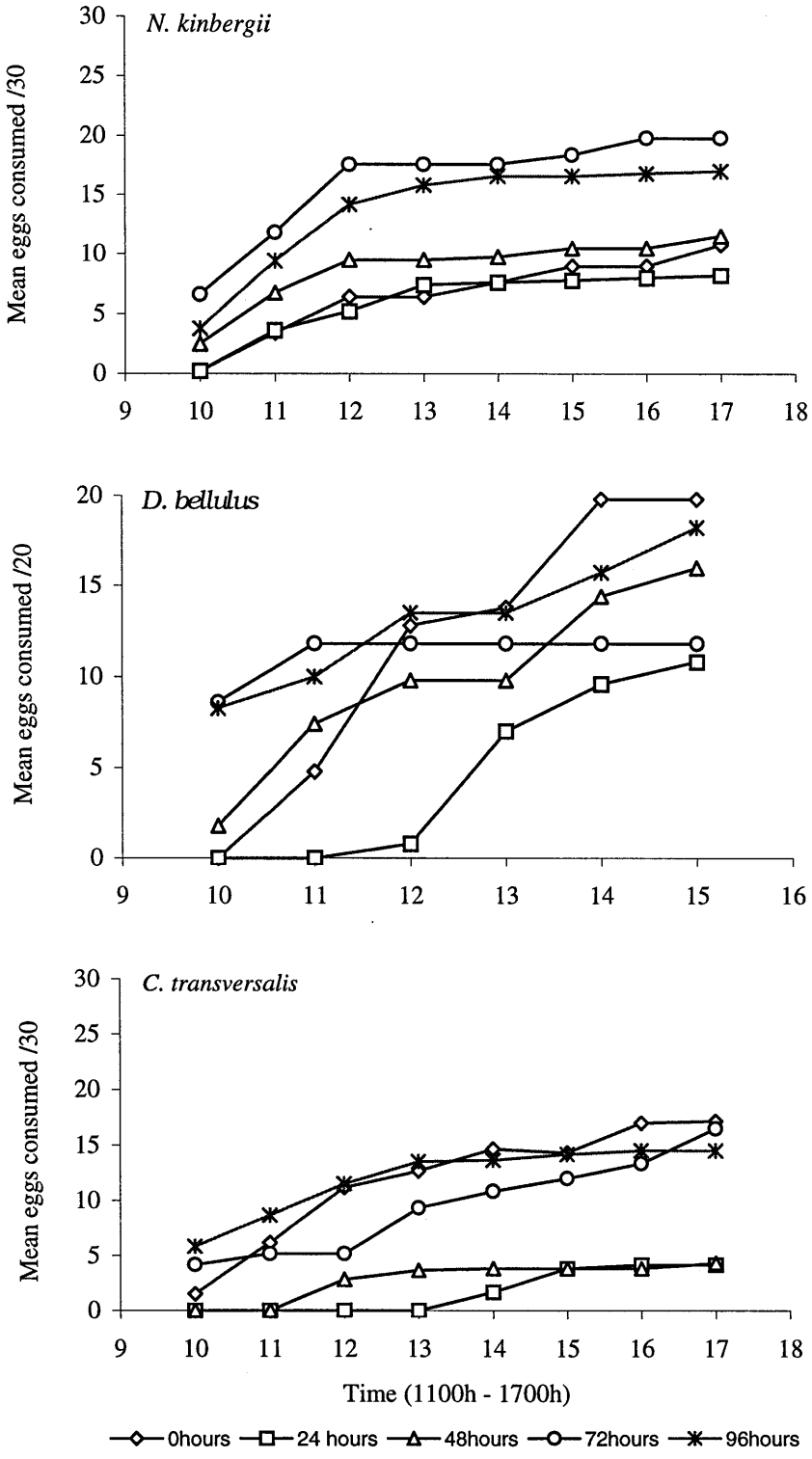

Figure 3.7 The mean cumulative number of heliothis eggs consumed over eight hours by predators that were pre-starved for different lengths of time. Error bars omitted to highlight trends.

At the end of the 24 hour experimental time, the analysis showed starvation time had no significant affect on consumption of eggs in any of the predator species (*N. kinbergii, D. bellulus* and *C. transversalis* (Figure 3.8).

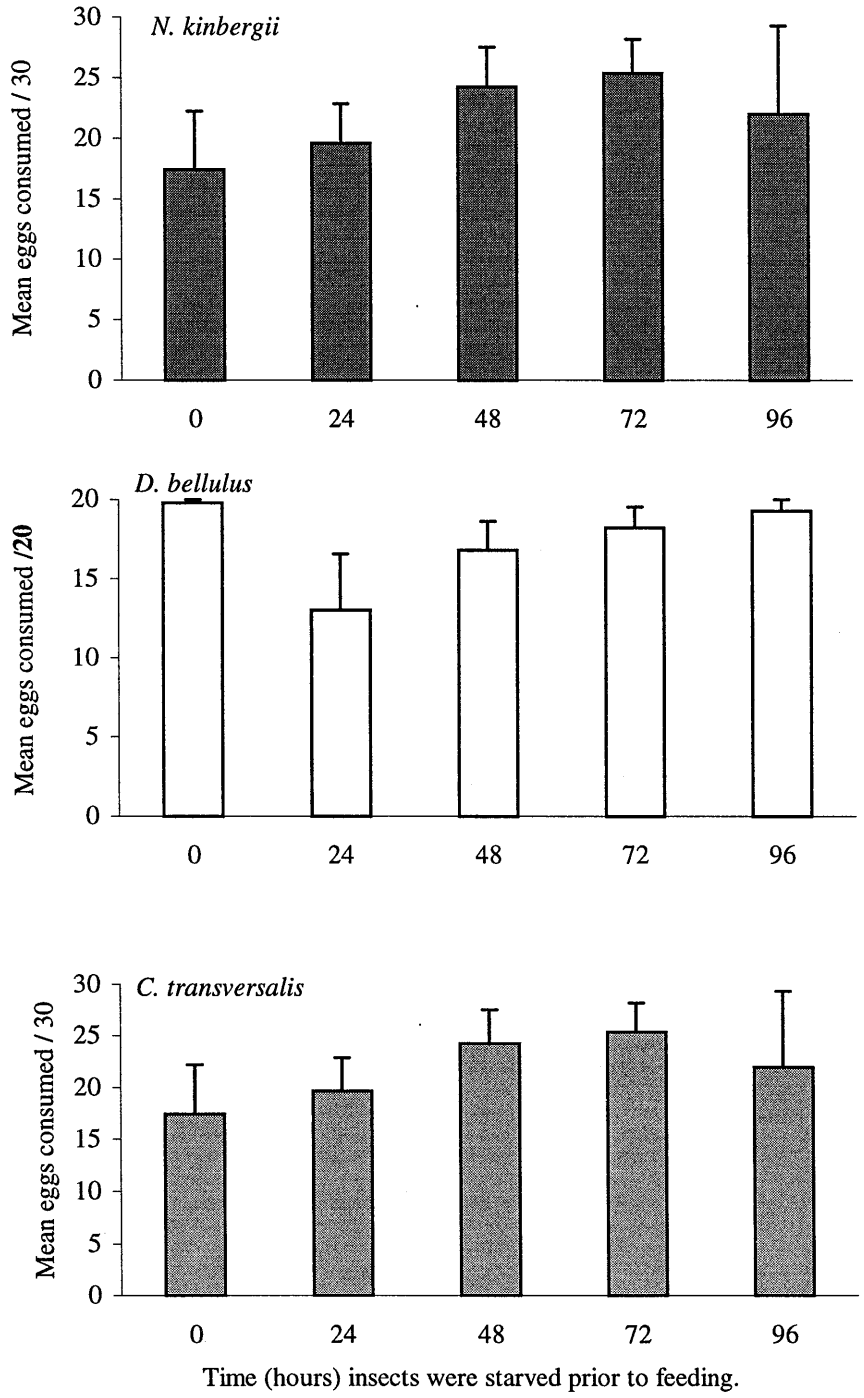

Figure 3.8 The mean number of heliothis eggs consumed after 24 hours by three predator species when pre-starved for different lengths of time. Bars indicate standard errors of the means.

The estimated time taken to consume 50% of eggs differed with starvation time in some of the predator species (Table 3.2). In all cases the longer starvation times of 72 and/or 96 hours appeared to speed the initial feeding rate up while 24 hours starvation appeared to slow initial feeding down.

Table 3.2 The estimated time taken (hours) by three predator species to consume 50% of *H. armigera* eggs when predators were pre-starved for different lengths of time.

Starvation time / Species	*N. kinbergii*	*D. bellulus*	*C. transversalis*
0 hours	6.77	2.94	3.24
24 hours	8.01	4.85	5.8
48 hours	9.09	3.56	N/A
72 hours	1.89	0.38	4.19
96 hours	2.46	1.87	2.49

Gender

Data from the gender experiment were analysed using GLM analysis. The final terms used for *D. bellulus* and *C. transversalis* were 'gender' and in *N. kinbergii* 'gender and density'. Figure 3.9 shows the number of eggs consumed by the gender of each predator species. In both *N. kinbergii* and *D. bellulus* there was a significant difference in the number of eggs consumed by males and females ($bf < 0.025$, $p = 0.0198$, $df = 44$ and $bf < 0.025$, $p = 0.015$, $df = 44$) respectively. There was also a significant density effect in *N. kinbergii*. Significantly more eggs were consumed at density four (20 eggs) than density one (one egg) ($bf < 0.01$, $p = 0.0042$, $df = 44$). There were no significant interaction effects. There was no significant difference between the number of eggs consumed by male and female of *C. transversalis* ($p > 0.05$).

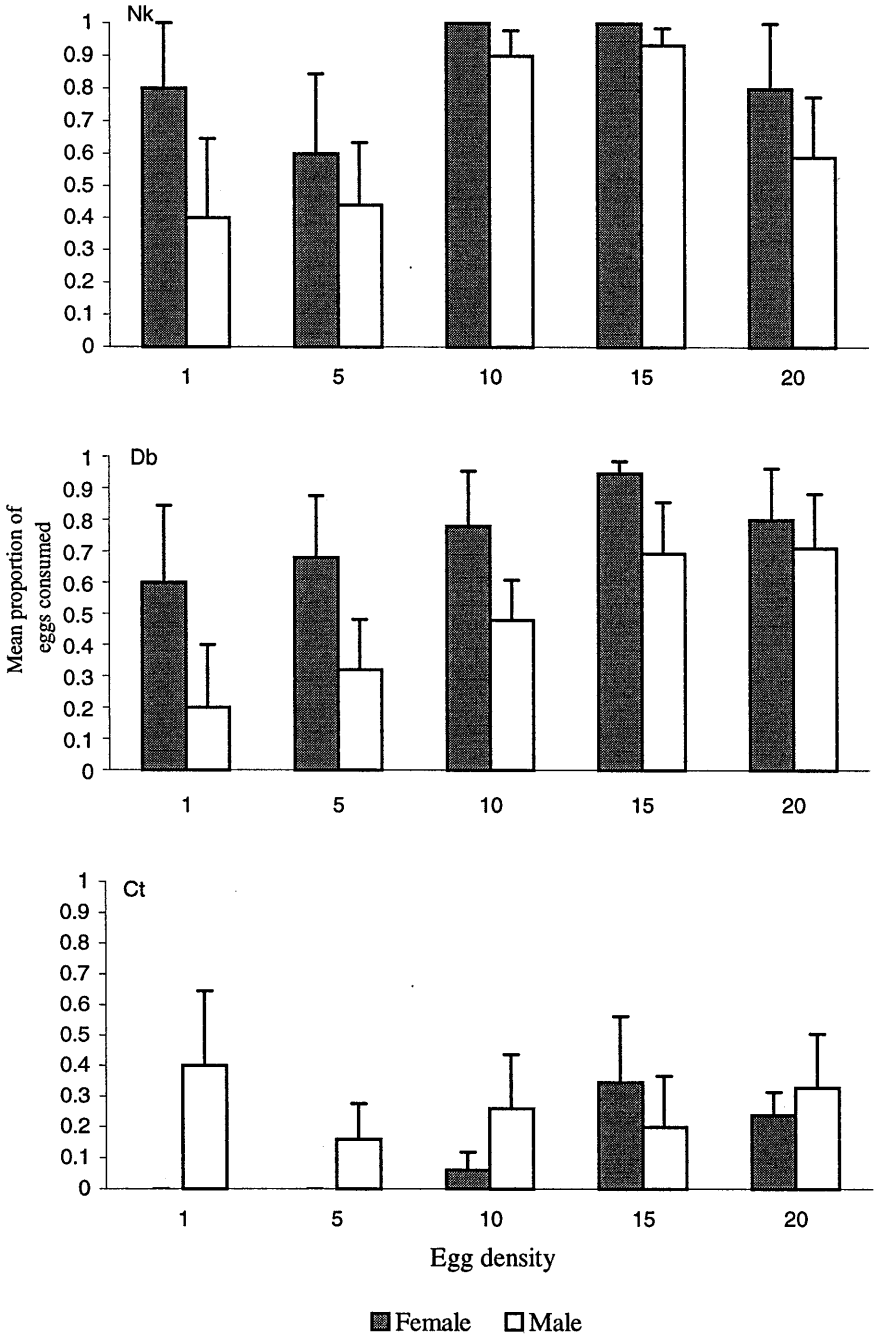

Figure 3.9 The mean proportion of heliothis eggs consumed at different prey densities by each predator species and gender. Bars indicate standard errors of the means.

Temperature

Temperatures inside the caged plants were higher than the temperatures set in the environment cabinet (15, 20, 25 and 30 °C). Realistically the mean temperatures (dry) inside

the caged plants were 20, 24.6, 30.2 and 36.8 °C. These are referred to as temperatures 1, 2 ,3 and 4 respectively in the following results. Figure 3.10 shows the wet and dry temperatures recorded in these experiments both in and outside caged plants over a 24 hour time period.

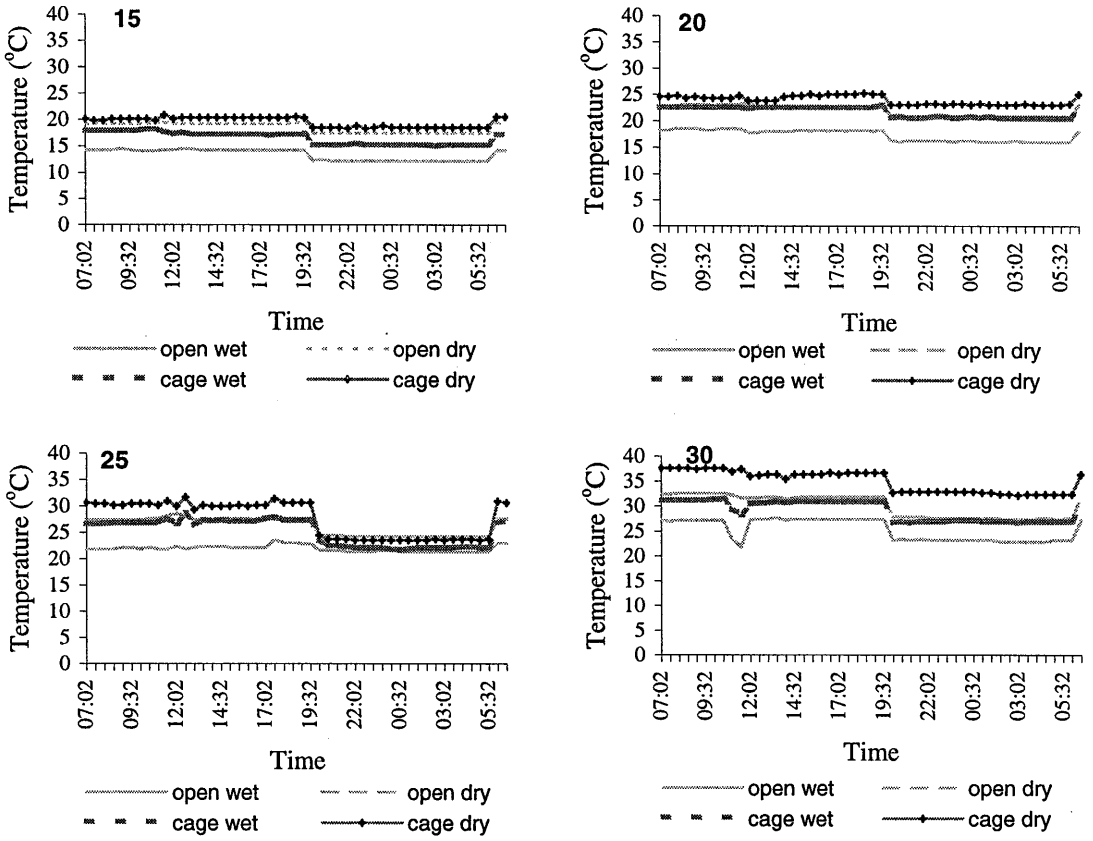

Figure 3.10 The mean wet and dry temperatures both in and outside the cages in the environment cabinet during the experiments on the effects of temperature on predation.

In all temperature experiments the variation was high (there were some individuals who ate nothing and some that ate everything) making statistical significance variable between treatments. To better illustrate the trends shown, data were plotted as 3D line surface graphs without error bars. The analysis for comparison of temperatures used GLM analysis. The amended model terms for *C. transversalis* females were 'temperature and density'. The amended terms for the model used for *C. transversalis* males were 'density and density * temperature'. For *D. bellulus* females and males the amended model terms were 'temperature and temperature^2'.

Temperature had an affect on the number of heliothis eggs consumed by female *C. transversalis*. Female *C. transversalis* increased consumption of eggs with increased

temperature in all egg densities (bf < 0.05, p = 0.0012, df = 95) and there were significant differences between the number of eggs consumed between temperature 4 and 1 (bf < 0.008, p = 0.007, df = 95) between temperature 4 and 2 (bf < 0.008, p = 0.0001, df = 95), and between temperatures 2 and 3 (bf < 0.008, p = 0.0001, df = 95) (Figure 3.11a). Temperature did not significantly affect the number of eggs consumed by male *C. transversalis* (Figure 3.11c). However, the graph indicates more eggs are consumed at temperature 3.

There was a significant linear and quadratic effect of temperature over all prey densities for female *D. bellulus* (bf < 0.05, p = 0.0008 and p = 0.0016, df = 97) (Figure 3.11b). There was also a significant difference in the number of prey consumed between temperatures 1 and 3 (bf < 0.008, p = 0.0004, df = 95). Overall the data for females indicates less eggs were consumed at temperature 1 and the most at temperature 3 (Figure 3.11b). There was no significant temperature effect on male *D. bellulus* (Figure 3.11d).

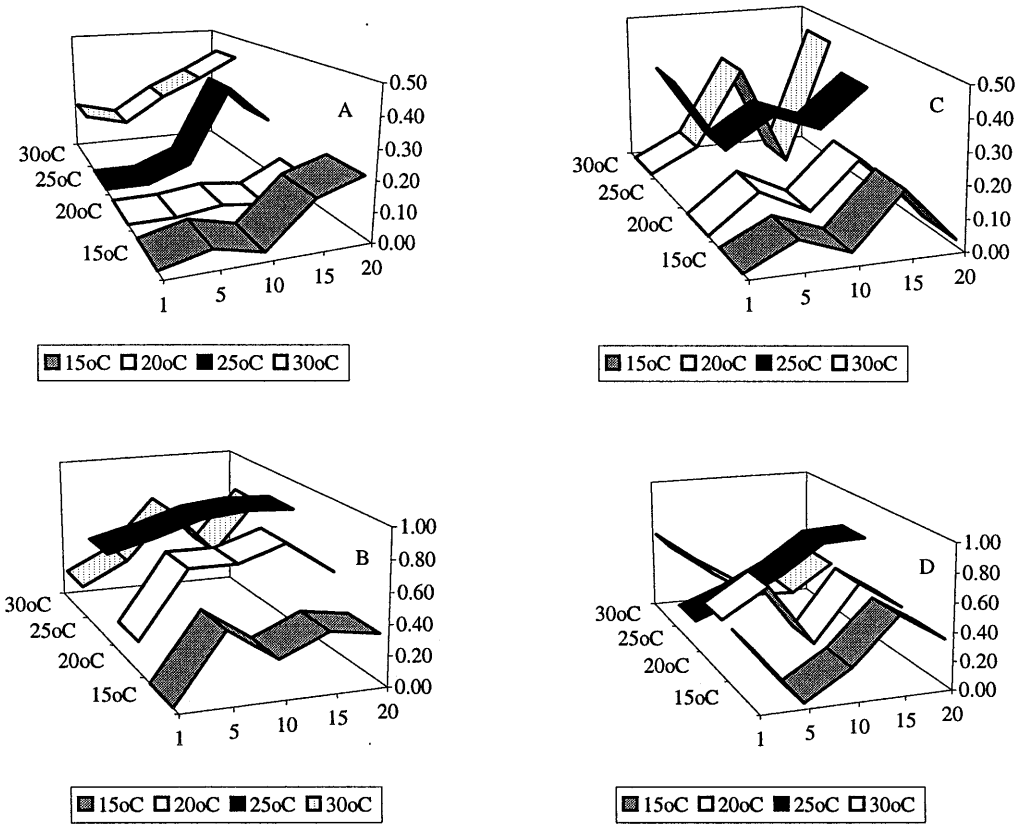

Figure 3.11 The mean number of heliothis eggs consumed at four temperatures and five prey densities by female *C. transversalis* (A) and *D. bellulus* (B), and male *C. transversalis* (C) and *D. bellulus* (D).

The proportion of each predator population that fed at each temperature and density was also plotted for each species and gender (Figure 3.12). Temperature appears to affect the proportion of *C. transversalis* individuals that fed with more females (3.12a) and males (3.12c) feeding at temperatures 3 and 4. Only temperature 1 had an effect on the proportion of female *D. bellulus* that fed (3.12c) and in male *D. bellulus* temperatures 1 and 4 affected the number of individuals that fed (3.12d).

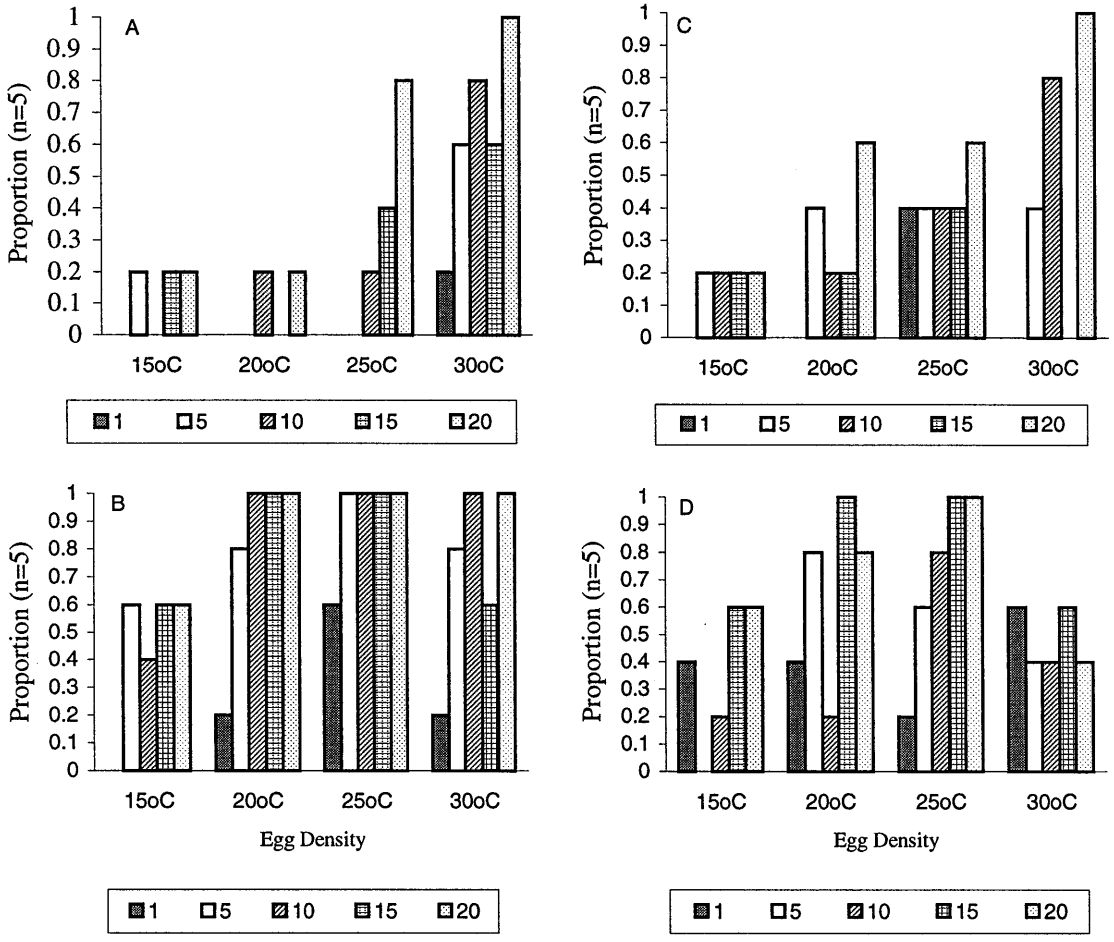

Figure 3.12 The proportion of each sample population that fed at four temperatures and five prey densities. A = Female *C. transversalis*, B = male *C. transversalis*, C = female *D. bellulus* and D = male *D. bellulus*.

3.4 Discussion

The parameters tested in this chapter influenced the number of eggs consumed by each predator species. Overall, the main differences occurred with the placement of eggs on cotton plants, the gender of each predator species and the temperature of the experimental arena (the environment cabinet). There were also some parameters that did not affect consumption by the predator species over 24 hours though in some cases they had significant effects over shorter time periods. These included the starvation time, prey sites on plants and the use of both *H. armigera* and *H. punctigera* eggs.

Heliothis species

The results indicated that no preference was shown between species of heliothis eggs. As the eggs are the same size (Zalucki *et al.* 1986) and age, and both species oviposit eggs in similar positions on plants (Mensah 1996) these results show that including data on prey consumption based on *H. punctigera* eggs only will not bias any comparisons of consumption by predators. Other authors do not discern between heliothis species. Butler and May (1971) used both *H. virescens* and *H. zea* eggs when testing predation by *Chrysopa carnea* Stephens. Lopez *et al.* (1976) did the same when comparing predator efficiency in laboratory studies. In the United States McDaniel and Sterling (1979) referred to predation as being on *Heliothis* spp. in general as did Room (1979b) (on *Helicoverpa* spp.) when screening potential predators in Australia. No papers discuss finding a difference in consumption of *H. punctigera* and *H. armigera*. However, in the United States Henneberry and Clayton (1985) refer to *C. carnea* larvae as consuming both *H. virescens* and *Pectinophora gossypeilla* (Saunders) and show a difference in the number of eggs consumed when both are offered. They ascribe this to differences in egg size and possibly oviposition sites. McDaniel and Sterling (1979) showed the number of predators scoring positive to radio labelled *H. virescens* eggs declined when native *Heliothis* spp. were laying heavily. Therefore, the density of a prey species may affect consumption preferences. With the exception of prey density the differences between prey types (such as size) used by each of these authors are not the same for the Australian heliothis species used in this study.

In an experiment using both *H. armigera* and *H. punctigera* the only factors that may influence predation would be egg age, oviposition sites and possibly chemical influences from oviposition. One day old eggs of *H. virescens* seemed to have a greater chance of predation than older eggs (McDaniel and Stirling 1979). The effect of egg age is addressed in Chapter 5 in this thesis. Secondly there can be an effect on consumption due to differing oviposition sites and behaviour by moths. As mentioned earlier the oviposition sites do not differ between the Australian heliothis species. Whether the different chemical stimuli associated with each species affects predator behaviour is unknown. Prey pheromones are known to influence predators. Sex pheromone from female coccids is thought to attract *Aphytis melinus* Debach (Sternlicht 1973) and *Nemeritis canescens* (Grav.) is attracted to aggregation pheromones of the caterpillar *Ephestia cautella* (Corbet 1971). In this study only one day old eggs were used and eggs were painted on so any effects of egg age or maternal chemicals would not have occurred. Likewise, as it was the egg stage used, responses of predators to volatiles emitted by an injured plant (McCall *et al.* 1994) are not applicable to this study, although they may affect studies where larvae are used as prey (Chapter 5).

Over a 24 hour time period the predators consumed all the eggs provided. Although there may be differences in the numbers of eggs consumed beyond 20 eggs, the temperature experiments where *H. punctigera* were used did not include numbers of prey greater than 20. Whilst the species of prey did not affect the numbers consumed, the position eggs were placed on the cotton plants did affect the predators.

Egg placement

In this study the coccinellids were most affected by the position of the prey on the plants. If finding prey is as a result of successful attack then the response of the predator is usually an increase in turning movements. This is true for coccinellids (Nakamuta 1985). In this study all species were observed intensifying their search upon consumption of a prey. Evans (1976) showed that *Anthocoris consusus* (Reuter) spent more time searching in regions where high prey numbers were available and intensified searching upon encounter of a prey. This search was usually restricted to one leaf. However, in this study the point at which 50% of eggs were consumed indicated that in the case of *N. kinbergii* and *D. bellulus* prey availability did not appear to influence the numbers consumed at each site. That is, they did not consume all the

prey at one site first. It is possible that if these two species exhibit a change to area-concentrated searching after encounter with a prey, the size of the search area is larger than one leaf only or, the time taken to encounter another prey changes their searching behaviour to an extensive search area.

After 24 hours the numbers consumed did not differ between sites with the exception of *C. transversalis* which had eaten more from the upper leaf surface. In *C. transversalis,* eating more on the upper leaf may reflect searching behaviour, in that they tended to run along the outer upper edge of the leaf more often than the other surfaces. This reflects similar behaviour to adult *Coccinella septempunctata bruckii* shown by Nakamuta (1985). He also showed that upon encounter of a prey coccinellids tend to intensify their search until no prey are found. As a result of the findings in this study, placing eggs on the upper leaf surface was considered to be suitable for the purposes of studying and observing consumption by predators.

Random versus fixed placement

Using plants provides a more realistic search arena than petri dishes. O'Neil (1989) pointed out that searching in insects is more likely to be a limitation to predation than handling of the prey. Coccinellids are generally considered as finding their prey randomly and by touch but may have limited detection abilities over a short distance (Hodek 1991 cited in Polgar *et al.* 1991, Stubbs 1980). Therefore, placing prey in one spot is likely to increase consumption rates. It was thought that the use of plants would also eliminate the cases where all the predators ate everything. However, the results from *C. transversalis* in the site preference trial showed that if eggs were all placed in one area (if found) they would all be eaten. In all experiments there was high variation within treatments, mainly due to some predators not feeding at all and others consuming everything. Although significantly fewer eggs were consumed by predators when the eggs were placed semi-randomly, the semi-random placement of eggs aided in reducing this variation. It is also more realistic to position eggs in individual spots on the plants as in the field heliothis do not often lay their eggs in one site on the plant (Kyi *et al.* 1991, Zalucki *et al.* 1986).

Predator preparation

Food deprivation may affect species in different ways if they have different modes of action (McIntyre and Vaughn 1997). They found hungry beetles contacted food more often and fed beetles would often pass over food even if it was contacted. In this study the number of individuals that initially fed differed between starvation times. More individuals starved for 72 and 96 hours fed in the first hour, than individuals starved for 0, 24 or 48 hours. The results from the time taken to consume 50% of the heliothis eggs support these findings.

In all cases the predators that were not starved of prey (0 hours) consumed as much or more than those that were starved for 24 hours or more. *N. kinbergii* and *D. bellulus* that were starved for more than 48 hours, initially fed on 50% of the eggs faster than the other starvation times. Starvation may alter the behaviour of a sit and wait predator (such as nabids) which may only start to move to look for prey if they are hungry enough (Hassell and Southwood 1978). This result was different for *C. transversalis*. Coccinellids are known to go into a quiescent stage if sufficient food is not found (Hodek 1984). Of interest is that those predators starved for 24 and 48 hours ate less and generally took longer to feed than those not starved and those starved for 72 and 96 hours. After 24 hours of being allowed to feed there was no effect of starvation on the numbers of eggs consumed in each species. Provided similar prey densities were used starving predators for 24 to 48 hours prior to use was not likely to influence the results of future trials. However, overdispersion of data is likely to occur when predators have differing hunger levels (Casas and Hulliger 1994). Considering that fewer predators ate when starved for 24 and 48 hours, starving them for more than 48 hours, would be better.

Gender

When offered eggs of heliothis both male and female predators ate the prey offered. However, both female *N. kinbergii* and *D. bellulus* consumed more eggs than the males of the species. It is likely that females when in reproductive state may consume more than males but, they may also spend more time looking for oviposition sites. The reproductive status of these predators was unknown. However, as none were observed to be ovipositing and no eggs were found oviposited in the dental wicks it is unlikely that the females were in a reproductive stage.

Donahoe and Pitre (1977) using field-collected predators of unknown age, found a difference between the sexes in consumption of *H. zea* larvae by *Reduviolus rosipennis* (Reuter) on cotton plants. Males consumed less than females. The results in this study agree with results from the heliothis prey preference experiment where female nabids consumed more than males and *C. transversalis* did not differ greatly. The exception was *D. bellulus* which did not show a gender difference in the heliothis prey type experiment. Stanley (1997) noted that it was only reproductive *D. bellulus* females that consumed more than males. This may explain the difference between results in this study. As a result of the findings in both these studies it was decided that gender should be separated in future experiments.

Temperature

Results showed that consumption by the predators increased with temperature from 15 - 25 °C (24.6 – 36.8 °C inside the cages) and then varied at 30 °C (36.8 °C inside the cages) with consumption by *D. bellulus* females dropping off. Low temperature can induce quiescence or diapause in coccinellids (Hodek 1984, Anderson and Hales 1984). Temperatures of 18 °C and 34.5 °C limited reproduction in *Coccinella repanda* (syn. *C. transversalis*) (Anderson and Hales 1984a). Reproductive females tended to enter diapause at low (<20 °C) temperatures. Their data indicated that temperature could induce *C. repanda* into diapause even when high prey densities were available. Prey density also influenced predation under different temperatures. The effects of prey density are further investigated in Chapter 4. The aim of this thesis was to observe predation not hamper it. Therefore, it was decided that future experiments should be conducted with the environment cabinet set to 25 °C (in cage 30.2 °C).

Using a 24 hour time period

In studies on the time of day that predation occurred there were differences amongst species. Peak foraging by *O. insidiosus* was between 10:00 am and 13:00 pm (Coll *et al.* 1997). Peak activity for *R. roseipennis* was at 19:00 - 20:00 pm and was considered a response to light stimuli (dusk/dawn) by Donahoe and Pitre (1977). Trials left for 24 hours as in Donahoe and Pitre (1977) showed peak activity at sunset and sunrise. The results from all trials in this study that included time did not show trends in feeding times other than if given 24 hours to feed the majority of predators eventually fed. The use of 24 hours also eliminated some of the

differences that occurred when an experimental time of eight hours was used. This experiment showed that the predators are all likely to find and consume eggs at common oviposition sites used in the field. Braman and Yeargan (1989) found that adult nabids differed in plant site preference. Only one species (*Nabis americoferus* (Carayon)) were largely located in the upper canopy of soybeans. However the majority of nabids (76.8%) collected from the field were found on leaves. Donahoe and Pitre (1997) showed that the majority of predators were observed on the upper plant parts of cotton and mostly on the squares and leaves over hourly observations for 24 hours. The majority of the time on the leaf was on the lower surface. Although these examples highlight that differences occur between species they also show that the leaf is a preferred site for predators on plants

How these effects might relate to field conditions

Whilst the aim of these experiments was to determine artificial experimental conditions it should be noted that some of these parameters are also applicable to field studies and to interpretation of laboratory results. For example heliothis lay single eggs in the field on individual sites on a plant so it is more appropriate to simulate the same in the laboratory and the fact that it takes predators longer to find them provides a more accurate assessment of predation. The effect of *H. armigera* and *H. punctigera* pheromones and host karomones on predation is not known. It would be of value to consider this when using naturally laid populations of eggs. When interpreting results where one gender (particularly reproductive females) of a predator species is used an over estimation of predation may occur.

Temperature will affect predation in the field. Beisner *et al.* (1997) found less *D. pulex* were consumed at 18 °C than 24 °C by *M. ehrenbergii*. Day to day variation in predation levels may be in response to temperature. Therefore, it is important to consider this factor when interpreting predation levels in field trials and when pooling data on day to day predation. This is supported by the findings in Chapter 8 on predation by *D. notescens* and highlights the importance of doing predation studies over a range of temperatures within a season. Also of consideration is the fact that prey density interacted with temperature.

3.4.1 Conclusion

Over a 24 hour time period two of the predator species consumed all the eggs provided. Although there may be differences in the numbers consumed beyond 20 eggs, the experiments where *H. punctigera* eggs were used did not include prey numbers greater than 20. All of the experiments that included *H. punctigera* were conducted in 24 hours or less and with 20 eggs so using either heliothis species of prey should not effect consumption by the three predator species. For future experiments it was determined that temperature should be set at 25 °C and the predators starved for over 48 hours prior to use. Also only female predators should be used in future trials unless time allowed for capture and use of males. The results showed heliothis eggs should be placed semi-randomly on the upper leaf surfaces of the cotton plants and consumption tested over 24 hours. As noted in the temperature and gender experiment the density of prey appeared to also affect consumption and the proportion of predator populations that fed. These parameters were tested in Chapter 4 – The consumption of heliothis.

Chapter 4

4. The consumption of heliothis eggs by three predator species.

4.1 Introduction

The basis on which a predator is considered to have potential as an effective biological control agent is supported by its numerical and functional response to prey numbers. The former assesses how predator numbers increase in response to prey numbers and the latter, how many prey the predators are able to consume given different numbers of prey. Numerical response is defined as the change in predator numbers due to variable prey populations (Crawley 1975). The change in predator numbers may occur as a result of reproduction, immigration or a change in life stage survival (Wratten 1987).

Functional response is the change in the number of prey consumed by a single predator as a result of prey density. Models for functional responses were largely derived first by Solomon (1949) and then from Holling's "disc equation" (Holling 1959). The relationship between the number of prey consumed and the number of prey captured changes because of handling time (the time taken to consume a prey). Handling time is then influenced by the cost of energy to the predator, gut saturation, search image formation, search arena, and emigration or interference from prey or other predators (Bell 1990, Wratten 1987). Handling time can vary with prey density and hunger levels of the predator. There are four types of models used to describe the functional response of predator to prey. These are shown in Taylor (1984). A type I curve represents a linear relationship, type II a decelerating curve, type III a sigmoidal relationship and type IV a dome shaped response. If prey consumption fits a type III model (sigmoidal functional response curve) then the predator is thought to be a good candidate for biological control as, theoretically, it provides a stabilisation of the prey population. This is because after an initial slowing of consumption the predators ability to search and find prey increases as a result of learning. Data normally collected for describing the functional response of a predator are the number of each prey consumed over a series of prey densities,

time to attack and the handling time. This enables functional response curves to be obtained and attack rates and handling times to be calculated (Chesson 1989).

Functional response models are generally based on results obtained from predator species with high host specificity and on prey that are considered 'r' strategists An 'r' strategist has a short rapid life cycle with high fecundity and occurs in high numbers. In most circumstances predators do not need to reduce these prey to very low numbers such as one or two per plant in order to be effective. These types of prey have been used extensively to determine the efficacy of a biological control candidate (Coll and Ridgeway 1995, Cohen *et al.* 1993, Congdon *et al* 1993).

Similarly the functional response has been obtained for generalist predators with prey that occur in low or patchy numbers in the field and on crops that require control to very low numbers per unit area (Stanley 1997, Wiedenmann and O'Neil 1991a, Nordlund and Morrison 1990, O'Neil 1989). Under these criteria assessing the type of functional response has little meaning as the numbers of prey required to reach an asymptote and therefore assess the functional response are not realistic. There are also other factors that affect generalist predator efficiency that are often not considered in these type of functional response experiments.

Generalist predators are polyphagous, may be opportunists (Hagler *et al.* 1992) and many have the ability to switch to the dominant prey type as other sources of prey become depleted. This is known as switching behaviour (Murdoch 1969). Switching and the influence of other prey types are discussed in Chapter 5 of this thesis. Other factors that influence predation include the searching ability of the predator, the number of prey available, the patch size and the predator behaviour towards prey numbers and prey stages. It is well known that these factors affect consumption rates (Coll *et al.* 1997, Frazer and Gilbert 1976, Lopez *et al.* 1976, Murray and Mensah 1996).

Searching ability is considered to be one of the most important of these influences. The cost of searching to a predator is loss of time for breeding, finding refuges and egg laying. Searching by the predator is also likely to be affected by the first encounter event. Upon encounter or consumption of a prey the predator behaviour may alter to an area concentrated search (Evans 1976). If few prey are encountered then the predator may increase the rapidity of searching over a larger area. The behaviour of the predator may occur due to its means of

locomotion, perception of sensory information, prey resources available and the risks involved in finding resources. For example, low temperature is known to cause coccinellids to spend more time being inactive even when capable of searching (Frazer and Gilbert 1976).

Predator searching can also differ amongst different plant species, different plant surfaces and prey distributions within those plants (Coll *et al.* 1997, Treacy *et al.* 1987). Furthermore searching in a patchy environment can influence how a low level of prey is maintained. For example Congdon *et al.* (1993) showed *Stethorus punctum picipes* Casey was able to locate and consume prey at low densities on plants. Conversely, the increase in heliothis survival in cotton late season was attributed to the loss of search efficiency of predators due to an increase in plant size Congdon *et al.* (1993).

Prey numbers also influence whether a predator is considered efficient. Numbers of prey are usually dynamic and, if the predator is to be used for biological control, the needs of the predator must be considered. These include knowing how many prey they need to consume (O'Neil 1988), whether they can find them and whether the target prey are the most dominant species in the crop. Hagler and Naranjo (1994a) showed that the proportion of predators consuming pink bollworm (*P. gossypiella*) and whitefly (*Bemisia tabaci* (Gennadius)) was fairly constant throughout the season despite some very low pest population levels. They suggest that the natural enemies were still able to find prey at very low numbers. Another important consideration is the number of prey the predator is required to consume if it is to reduce prey numbers to below economic thresholds.

Economic thresholds

Where the predators are to be used as the main form of control they need to be able to consume prey to below the economic thresholds that are part of the crop management (Luff 1983). Economic thresholds are based on the point at which action must be taken on the prey before they cause damage to the crop (Stern *et al.* 1959). Sterling *et al.* (1989) describes the concept of 'inaction' in a management program as the density of natural enemies sufficient to maintain pests below the action level (economic injury level). This assumes an understanding of the natural enemies' contribution to control over space and time. If this is understood a decision to act or not on a pest population can be made. The threshold is usually described as the number of prey per unit of habitat. In cotton in the 1994/1995 season these thresholds

were two heliothis eggs or small larvae per metre. If predators are to be used as the main focus of control in cotton then they need to be able to reduce heliothis to below these thresholds.

Direct and indirect methods of evaluation

The way in which predation studies are done can vary. As mentioned in Chapter 1, the impact of predators on prey populations is often inferred by numbers of both predator and prey in the field. Although this may help to identify which predators are worth considering for further investigation it does not provide information on the direct impact of each predator species (Wratten 1987). Direct impact can be measured by exclusion studies of prey or predators in small arenas such as a Petri dish and large cages (in laboratory or field situations). Studies may also be done with exclusion of prey or predators by pesticides, through direct observation of predation in the field and by serological or labelling techniques that detect predation of a specific prey. These methods, and their limitations, are described in detail in Seymour and Jones (1991), Luck *et al.* (1988), Sunderland (1988), Wratten (1987) and, Kiritani and Dempster (1973). Serological techniques are further discussed in Chapters 6, 7 and 8.

In theory to assess a predator species we need to conduct a field survey on predator and prey populations, direct observations on feeding, exclusion trials, behaviour studies and serological trials. The efficiency of a predator species can then be ranked and used to aid in management decisions (Mensah and Singleton 1998, Dent 1991, Breene *et al.* 1990). Wherever possible all these aspects should be combined to assess a predator's use as a biological control agent. However, in reality resources often do not allow all these aspects to be studied. Given small predators that are difficult to see, low abundance of prey and predators and short duration of study time, means field studies on direct predation are difficult. For this reason, much reliance has been placed on cage studies conducted in artificial environments (Luck *et al.* 1988). Although they are useful, there are some limitations to cage studies. If cages are used then understanding the preferences of prey oviposition, feeding sites, prey dispersion and predator searching behaviour are important. These issues have been discussed in Chapter 3. Cages also change the micro-climate on the plant surface which may alter predation behaviour (Hand *et al.* 1967). Confining prey and predators is likely to increase predation rates (especially at low densities) as predators do not emigrate and therefore tend to re-search areas (Luck *et al.* 1988).

Despite these limitations, cages are useful in providing information on the preferred prey of the predator, potential consumption, the effect of search area on predation rates and enable the comparison of individual predator species. Using caged plants Isenhour and Yeargan (1981) found leaf size affected predation by *O. insidious*. Propp (1982) used cages to show that search area will alter consumption by *N. kinbergii*. In a comparison of predator species in cages Lopez *et al.* (1976) showed there were differences between predator stages and species on consumption of *H. virescens*. Lingren *et al.* (1968) used cages to compare predators of *H. virescens*. Using Petri dishes DeClercq and Degheele (1994) compared life stages of two predators (*P. maculiventris* and *P. sagitta*) on all life stages of beet armyworm (*S. exigua*) and found that predation differed with life stage. They also concluded that it was important to measure predation using realistic prey numbers and suitable search arenas.

4.1.1 Purpose of this study

Current studies have shown that in Australia predators consume heliothis (Mensah and Singleton 1998, Stanley 1997, Room 1979b). However, few have attempted to quantify the direct impact that individual predator species have on heliothis. Room (1979b) provided predator species with heliothis eggs in Petri-dishes and found the mean consumed per day for *N. kinbergii, D. bellulus* and *C. transversalis* were 3.8, 6.4 and 1.4 respectively. Similarly, Stanley (1997) showed that in Petri dishes *D. bellulus* fed on an average of 6.3 eggs per day over 12 days. Stanley (1997) also showed in one field-cage study that the consumption of *H. punctigera* eggs and larvae by *D. bellulus* was not affected by low or high prey density. Similar results were obtained for *M. signata* larvae, *N. kinbergii* and *C. transversalis* on *H. punctigera* larvae. The effects of prey density on the number of heliothis eggs consumed *by N. kinbergii* and *C. transversalis* are unknown for cotton in Australia. This chapter compares consumption of heliothis eggs by *N. kinbergii, D. bellulus* and *C. transversalis.*

As mentioned in Chapter 1 the way in which a study of predation is done depends on logistics and resources available. For this study these are explained in Chapter 2. Cage studies described in Chapter 3 showed how parameters such as location of prey and temperature affect predators in cages. It did not attempt to measure the effectiveness of each species as a biological control agent. The aim of this chapter was to determine the maximum number of prey consumed by an individual, the point at which prey density saturates the predators

feeding and, given that heliothis lay eggs in low numbers in the field, the ability to find prey at realistic numbers on different sized plants. Limitations to the predators' finding prey were also investigated by using different sized search arenas.

4.2 Methods

Following the information gained from the experiments in Chapter 3, the environment cabinet used for cage studies was set at 25 °C and the predators were starved for at least 48 hours and kept in the environment cabinet for 24 hours prior to use. *Nabis kinbergii, D. bellulus* and *C. transversalis* were used in each experiment. Unless specified all experiments were done on potted plants in the environment cabinet as explained in Chapter 2. With the exception of the proportion of predators that fed data, all analyses in this chapter were done using generalised linear models (GLM) as described in Chapter 2. The amended models used in each analysis are given below in the result section. Data from the proportion of a population that fed were analysed using Chi-square tests with a Pearson correlation (Anon 1996b). As in the previous chapter non significant results are expressed as $p > 0.05$, some data were not analysed as results are obvious and 'bf' shows the value used to determine significance where multiple t-tests occurred.

4.2.1 Prey density

Maximum consumption

The results from Chapter 3 showed that in some cases the predators ate all the prey offered. In order to see how many eggs could be consumed by three species of predators, per day, over five days, when no constraints such as searching, competition and predation were present, predator consumption was tested in Petri dishes (8 cm diameter). Five females of each predator species were presented with 100 *H. armigera* eggs per day in individual Petri dishes that contained a wet dental wick for supply of H_2O. Early morning (6:00 - 8:00 am) fresh *H. armigera* eggs were painted (using a camel-hair brush) on to the base of the dish so that they were spread evenly over the whole area. The experiment was done in a temperature controlled room set at 25 ± 2 °C. Each day (allowing for 24 hour feeding time) the number of eggs eaten by each predator were recorded and a new dish containing another 100 fresh eggs was

supplied for each individual. The dental wicks were examined each day for predator eggs and replaced with a fresh wick.

This experiment shows the potential consumption for each predator when unlimited eggs are supplied. It does not include parameters such as searching ability and prey density. Therefore, two further sets of experiments were conducted, one to look at the effects of prey density on consumption and the other to investigate how the size of the search area affected consumption.

Low egg density

Using unrealistically high prey densities in laboratory functional response experiments often do not provide useful information for the field situation. The logical step is to look at the effects of prey densities that reflect field populations. As shown in Chapter 1 the number of eggs found on a cotton plant vary according to the literature from one to hundreds but the general consensus is that in sprayed cotton numbers of eggs are generally below ten per plant. Therefore, the prey numbers chosen for this experiment were 1, 5, 10, 15, 20 eggs per plant as this reflected normal to high levels.

Heliothis eggs were brushed onto the plants by a wet camel-hair brush. In accordance with results from the preferred feeding position on the plants (Chapter 3 – egg position) the eggs were placed on the top of the leaves on the plants. Five female *N. kinbergii*, *D. bellulus* and *C. transversalis* were compared for each prey density. As the environment cabinet was not large enough for all treatments and species to be tested at once, predator species and prey density treatments were mixed over three days. To ensure no eggs were lost due to factors other than predation, three control plants with 20 eggs and no predators were also included. The number of eggs consumed after 24 hours were recorded for each of three predator species.

High egg density

In the previous experiment the number of heliothis prey used reflected realistic levels found in the field. However, as these are generalist predators and there are occasions when heliothis numbers do increase beyond 20 per plant, the point at which prey consumption is limited by density was investigated. Once again, females of the three predator species were used. In

order to increase replication within treatments only four egg densities (20, 40, 80 and 160) were chosen. This allowed for eight replicates of each species and prey density and a range of prey both lower and higher than the maximum consumption seen in the first experiment in this chapter. The eggs were placed on plants in the same way as the low prey density trial and the predator species and prey density treatments mixed over three days. Three control plants with 20 eggs and no predators were also included. The numbers of prey consumed were recorded over 24 hours.

The proportion of individuals that feed

Due to the fact that egg density also seemed to affect the proportion of individuals that fed (Chapter 3 – temperature experiments) the proportion of individuals that feed was investigated using a larger number of individuals. Four egg densities were used (1, 5, 10, and 20) on potted caged plants. These were set up the same way as the previous two experiments in this chapter. Female *N. kinbergii*, *D. bellulus* and *C. transversalis* were tested at each density with one individual per cage. A total of 24 replicates were used for each species and treatment. The species and prey density treatments were mixed over 12 days. The number of eggs consumed and the number of individuals that fed were recorded over 24 hours. In order to determine if there were significant differences between the proportion of predators that fed data were analysed using Chi-square tests (χ^2) with a Pearson correlation test to indicate if there was a linear increase with prey density (linear association) (Anon 1996b).

4.2.2. Search arenas

Single plant search arenas

Whilst using potted plants is more realistic that Petri dishes, the size of cotton plants changes in the field. This is important as the stage of the plants in the field affects the spray program for heliothis. If the predators are more readily able to find eggs on smaller plants they may be more effective as biological control agents early in the season, thus reducing the number of pesticide applications. Consequently, the effect of plant size and the number of plants was investigated further. Two experiments were done. Firstly, the consumption of eggs on three plant sizes was compared and secondly, the consumption of eggs on one meter of cotton with varying egg densities per plant was observed and recorded. In the first experiment the stages

of plants selected were seedling, pre-squaring to squaring and, squaring to boll formation. These equates to early, mid and late season crop production phases (Wilson 1981). Twenty eggs were placed on the upper leaf surfaces on the top third of each plant. Twenty-four females of each predator species were tested providing a total of eight replicates per species, per plant size. The number of eggs consumed after 24 hours were recorded.

Multi- plant arenas

In the larger arena (dimensions detailed in Chapter 2), cages were made to house one metre of cotton (4 potted plants). This allowed four cages to be set up per day in the environment cabinet. Each plant in the cage had 0, 1, 10, or 20 eggs painted on it. The number of eggs were randomly assigned to a plant within the metre by pulling tags out of a bag so that each metre contained a total of 36 eggs. Each day, over seven days, one predator was placed on the top first open leaf of the second plant in each cage. Female predators of all species were observed continuously for 11 hours (0800h – 1800h) in the environment cabinet and the time they spent on each plant, the activity (moving, feeding, preening or resting) and the numbers of prey consumed were recorded. The number of prey consumed over 24 hours were also recorded for each individual. The total numbers of eggs consumed were expressed as a proportion of the total over the metre. The time of day that predators fed was also recorded over the 12 hours. A total of 27 predators (nine of each species) were observed.

4.3 Results

4.3.1 Prey density

Maximum prey consumed

The mean number of heliothis eggs consumed when predators were provided 100 eggs each day are shown in Figure 4.1. Over the five days *D. bellulus* fed on an average of 34.4 ± 3.59 eggs per day and *N. kinbergii* fed on an average 51.3 ± 4.59 eggs per day. *Coccinella transversalis* fed on an average of 30.8 ± 6.63 eggs per dish over three days. One coccinellid died on the first day due to parasitism so a replacement was included the following day. The rest of the coccinellids started to die over the five days due to parasitism so only three days data were used. On the second day of feeding there was a marked decrease in egg

consumption by *C. transversalis* (an average of 28.4 eggs less than the first day) and *D. bellulus* (35.8 less eggs). Only a slight decrease in consumption occurred in *N. kinbergii* (5.4 less eggs) although the nabids fed on consistently high numbers over the five days (Figure 4.1). The range of prey numbers consumed over the five days was high for all species. The numbers consumed by *N. kinbergii* ranged between 11 and 88, *D. bellulus* between 11 and 86 and *C. transversalis* 0 and 71. No eggs were laid by any of the species suggesting that these were not reproductive females.

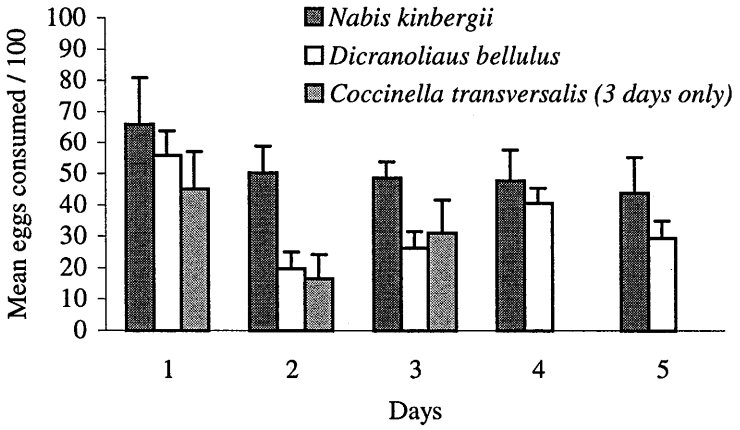

Figure 4.1 The mean number of heliothis eggs consumed in Petri dishes over five days by three species of female predators. Bars indicate standard errors of means.

Prey density

In the first experiment, using 'low' (realistic) prey densities, the effect of density was not significant in the analysis using a saturated model. This shows low prey densities did not limit the predators' consumption rate. This is shown in Figure 4.2 where the proportion consumed at each density forms a line parallel to the x axis. This indicates a type I (linear) functional response. The amended model used for the low density trial was "species". The results from the final analysis showed that there was a difference between species. *Coccinella transversalis* ate less than the nabids (bf = 0.016, p = 0.0042, df = 86). The data also show that *N. kinbergii* and *D. bellulus* were able to find and consume heliothis eggs at all densities including one egg on a plant (Figure 4.2).

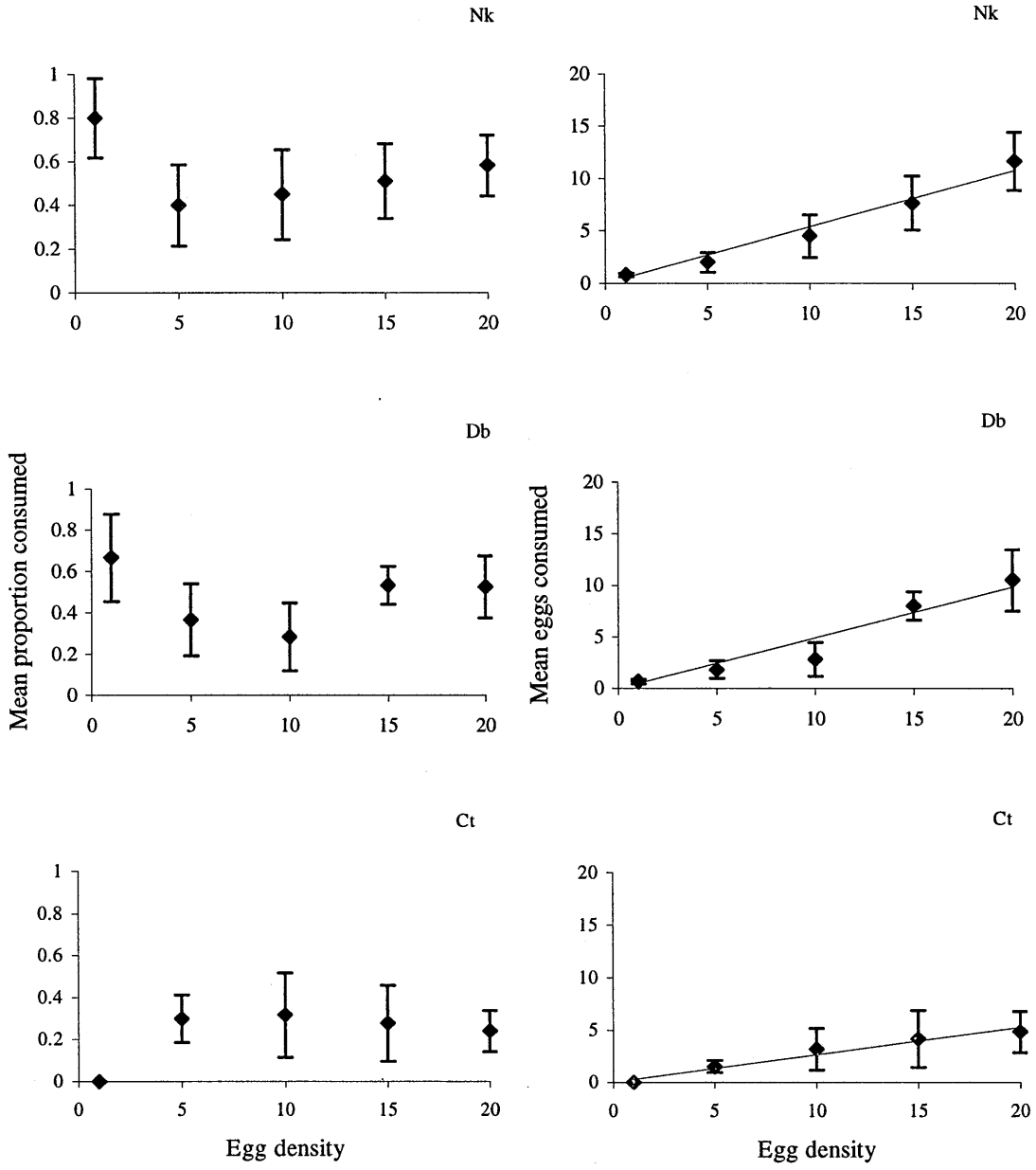

Figure 4.2 The effect of low heliothis egg density on the number of eggs consumed by three predator species. Nk = *N. kinbergii*, Db = *D. bellulus* and Ct = *C. transversalis*. The graphs of the left hand side show the proportion of prey consumed at each prey density. On the right hand side the line indicates the functional response. Bars indicate standard errors of the means.

High prey densities did not significantly affect consumption rates, nor was there a difference between species. The final terms used in the model for high prey densities were "species + density". The data indicate a type I (linear) functional response (Figure 4.3).

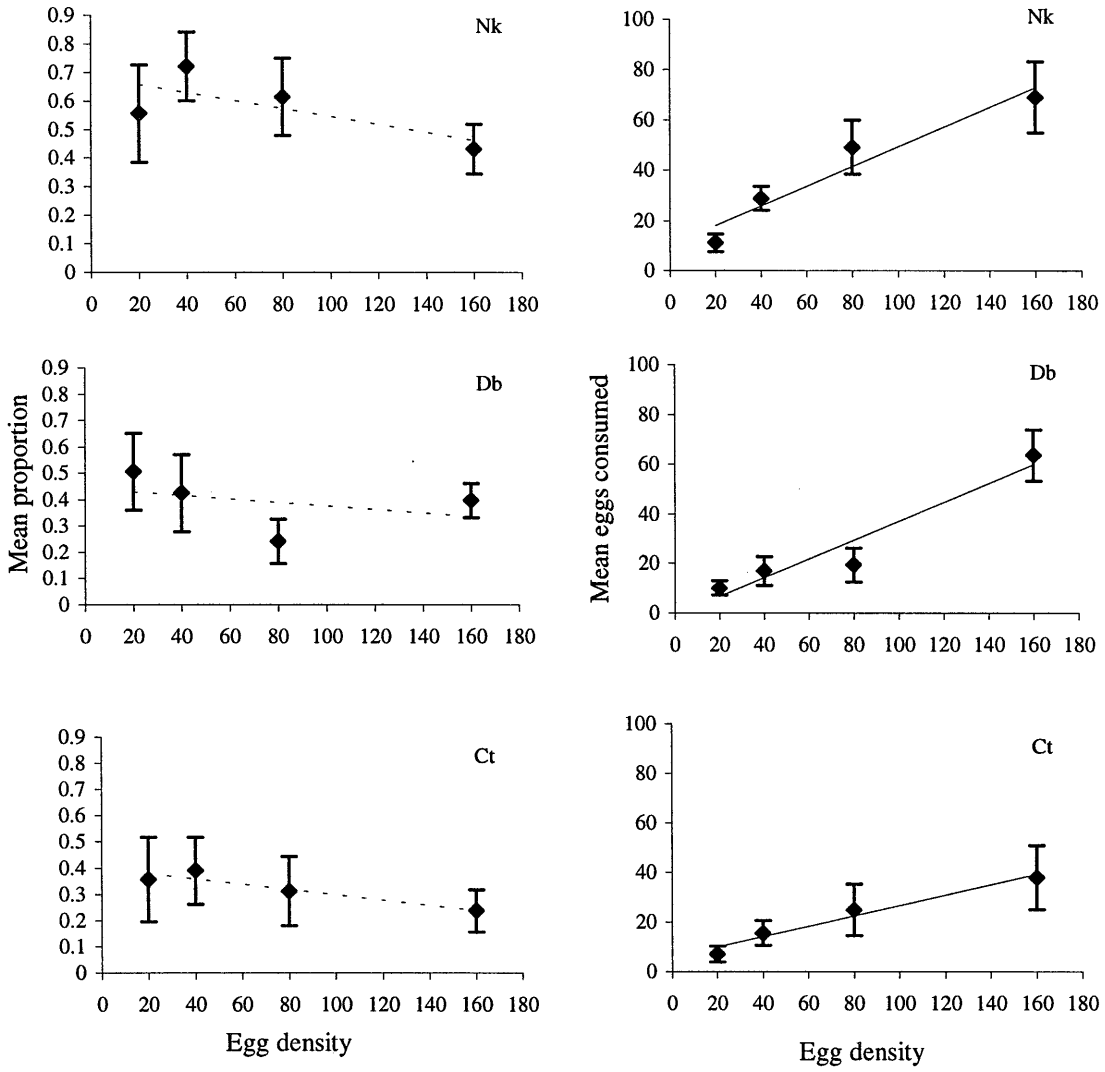

Figure 4.3 The effect of high heliothis egg density on the number of eggs consumed by three predator species. Nk = *N. kinbergii*, Db = *D. bellulus* and Ct = *C. transversalis*. Bars indicate standard errors of the means.

The proportion of individuals that fed

If a predator found the prey it tended to consume all or most of the eggs available on the plant. This meant the proportion of eggs consumed when a predator found them remained similar indicating that prey density did not affect the proportion consumed over the 24 hours

(Figure 4.4). If an egg was found on the plants with a density of one egg then 100% of the density was consumed. Therefore, this data was not included in Figure 4.4. There is a downward trend in the coccinellids that may show that as the density of prey increased the number of eggs consumed declined (not analysed). For the other two species this experiment supports the findings in the low prey density experiment: the predators' ability to consume prey are not limited by prey density. This trial does show that the number of individuals finding prey is affected by prey density. Analysis of the data showed there was a significant density affect for *N. kinbergii* (χ^2 = 10.55, df = 3, p = 0.014) and *D. bellulus* (χ^2 = 9.00, df = 3, p = 0.29) and that the relationship between the proportion that ate and the prey density was linear for both these species (linear association = 7.3, df = 1, p = 0.007 and linear association = 4.96, df = 1, p = 0.026 respectively). Figure 4.5 shows that there is a marked increase in the number of individuals finding eggs as the density increases from one to five eggs per plant and in *N. kinbergii* and *D. bellulus* the increase continues with higher densities of prey. The affect of density on the proportion of *C. transversalis* that ate was not significant (χ^2 = 3.50, df = 3, p = 0.321).

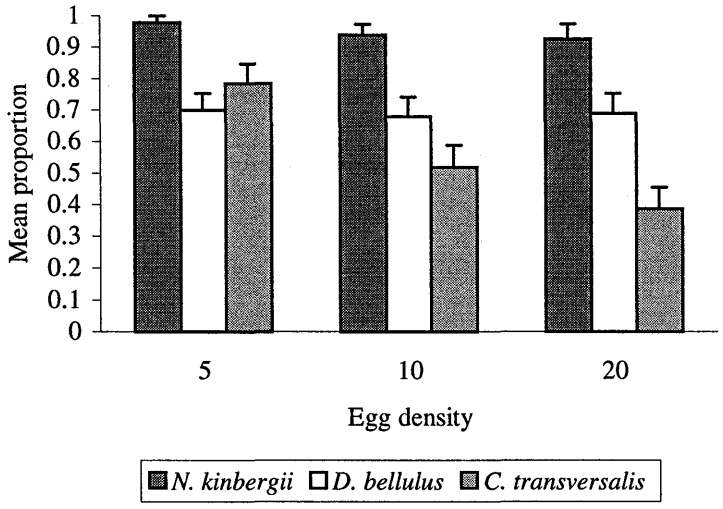

Figure 4.4. The mean proportion of heliothis eggs consumed by three predator species that ate when offered different prey densities. Nk = *N. kinbergii*, Db = *D. bellulus* and Ct = *C. transversalis*. Bars indicate standard errors of the means.

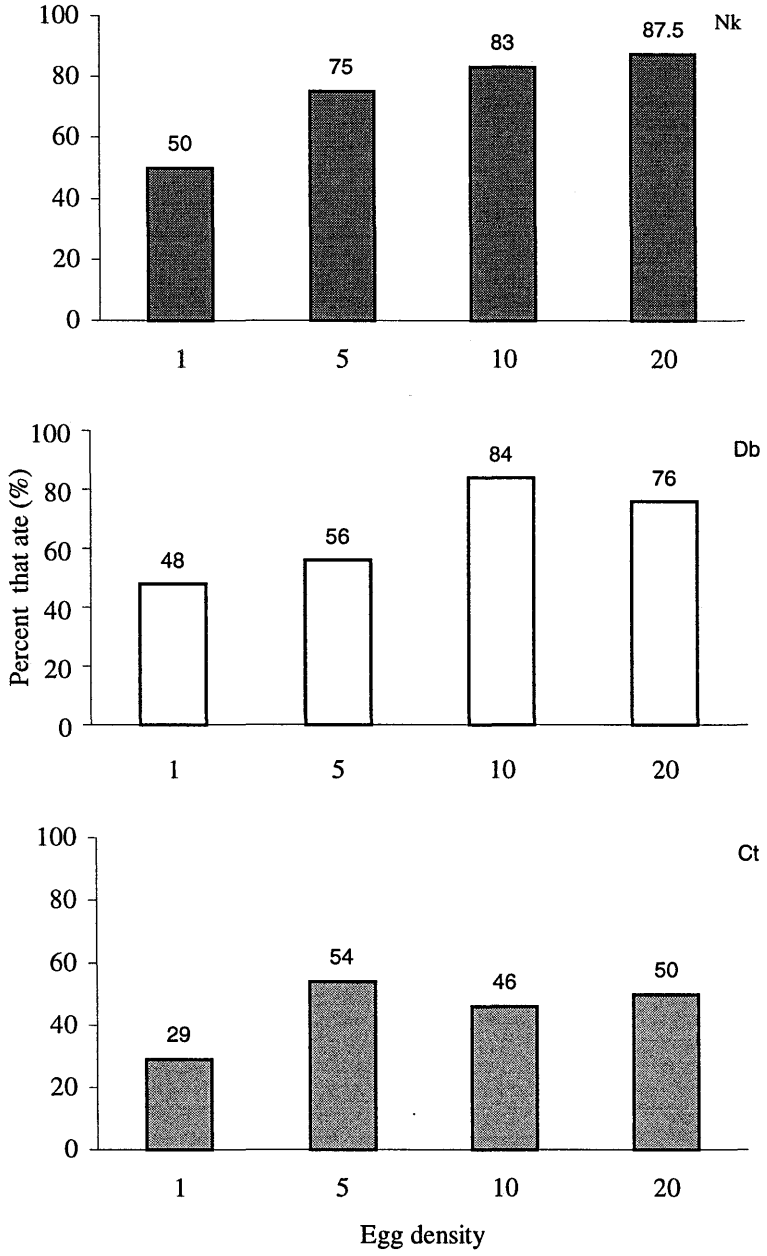

Figure 4.5. The percent of individuals that found and consumed prey at different heliothis egg densities. Nk = *N. kinbergii*, Db = *D. bellulus* and Ct = *C. transversalis*

4.3.2 Search arenas

Single plant search arenas

The final terms used in the model fitted to the data from the experiment using single plants of various sizes were "treatment + species" where treatment = plant size. There was a significant

treatment effect. Less eggs were eaten on large plants than small (bf = 0.016, p = 0.0035, df = 67). However, within predator species there were no significant differences in the number of eggs consumed on each plant size (Figure 4.6).

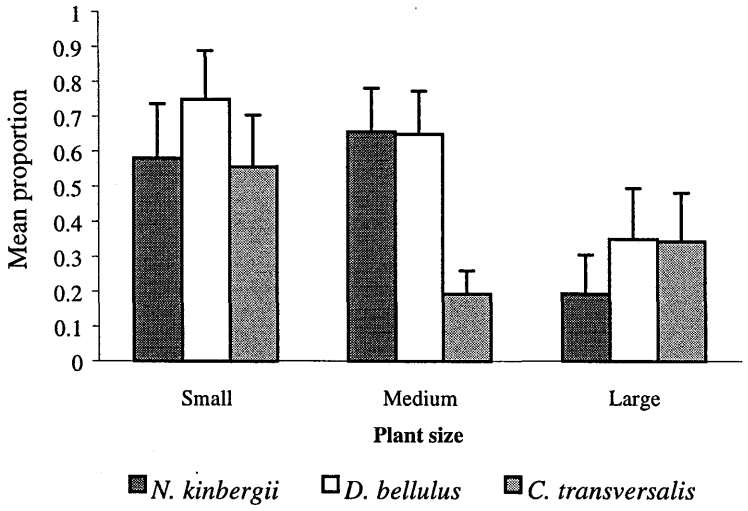

Figure 4.6. The mean proportion of heliothis eggs consumed by three predator species on small, medium and large cotton plants over 24 hours. Bars indicate standard errors of the means.

Multi plant arenas

Results from the one metre of cotton arenas showed that the predators were still able to find and consume eggs over 24 hours, when there were densities of between 1 and 20 eggs on a plant (Figure 4.7). Over the time period three of the nabids died. The data from these were not included in the results. Observation showed the predators tended to return to sites where eggs had been found. The number of eggs originally painted on to a plant and the time spent feeding on that plant during the 11 hours was recorded for each species. During the day the majority of predators were observed feeding on plants with 10 or 20 eggs on them (Table 4.1). Unfortunately, there were too few individuals that were observed for the entire time to enable detailed analysis of the time spent feeding, resting, preening and moving. This was due to some predators being lost throughout the trial (probably in the base of the pots or in the soil) making continuous observation difficult. There were no trends in the time of day that the predators fed for any of the species although, *D. bellulus* tended to feed from midday onwards

(Table 4.1). The number of eggs consumed after 24 hours indicated more eggs were consumed after observations ceased at 1800h.

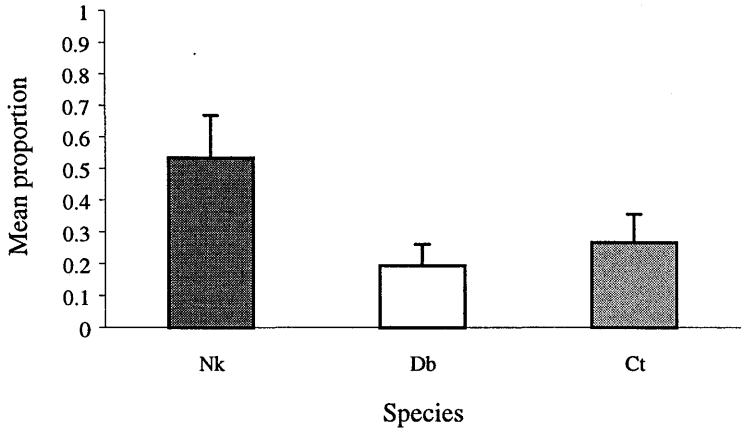

Figure 4.7. The mean proportion of heliothis eggs consumed in 24 hours on one metre of cotton in large cages by *N. kinbergii* (Nk), *D. bellulus* (Db) and *C. transversalis* (Ct). Bars indicate standard errors of the means.

Table 4.1 The time (minutes) spent feeding on a plant in each hour by a predator (bold) and the number of heliothis eggs originally placed on each plant.

Time / Species	7-8	8-9	9-10	10- 11	11-12	12-13	13-14	14-15	15 - 16	16 - 17	17 – 18
N. kinbergii	20-**10**	20-**25**	20-**20**								
N. kinbergii		20-**5**	10-**10**	10-**4** 20-**11**	20-**10**	20-**16**	20-**20**	20-**33**			
D. bellulus										20-**6**	20-**5**
D. bellulus								20-**5**			20-**10**
D. bellulus							20-**2**				
D. bellulus	10-**4**	10-**4**				5-**1**	5-**3**				
D. bellulus						5-**1**		10-**3**	20-**5** 10-**2**		
C. transversalis			20-**6**	20-**5**			10-**1**	10-**3** 20-**10**	20-**8**		
C. transversalis			20-**7**	20-**8**	10-**5**		20-**2**	20-**1**			10-**1**
C. transversalis									5-**2**		5-**2**
C. transversalis									20-**4**		20-**3**
C. transversalis					10-**6**	10-**2**					

4.4 Discussion

4.4.1 Prey density

Maximum prey consumed

The high consumption of heliothis eggs in a simple arena (Petri dishes) shows individual predators are capable of feeding on higher numbers of heliothis eggs than those normally found in field grown cotton. In comparison with results of similar trials by Stanley (1997) on *D. bellulus* and Room (1979b) on *C. transversalis* and *N. kinbergii*, the numbers of eggs consumed in this study were far higher. The female predator species used in this study were not reproductive (they didn't lay eggs) which indicates that the numbers consumed here may be even higher when reproductive females are used. Stanley (1997) found that overall *D. bellulus* ate an average of 6.3 eggs per day but reproductive females ate an average of ten eggs per day over 12 days, almost twice as many eggs as non laying females. Room (1979b) found *N. kinbergii* and *C. transversalis* consumed an average of 3.8 and 1.4 heliothis eggs per day. The results from this study show a 13 and 27 fold increase respectively for these two species. There is need to consider why there is such a discrepancy between studies. The difference between this study and Stanley (1997) and Room (1979b) may be due to the original source of the predators and the time of year that they were collected. As the predator species in all studies were field collected the feeding history and age of each was unknown. Both these factors can affect feeding rates (Donahue and Pitre 1977, Holling 1959). These differences highlight the need to do these studies both within season times and between seasons, using different cohorts of predators.

The results of this trial also agree with the work of Frazer and Gilbert (1976) on *Coccinella trifasciata* Mulsant predation on the aphid *Acyrthosiphon pisum* (Harris) that a simple search arena without searching limitations will provide higher consumption rates than those found in the field. It is possible that the more prey offered the more are eaten. Henneberry and Clayton (1985) showed *Nabis* spp. fed on average on 39 *P. gossypiella* (pink bollworm) eggs per day over eight days when offered prey in excess of potential consumption. Whether the coccinellids ate more than in field situations was unknown. The mortality of the coccinellids in this study was due to parasitism. The effects of parasitism on consumption are not known for *C. transversalis* but may either increase or decrease feeding activity and thus affect

consumption rates. Therefore, the results here should be viewed with caution. The reason a lower number of eggs were consumed on the second day by *D. bellulus* and *C. transversalis* was not known but may have been a direct response to initial starvation all species initially feeding on more eggs. Stanley (1997) found a similar result for *D. bellulus*.

The results from this study show that without limitations such as prey density and search area the predators are able to consume numbers of heliothis that are higher than those normally found in the field. As predators do not consume all heliothis in the field (see Chapter 1) it suggests there must be limiting factors that affect the predators' ability to find prey at lower numbers than those provided in this experiment.

Low prey density

The fact that there was no difference between the proportion of eggs consumed when given a range of low prey densities would indicate that these predators have a type I functional response (Figure 4.2). However, two aspects need to be considered before assuming this to be the functional response. Firstly, for response curves to be fitted greater densities of prey would be needed to enable consumption to reach an asymptote, and more data points at the lower end of the density treatments would be required to determine the difference between type II and III curves (Casas and Hulliger 1994). It was beyond the resources of this study to do this type of experiment.

Secondly, it is likely that with low prey numbers (realistic numbers) an asymptote would not be reached. Nordlund and Morrison (1990) found a linear functional response for *Chrysoperla rufilabris* (Burmeister) larvae when given low numbers of *H. virescens* eggs on cotton seedlings. This was thought to be a type II functional response, as a plateau had not been reached due to low prey densities. Hutchinson and Pitre (1983) also showed a type I functional response by *G. punctipes* to *H. virescens* eggs on cotton when realistic prey numbers were used. Similarly, Wiedenmann and Smith (1993) suggested that a linear response found for parasites of *Diatraea saccharalis* (F.) was due to using realistic host numbers. These examples indicate that when using realistic prey levels an asymptote will not be reached. Therefore, the point at which the predators in this study are affected by handling time (reaching an asymptote) is not relevant to the consumption of prey that would occur in the field. At the densities used in this experiment the relationship between predator and prey

can be considered linear. That is, as prey numbers increase the number of prey consumed increases. This shows that the number of prey consumed by the predator is not limited by prey numbers normally found in the field. In such cases how the availability and dispersal of prey affects predation rates becomes an important consideration.

Proportion of predators finding prey at low densities

The lower the prey numbers, the smaller the proportion of predators was that found and consumed heliothis eggs (Figure 4.5). If searching by predators remains constant then the chance of finding prey is greatly reduced at low prey densities. However, it is unlikely that predator behaviour remains constant. Although the area searched by a predator increases when prey are scarce and lasts longer than when prey occur in high densities, predators that are unsuccessful spend more time resting than searching. This was found by Wiedenmann and O'Neil (1991b) when investigating searching behaviour of *P. maculiventris* on plants and supports the findings here that the lower the prey density the lower the proportion of predators that consume the prey. In a field situation it may be more feasible for a predator to feed on other food sources such as honeydew or pollen when prey are low rather than expend time looking for scarce prey (Vohland 1996). Wiedenmann and O'Neil (1991b) found the predator, *P. maculiventris,* was also able to find prey at low densities but Heimpel and Hough-Goldstein (1994) showed they were much more efficient when prey were at high densities due to area concentrated searching. They ate more per hour than at low densities. It may be an advantage for optimising predation to have higher densities of the target prey in the field.

Higher prey density

As an asymptote was not reached when comparing prey density to the numbers consumed, the predators' response to high prey densities showed they were not limited by the prey densities provided. We know that when placed in a Petri dish with unlimited eggs the predators *C. transversalis, D. bellulus* and *N. kinbergii* will feed on an average of 30, 34 and 51 eggs per day, respectively. One would expect that when given prey numbers greater than these on a plant less would be consumed due to higher searching time and eventually a saturation level would be reached. This was not the case for high prey numbers used in this study indicating that the predators are voracious feeders, capable of feeding on heliothis egg numbers far

greater than would be found in the field. Nordlund and Morrison (1990) suggested the reason *C. rufilabris* were not limited by high numbers of prey (*H. virescens*) was that they were voracious feeders. There was a slight negative trend in all species as prey density increased to very high levels. This may indicate that with greater replication and reduced variance the negative relationship may have been statistically significant. If consumption of prey by predators is not limited by low or high prey density then finding realistic prey numbers in a larger arena is most likely to affect the numbers of prey consumed.

Search area

Although there were no significant treatment affects as a result of plant size for individual species there was an overall effect on numbers of eggs consumed on different plant sizes. Figure 4.6 shows that the mean number of heliothis eggs consumed between small and large plants was lower for all species when they had to find eggs on larger plants. It is likely that plant size does cause a reduction in the predators' ability to find prey but that this effect is only seen when the arena size is sufficiently large to affect the predator searching. O'Neil and Stimac (1988) found that the leaf area of soybean plants only affected per capita predation rates on predators when up to four fold increases in leaf area occurred suggesting the predators adjusted their searching rate to leaf area. Other studies have shown plant size affects predation. Donahoe and Pitre (1977) showed that plant age and size decreased predator effectiveness of *R. roseipennis* on *H. zea* on cotton plants. Lingren *et al.* (1968) found that adult *G. punctipes* consumed up to 18 *H. virescens* eggs per day but this declined to 1.9 on the second day due to larger search areas being provided. Nuessly and Sterling (1994) showed predation rates on *H. zea* declined with plant growth due to fewer predators per unit area. Coll and Ridgeway (1995) suggested lower rates of predation than previously recorded by *O. insidiosus* on thrips was due to the use of a whole plant as a search arena. Congdon et *al.* (1993) found the coccinellid *S. punctum picipes* was still active when prey densities were low. They suggest that the searching ability and dispersal of the predator is more important than the numerical response to prey. The view that a predators searching ability and arena will alter prey consumption is supported by Wiedenmann and O'Neil (1991a) who maintain that the effect of searching is often overlooked in functional response studies and that searching changes with prey density. Certainly in this study plant size and arena size affected consumption levels.

Species differences

There was a species difference in the number of eggs consumed when prey densities were low. *Coccinella transversalis* ate less than *N. kinbergii.* However the same result was not seen at higher prey densities. There may be several reasons for the difference in consumption rates. Nuessly and Sterling (1994) suggest that sucking predators are more efficient than those with chewing mouth-parts. This may partially explain consistently higher predation by *N. kinbergii* in many of these experiments but does not explain the sometimes equal consumption between *D. bellulus* (which has chewing mouth-parts) and *N. kinbergii.* If *C. transversalis* consistently consumed less it may be due to them being aphidophagous or coccidophagous (Hodek 1973). This is addressed in the next chapter. If they are aphidophagous their searching behaviour may differ as aphidophagous insects tend to search along mid veins underneath leaves where aphids are found more than the upper leaf areas. Results from the egg position sites experiments in Chapter 3 showed the underneath of leaves were not the preferred feeding site of this species. Coccinellids may also require more resting time in order to optimise water loss, oxygen consumption and metabolic rates (Wiedenmann and O'Neil 1991b). Spending more time under leaves may also avoid predation by enemies of the coccinellids. These reasons would explain a consistent lower consumption rate by *C. transversalis.* However, the consumption rates between species were not consistent. This may in part be explained from using different cohorts of insects. The age of cohorts may vary with the time of year. Donahoe and Pitre (1977) showed that feeding by adult *R. roseipennis* decreased with age. The age of these predators was unknown. As there is not a consistent trend in coccinellids eating less prey than nabids the most likely explanation to few prey being eaten at low densities is the one that coccinellids may be less able to find eggs at lower density. Coccinellids need to come into contact with their prey to perceive them (Hagen 1962). They may, therefore, have to spend more time searching at lower densities before they find their prey. Hodek *et al.* (1984) showed that the coccinellid *Cheilomenes sulphurea* (Ol) was affected by prey density more than search arena size.

Translation of results to field situations

The predators studied in these experiments were able to find and consume heliothis eggs and they can consume large numbers, larger in most instances than they would find in the field. It is the low numbers of prey that affect predation. The significance of fewer predators finding

low prey numbers is important when considering predator to prey ratios such as those used by Murray and Mensah (1996). These may need adjusting not only for each predator species but also for prey numbers. Although the predators find low prey densities on both single plants and one meter of cotton none were able to reduce prey numbers to below economic thresholds. Van den Berg and Cock (1995a) reached the same conclusion in their cage studies in Africa. However, they also state that the use of natural enemies is still important for natural mortality in sustainable management systems.

Frazer and Gilbert (1976) suggest the level of predation found in laboratory studies is likely to be an overestimation of predation that would be found in the field. This was shown by O'Neil (1989) in a comparison of field and laboratory functional responses of *P. maculiventris*. In the laboratory *P. maculiventris* were able to consume high numbers of prey but in the field consumption was limited due to prey density being extremely low in a larger arena. Similarly, the predators in this study are likely to be limited by larger plants so late season control will not be as efficient as on early season (stage one) plants. The implications of these findings are that the predators may be of greater use in early season control when plants are small and pesticides have not reduced predator numbers. This was shown by Deutscher and McKewen (1996) who suggested early season control of heliothis by pesticide was not required on cotton, as predators were present in sufficient numbers. As Frazer and Gilbert (1976) and O'Neil (1989) suggest the results from these laboratory trials need to be verified in the field. Chapter 8 of this thesis investigates field predation.

4.4.2 Conclusion

These results are useful in that they confirm that searching ability, size of arena and prey density do affect the ability of predators to find and consume prey but that prey density alone does not limit consumption by a predator. Rather, it limits the number of a population finding the prey. There is no doubt these predators can consume large numbers of prey and that these experiments showed the predators consume heliothis eggs finding them at realistic prey numbers and even on large search arenas (1 metre of cotton). However, as mentioned in the introduction these are generalist predators. Therefore, it is likely a choice of prey will also affect the number of heliothis eggs consumed. This is investigated in the next chapter.

Chapter 5

5. Prey preference and the effects of alternative prey.

5.1 Introduction

Generalist predators are polyphagous. Therefore, they are likely to consume a range of prey types and, in some cases, may also feed on plants and pollen. In Australian cotton the main prey types available to the insect predators include different stages of heliothis, aphids, thrips, mites and jassids (Wilson *et al.* 1998a, Stanley 1997, Fitt 1994, Room and Wardhaugh 1977). In studies on the efficiency of generalist predators the effects of other prey on consumption of the target prey are often not included. In spite of this, the effects of alternate prey on predation are usually referred to as an important consideration in measuring the effectiveness of generalist predators (Nordlund and Morrison 1990, Chesson 1989, Hutchinson and Pitre 1983, Room 1979b, Hassell and Southwood 1978).

The type of prey consumed by a predator may be dependent on the prey complex and availability, size of the prey, prey behaviour, searching behaviour of the predator, and nutritional requirements of the predator. For example, Siddique and Chapman (1987a) showed that in comparison of pea aphids (*A. pisum*) or diamond back moth (*Plutella xylostella* (L.)) development, survival and fecundity of *N. kinbergii* were significantly higher when they were reared on diamond back moth. Hodek (1973) provides examples of the effects of different prey types on the development and fecundity of several coccinellid species. In general, the examples show the species of prey provided affects development and survival of the coccinellids. Consequently, if the prey species affects survival of the predator it is an advantage for the predator to consume certain prey types. If a predator is required for control of a target pest then its possible preference for other prey needs to be assessed.

A predator species may be opportunistic feeding on whatever it encounters, may prefer a particular prey species or, may 'switch' to certain prey types. Switching is a behaviour found in predators where, as a population of a preferred prey type becomes available the predator may switch to searching for a particular prey thus reducing predation on the target prey (Murdoch 1969, Southwood 1978). Some coccinellid species are known to switch when target

prey are scarce (Hodek 1973). However, coccinellids are also capable of starving for long periods or consuming small amounts when a particular prey is low in numbers (Hodek 1973). The question is, are generalist predators likely to switch to larger populations of a species of prey, prefer one to another for nutrient or attack reasons or, are they just opportunists feeding on whatever they come across?

Preferences and switching have been shown for some predator species. Sloderbeck and Yeargan (1983) showed *N. americoferus* and *Nabis roseipennis* Reuter both consumed eggs and all larval stages of the green clover-worm (*Plathypena scabra* (F.)). Adults of both species consumed more first instar larvae than eggs. Wiedenmann and O'Neil (1990) showed *N. roseipennis* preferred boll weevil (*Anthonomus grandis* Boheman) larvae to Mexican bean beetle larvae (*Epilachna varivestis* Mulsant) when the prey species were isolated or together. They suggest spines on *E. varivestis* may deter the predators from feeding on one type of larvae. Lawton *et al.* (1974) showed that switching occurred in the aquatic hemipteran predator *Notonecta glauca* L. when provided with two prey types. Hunger of predators and density of prey affected the functional response of damselflies when alternative prey types were present (Akre and Johnson 1979). They suggest switching occurred due to searching modes of the predators. If the predators were in ambush mode it affected the encounter with a particular prey type more than if they were in walking mode. This is because motile prey would be more likely to be attacked than stationary prey. The searching mode of the predator was likely to be selected to reduce the risk of predation on the predator or by the availability of a particular prey type. Therefore, the response of the predator to prey is likely to differ with different types of feeding behaviour exhibited by each predator species.

Types of feeding behaviours exhibited by predators

The choice of prey by a predator may be dependent on the availability of prey and the predator species' searching behaviour and ability. Sterling *et al.* (1989) highlighted these effects. They showed work on four generalist predators of heliothis resulted in differences in their efficiency due to their searching behaviour. *Orius* and *Chrysopa* species appeared to adjust their behaviour to changes in prey types, numbers and distribution by changing search patterns. *Geocoris* and *Collops* species did not, preferring to continue searching in the same area of a plant. Similarly, prey movement may also be important. For example, nabids are considered ambush predators so motile prey may be preferred. In some cases the prey species

is also important to predators. Murdoch and Marks (1973) found that in some cases the previous diet of coccinellid larvae affected their prey preference. Predatory coccinellids are known to be either acarophagous, coccidophagous, aphidophagous, mycophagous or feed on a range of food types that include plants (Hodek 1973). As a result, the location and behaviour of the predators preferred prey would affect the areas searched by a particular type of predator. As well as searching particular areas on a plant, predators often use cues to find prey.

Rani and Wakamura (1993) showed faeces of *Spodoptera litura* (F.) larvae caused prey searching behaviour in the pentatomid *Eocanthecona furcellata* (Wolff). They suggest the rostral tip contains chemotactile sensillae that are used for prey detection. In some cases injured plants may also attract predators (McCall *et al.* 1994). Evans and England (1996) showed the presence of honeydew caused the ladybird *Coccinella septempunctata* (L.) to aggregate. Heimpel and Hough-Goldstein (1994b) found predatory stinkbugs differed in the cues they used to find prey. They found *P. maculiventris* nymphs responded to movement whereas *Perillus bioculatus* (F.) used primarily touch to recognise prey. Coccinellids are generally considered random feeders that intensify local searching upon eating and in most cases (but see Stubbs 1980) use contact to identify their prey (Hagen 1962). However, they are also known to search along raised veins and outer edges of leaves, as this is where aphids are found (Dixon 1959). Other cues may include prey size, shape, colour, and chemical stimuli such as gland secretions or pheromones (Dent and Walton 1997). The cues used for searching often depend on the type of prey the predators feed on.

5.1.1 What do predators in Australian cotton feed on?

Room (1979b) showed that many predators found in cotton ate both eggs and small larvae of heliothis when tested in laboratory conditions. Of these, *N. kinbergii* was also observed feeding on both larvae and eggs in cotton fields. Stanley (1997) added to this by showing *D. bellulus* adults and *M. signata* larvae fed on small heliothis larvae (neonates) in field cages and *C. transversalis* and *D. bellulus* fed on aphids. Yee (1998) found various pollen types were present in the guts of several predator species normally found in cotton crops in Australia; these included *D. bellulus*. In rice crops, Schicha (1974) found pollen was the dominant food in *D. bellulus* guts. Little is known about the food requirements of *D. bellulus* although Stanley (1997) showed they eat aphids as well as heliothis and pollen. This species is also considered a soil dweller so they may consume small soil fauna. Wilson *et al.* (1998a)

suggested *D. bellulus* may be important mite feeders. Pope (1988) cites *C. transversalis* as being obligate aphid feeders and Anderson and Hales (1984a) state they are an important predator of aphids in cotton. Whether any of these predator species show a preference for certain food types is unknown.

5.1.2 Purpose of this study

Room and Wardhaugh (1977) associated aphid (*A. gossypii*) decline with abundance of coccinellids in cotton. According to Stanley (1997) there is no correlation between the abundance of heliothis eggs and predator abundance in cotton fields. Indeed large numbers of predators can often be seen when aphid populations occur at the beginning and late in the cotton-growing season. Consequently, it is important to know whether the predators feed on aphids rather than heliothis eggs. Aphids are also considered pests in cotton (Fitt 1994) as they cause damage to cotton leaves and to the lint by production of honeydew.

The aim of this chapter was to test if the predators *N. kinbergii, D. bellulus* and *C. transversalis* preferred a stage of heliothis egg or larvae and what the effects of different densities of aphids were on their ability to feed on heliothis eggs.

5.2 Methods

5.2.1 Comparing heliothis stages

White versus brown eggs

In this thesis most experiments were done over a 24 hour time period. If brown heliothis eggs were used for prey they tended to hatch before the end of the experimental time. As a result the white eggs were used in all experiments. This enabled eggs to remain on plants without hatching during the experimental time (usually 24 hours). As both stages of heliothis eggs (described in Chapter 1) are present in the field and the thresholds at the start of these experiments was based on two eggs per meter of cotton row, the difference in feeding between white and brown eggs needed to be determined. There was also some evidence to suggest that older eggs were less palatable (McDaniel and Sterling 1979). This may affect the predators' consumption rates. To check if there was a difference in the number of white and brown heliothis eggs consumed by predators the following experiment was established.

The experiment was conducted in Petri dishes in an environment cabinet set at 25 °C. Ten white and ten brown eggs were painted with a camel hair brush on to a Petri dish containing a moist dental wick. The eggs were painted in two rows, each containing five eggs. White and brown eggs were alternated along the row 1 cm apart. There were eight replicates of female *D. bellulus* and *C. transversalis* and 15 replicates of female *N. kinbergii*. The number of eggs eaten each hour for eight hours were recorded.

Neonates versus eggs

Just as there are different stages of eggs in the field, there are also different life stages of the prey. These experiments tested whether newly hatched larvae (neonates) were preferred over heliothis eggs as a prey source by *N. kinbergii, D. bellulus* and *C. transversalis*. Using a camel hair paintbrush ten eggs were placed in a row in Petri dishes that contained a moist dental wick. At the same time ten neonates were placed in the centre of the dish. Immediately after dispersal of the neonates a single female predator was released into each dish. Another treatment containing neonates without eggs was included to ensure that the number of neonates was not saturating the predators appetite. One other treatment containing ten eggs was included to see if more eggs were consumed without neonates present. A control of eggs and neonates with no predators was used as neonates escaped easily and also ate eggs. The dishes were wrapped in Gladwrap® (plastic wrap) to stop neonates escaping. The numbers of neonates and eggs consumed each hour for seven hours (10:00 to 17:00) were recorded for females of each predator species. There were six replicates of each treatment and species. Each species was tested on different days. The experiment was conducted in the environment cabinet set at 25 °C and 14:10 day/night photoperiod.

5.2.2 Aphids as alternative prey

Aphids and heliothis eggs in equal numbers

Females of each predator species (*N. kinbergii, D. bellulus* and *C. transversalis*) were used in this set of experiments. The aphid species was *A. gossypii* and the eggs were *H. armigera*. The treatments were:

plants containing 20 aphids, 20 eggs and one predator,
plants containing 20 aphids and one predator (excluding the species *D. bellulus*),
plants containing 20 eggs and one predator and,
plants containing 20 aphids only.

There were 7, 8, and 9 replicates of each treatment for *N. kinbergii, D. bellulus* and *C. transversalis* respectively. A control of 20 eggs without predators was not added due to area constraints within the environment cabinet and results from previous experimental chapters had shown no eggs went missing when predators were not present. Aphids were originally field collected from cotton grown at the Australian Cotton Research Institute (ACRI), then taken to the University of New England (UNE) and reared on caged cotton plants (sicala V2) in a glasshouse. Immature aphids of visually equal size were placed on the first fully opened new leaf of a cotton plant using a camel-hair brush. These were allowed to settle for 24 hours and counted to make sure 20 were present prior to the predators being included. The eggs were also placed on the same leaf using a camel-hair brush. The arena used was caged cotton plants in the environment cabinet set at 25 °C and 14:10 day/night photoperiod. Each predator species was tested on different days.

The number of eggs and aphids consumed were recorded after 24 hours. Because the aphids were difficult to recover from plants, the number of aphids recovered from control plants was recorded as well. The mean number of aphids not recovered from control plants was deducted from the number recorded as consumed in the other treatments that contained aphids.

Different densities of aphids

If predators switch to a different prey type as a result of prey availability, then the density of the prey types available is important to consider in predation studies. The previous experiment used equal numbers of prey types. This experiment looked at the number of heliothis eggs consumed by *N. kinbergii, D. bellulus* and *C. transversalis* in the presence of different densities of aphids that would normally be found in the field.

For each predator species tested, ten plants were infested with aphids in the same manner as the previous experiment. These aphids were then allowed to multiply until high numbers of aphids (c. 200+ per plant) were present. At the time of use honeydew was present on these

plants. Another ten plants were infested with low aphid numbers (c.50 per plant). These were painted on to the top of leaves in the upper third of the plants and allowed to settle for 24 hours. Prior to use 20 heliothis eggs were painted on the upper leaf surfaces on the top third of each plant. Another ten plants contained only eggs with no aphids. This acted as a control to make sure that the predators were feeding when the experiment was conducted and allowed a comparison of consumption of heliothis eggs without the presence of aphids. A control using aphids or eggs only (without predators) was not included in this experiment as the aphid consumption was not being measured and no eggs had been lost in any previous experiments due to any factors other than being consumed. The consumption of eggs in each treatment was recorded after 24 hours. Results from each species were analysed separately as they were tested on different days as plants and aphids became available.

5.2.3 Analysis

The analyses used are described in Chapter 2. Repeated measures were analysed using generalised linear mixed models (GLMM) and the other data sets using generalised linear models (GLM). The data from the neonate and first aphid experiment were analysed as two separate experiments with eggs and neonates or eggs and aphids for each predator species. Therefore, the results did not need adjusting using the Bonferoni method. The significance level for these experiments was $p < 0.05$. The t value is presented in these results. The results for the other experiments in this chapter were adjusted using the Bonferoni method (bf) when multiple t tests occurred. All results that are referred to as non-significant were $p > 0.05$.

5.3 Results

White versus brown egg consumption

In all cases the predators did not commence feeding until after 11:00 am. As a result the data were only plotted from 12:00 to 18:00 hours. The graphs showed that during this time there were no obvious differences between the consumption of white and brown eggs by any of the three predator species tested (Figures 5.1).

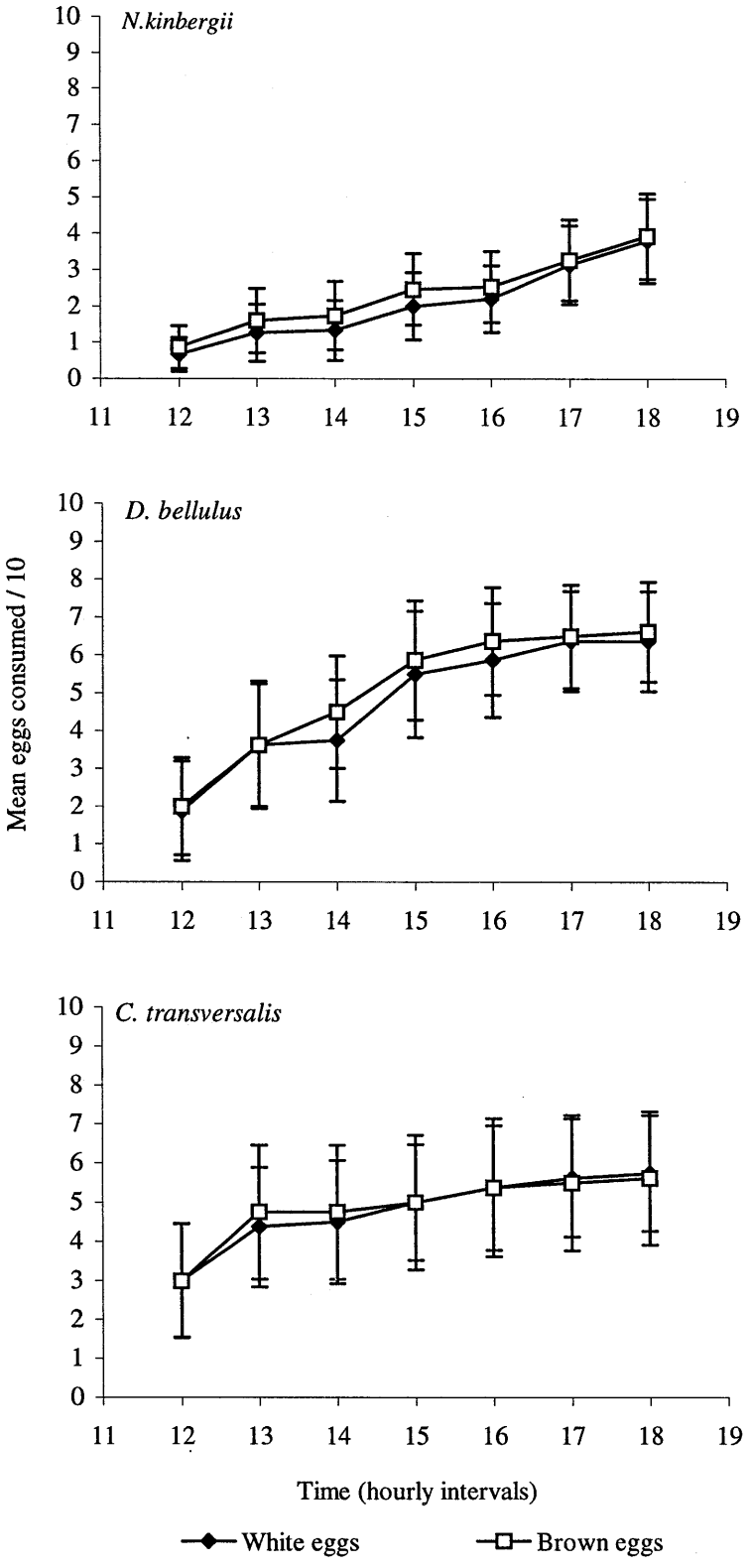

Figure 5.1 The mean number of heliothis eggs consumed by three predator species when both white and brown eggs were provided. Bars indicate standard errors of the means.

Neonate versus egg consumption

All predator species ate both neonates and eggs. The controls showed a mean of one neonate escaped from each dish so this was deducted from the other treatment data. Within all species, data were analysed as the difference in the number of neonates consumed with and without eggs, the difference between eggs eaten with and without neonates and, the difference between eggs and neonates consumed when in one arena. The amended model used in the analysis for neonates for *N. kinbergii* was [{(treatment + time) + time^2} + (treatment + time) + treatment*time^2] where treatment was plants with or without eggs. There was no significant difference in the number of neonates consumed whether eggs were present or not (Figure 5.2A). However, the results showed there was a significant treatment by time interaction ($t = 2.318$, $p = 0.0214$, $df = 10$). More neonates were initially consumed when eggs were present (Figure 5.2A). The amended model used for egg differences was (treatment + time) + time^2 where treatment was with or without neonates. There was a significant difference in the number of eggs consumed by *N. kinbergii* with and without neonates present ($t = 5.4564$, $p = 0.0001$, $df = 79$). *Nabis kinbergii* ate more eggs when neonates were not present (Figure 5.2B). The final model used for analysis of eggs and neonates together was [{(treatment + time) + (treatment * time)} + time^2 + (treatment * time^2)]. There was a significant difference in the number of each prey type consumed when both were in the same arena. More neonates were consumed than eggs ($t = 3.61433$, $p = 0.0003$, $df = 76$) (Figure 5.2C).

The main terms used in the analysis of neonates for *D. bellulus* were (treatment + time) + time^2 and for eggs (treatment + time) + (treament*time) and for both prey in one arena [{(treatment + time) + (treatment * time)} + time^2] + (treatment * time^2). There was no significant difference between the number of neonates consumed with or without the presence of eggs. However, there was a significant treatment*time effect ($t = 2.356$, $p = 0.0201$, $df = 10$) showing that more neonates were consumed in the presence eggs as numbers of prey declined (Figure 5.3A). There was no significant difference in the number of eggs consumed by *D. bellulus* with or without neonates present (Figure 5.3B). When both prey were in the same arena there was a significant difference in the number of each type consumed. More neonates were consumed than eggs ($t = 3.4284$, $p = 0.0005$, $df = 76$) (Figure 5.3C). There was also a significant interaction between treatments and time ($t = 2.7631$, $p = 0.0198$, $df = 5$) showing again that as fewer neonates were available more eggs were consumed.

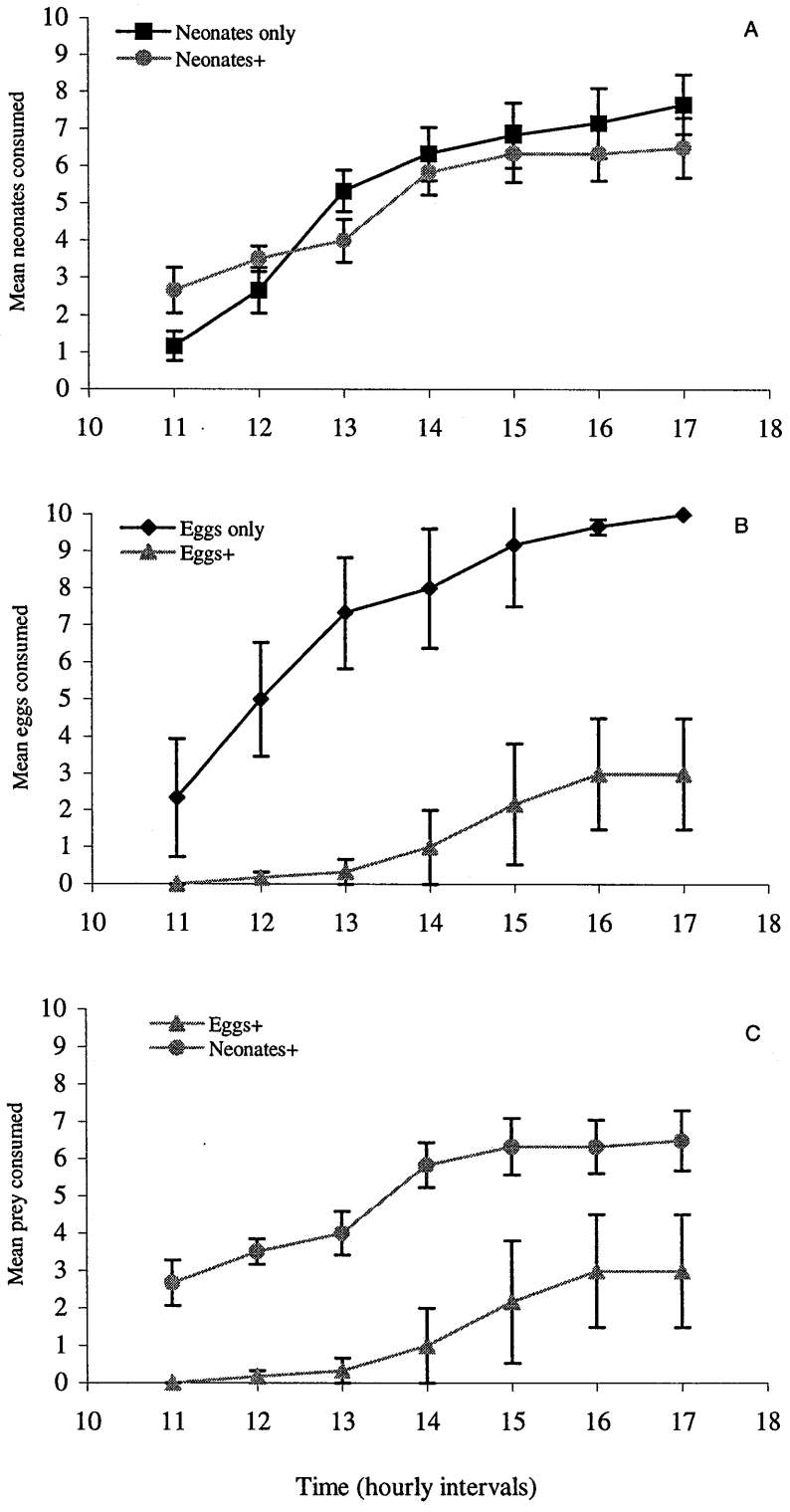

Figure 5.2 The mean cumulative number of prey consumed during each hour by *N. kinbergii* when heliothis eggs and/or neonates were present. A = neonates consumed with and without eggs, B = eggs consumed with and without neonates and, C = eggs and neonates consumed in one arena. Bars indicate standard errors of the means.

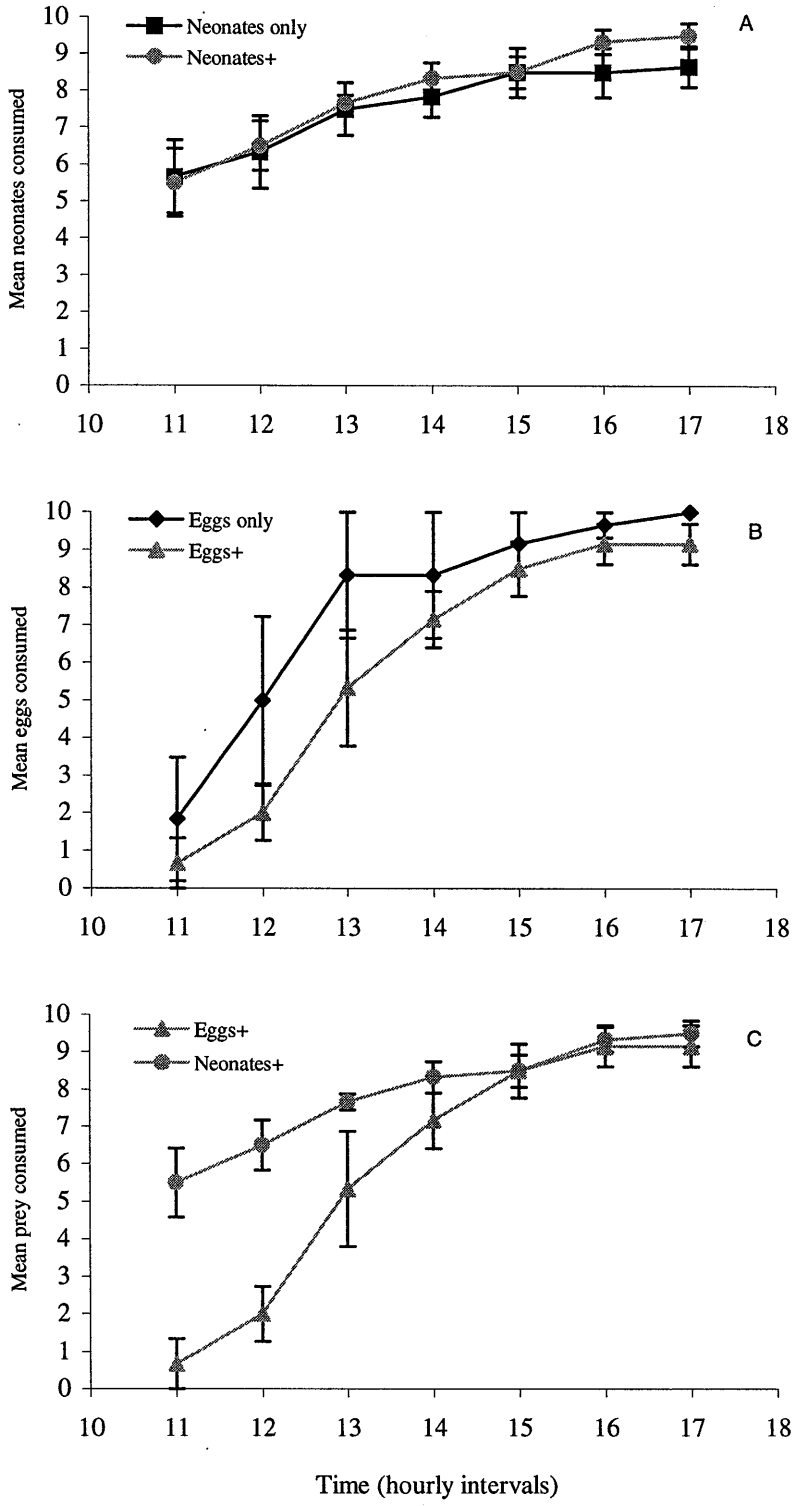

Figure 5.3 The mean cumulative number of prey consumed during each hour by *D. bellulus* when heliothis eggs and/or neonates were present. A = neonates consumed with and without eggs, B = eggs consumed with and without neonates and, C = eggs and neonates consumed in one arena. Bars indicate standard errors of the mean.

Results for *C. transversalis* showed there were no significant differences between eggs or neonates consumed in the presence of either prey type, nor when both prey types were in one arena (Figures 5. 4 A, B and C).

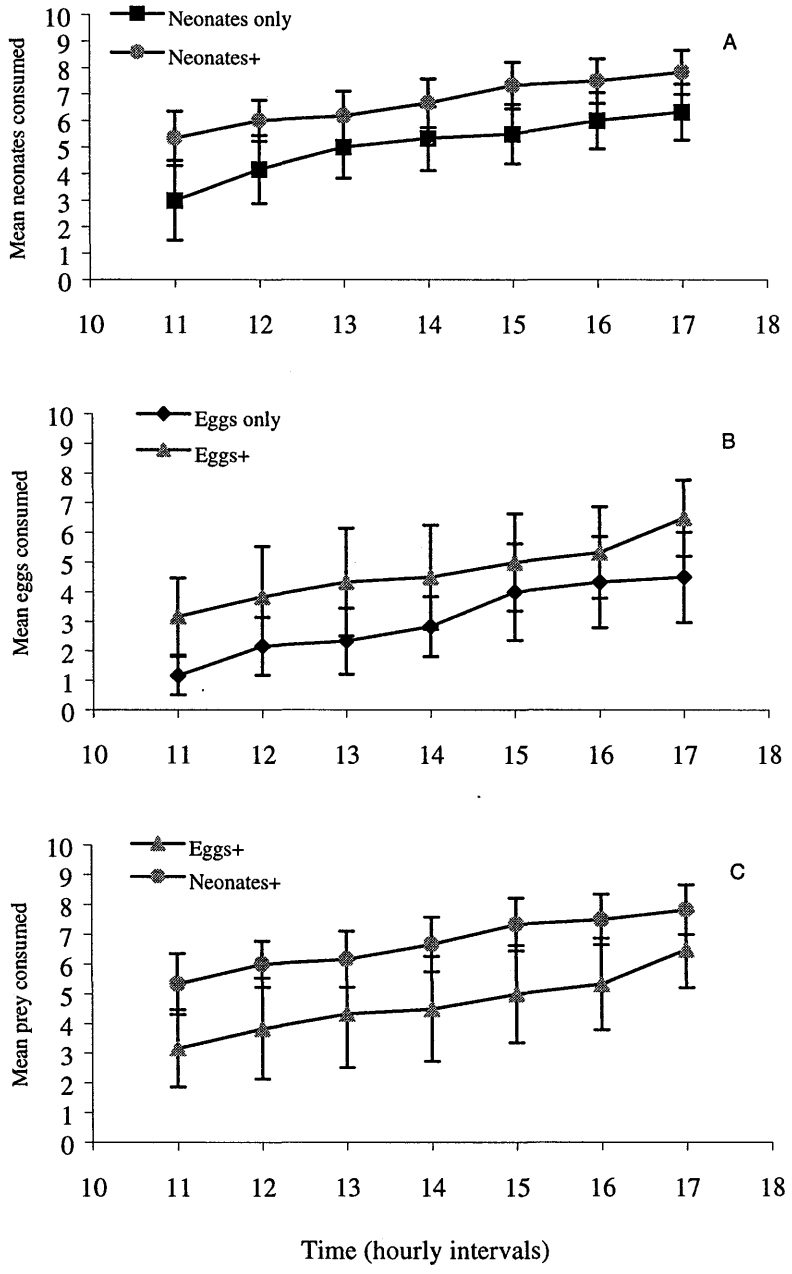

Figure 5.4 The mean cumulative number of prey consumed during each hour by *C. transversalis* when heliothis eggs and/or neonates were present. A = neonates consumed with and without eggs, B = eggs consumed with and without neonates and, C = eggs and neonates consumed in one arena. Bars indicate standard errors of the mean.

5.3.1 Aphids as alternate prey

Aphids and eggs in equal numbers

After a settling time of 24 hours the aphids had dispersed over the leaf surface and to other leaves on the top third of the cotton plant. Eighty five percent (±5) of all aphids were recovered from the cotton plants used during these experiments. In all cases the predators ate aphids as well as heliothis eggs. The data were analysed in three ways: firstly to see if there was a difference between eggs consumed with and without aphids present, secondly to see if the number of aphids consumed differed with and without the presence of eggs and thirdly to see if more eggs were consumed than aphids on the same plant. The difference in aphids numbers consumed by *D. bellulus* could not be analysed as there was not an aphid only treatment. With one exception, there were no significant differences in the number of aphids or eggs consumed in the presence of the other prey type or when on separate plants (Figure 5.5). The exception was the number of aphids eaten by *C. transversalis* when eggs were present on the same plant. More aphids than heliothis eggs were consumed when both were present on the same plant ($t = 3.4041$, $p = 0.0047$, $df = 8$) (Figure 5.5).

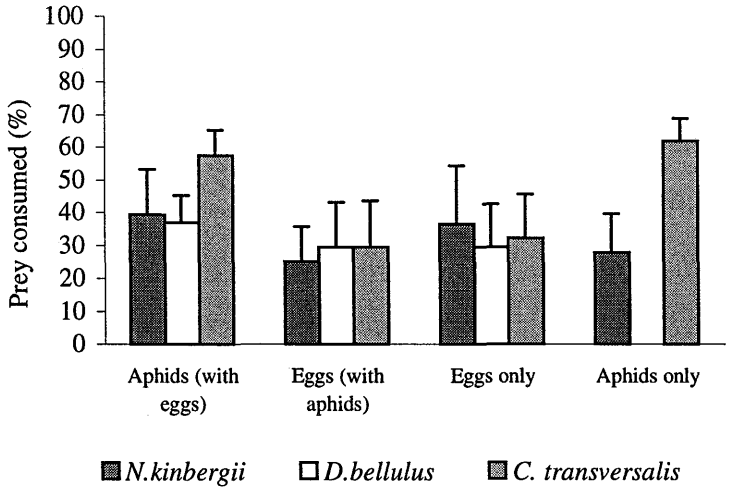

Figure 5.5 The percent of aphids and heliothis eggs consumed by three predator species. Bars indicate standard errors of the means.

Different densities of aphids

For each analysis the full model (treatment) was used. All predator species ate heliothis eggs despite different numbers of aphids being present (Figure 5.6). However, there was a significant difference in the number of eggs eaten in the presence of aphids for *D. bellulus*. Fewer eggs were eaten when 50 or 200+ aphids were on the plants than if no aphids were present (bf = 0.016, p = 0.003, df = 27) and (bf = 0.016, p = 0.0003, df = 27) respectively. This showed the presence of aphids at two densities affected consumption of heliothis eggs by *D. bellulus* (Figure 5.6).

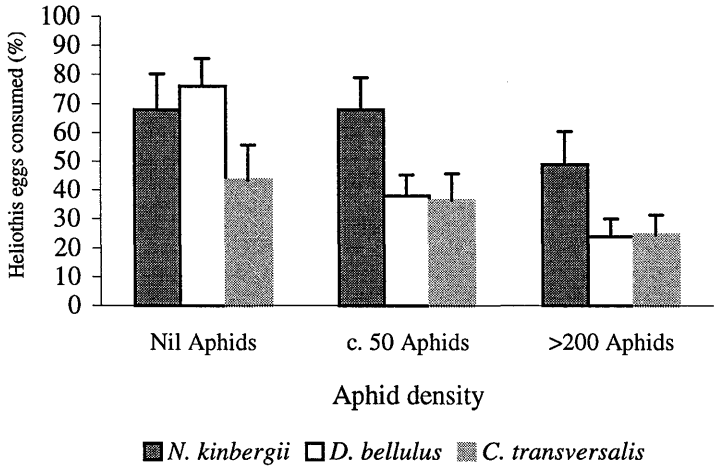

Figure 5.6 The percent of heliothis eggs consumed by three predator species when different densities of aphids were provided. Bars indicate standard errors of the means.

5.4 Discussion

Egg stage

The stage of heliothis egg does not appear to affect consumption by these predator species in Petri dishes. Unless there are effects from dissipating kairomones or reduction of moth scales through wind or rain it is likely these predators will consume both egg stages in the field. Stanley (1997) showed the predators found and consumed older heliothis eggs on field caged cotton.

Neonates versus eggs

The results from testing for preferences using neonates differed for each predator species. *Nabis kinbergii* obviously prefer heliothis neonates to eggs but consume eggs as well. If no neonates are present in the field these predators will probably increase the number of eggs they consume. As heliothis eggs start to hatch in the field, *N. kinbergii* are likely to prefer consuming the emerging neonates. Araya *et al.* (1997) showed nabids preferred small and medium larvae rather than eggs of the noctuid *Rachiplusia nu* (Guenee). Nabids may prefer neonates to eggs due to the motile behavior of the prey. Siddique and Chapman (1987a) suggested that, as *N. kinbergii* spent 30% of their time being inactive, the motility of prey was important for capture rates.

In the field, one needs to consider escape tactics of the prey on plants. Neonates can fall off quickly thus avoiding attack. Stanley (1997) did not detect additional mortality of young heliothis larvae due to predation by nabids compared with background mortality when using field cages. In this study the reason Petri dishes were used was because neonates were extremely difficult to recover from plants. Certainly the results here indicate nabids will both attack and consume neonates in preference to eggs. Other studies suggest predators (including nabids) will consume larvae in the field. Lingren *et al.* (1968) found adult female *N. alternatus* fed on 36.7 eggs and 45 first instar *H. zea* per day on cotton. Similarly, Van Den Bosch *et al.* (1969) showed that predators (including nabids) of *H. zea* reduced both eggs and larvae populations. Donahoe and Pitre (1977) found female *R. roseipennis* fed on average on 39 first instar *H. zea* per day but this varied with predator age. Young adults consumed almost double the average. Ridgeway and Jones (1969) showed *C. carnea* were better at controlling heliothis larvae than eggs in cotton.

Consumption of heliothis eggs and neonates by *D. bellulus* differed from *N. kinbergii*. The results agreed with those of Stanley (1997) who showed *D. bellulus* ate early instar heliothis. However, unlike *N. kinbergii*, when both prey were in one arena consumption of each prey type by *D. bellulus* changed with time indicating that this species may exhibit switching behavior. A similar decline in numbers of heliothis eaten when aphid numbers increased was also exhibited in the experiment on aphid density in this study. Ables (1978) showed that as numbers of one prey type declined, the assassin bug *Zelus renardii* Kolenati increased consumption of the other prey types available.

There were no significant effects of the presence of alternative prey on heliothis egg consumption for *C. transversalis*. In most cases coccinellids need to encounter their prey to perceive them (Hodek 1973). As each coccinellid in this study had a similar chance of encountering each prey type in the Petri dishes, the equal numbers of each prey type consumed may be explained by the coccinellids' need to encounter prey. Also in this type of arena (Petri dish) the differences in size and behaviour of the neonates compared to eggs did not significantly affect the consumption by coccinellids of either stage, although there was a trend towards greater consumption of neonates.

The numbers of larvae consumed in this study are likely to also be an overestimation of those consumed on plants. Awan (1990) found more *H. punctigera* larvae were consumed by predators in Petri dishes than on plants. In most cases in this study, the predators ate more neonates than eggs when offered both in the same dish. Therefore, the size of the prey at a density of 10 did not affect the number of prey consumed. As each predator species tested consumed both heliothis larvae and eggs their use as biological control agents warrants further investigation as both pest stages are found in the field.

5.4.1 Aphids as alternate prey

Differences occurred between predator species in the number of heliothis eggs consumed when aphids were present. When equal numbers of aphids and heliothis eggs were present on a cotton plant, *N. kinbergii*, *D. bellulus* and *C. transversalis* consumed the same numbers of eggs whether aphids were present or not. Therefore, aphids in low numbers are not likely to affect the number of heliothis eggs consumed in the field. However, *C. transversalis* appear to feed on more aphids than eggs when both prey types were on the same plant.

The species significantly affected by aphids at higher densities was *D. bellulus*. Stanley (1997) suggested this species might exhibit switching behaviour. However, neither study set out to measure switching behaviour. A possible explanation for the reduced number of heliothis eggs being consumed is that, as one prey is present in higher numbers there may be less chance of the other being found. Although the presence of aphids in high numbers affected the numbers of heliothis eggs consumed it did not stop consumption of heliothis eggs. Other studies show predators may be affected by alternative prey species. Ridgeway and Jones (1968) attributed differences in consumption of *H. zea* and *H. virescens* by *C. carnea*

larvae in part to the presence of *A. gossypii* in cotton. However, they did note that even when high numbers of alternative prey were present, eggs and larvae of heliothis were still consumed. They attributed this to the searching behaviour of *C. carnea* as they tended to search in areas where heliothis were found. In this study *D. bellulus* were similar to *C. carnea* in the Ridgeway and Jones (1968) study, as they were affected by the presence of aphids but still consumed heliothis eggs.

The reduction in numbers of target prey eaten may be a result of handling time of the other prey (Chesson 1989). However, as there was not a reduction in number of target prey eaten in this study when aphids were present in equal numbers it is unlikely that the handling time of the prey affected the predators finding heliothis eggs. Size of prey was found by Siddique and Chapman (1987b) not to matter when adult *N. kinbergii* were fed either pea aphids (*A. pisum*) or mirids (*Sidnia kinbergi* Stal).

Coccinella transversalis ate more aphids than eggs when equal numbers of prey were present. This may be due to a preference of prey but considering half as many eggs were eaten as aphids it is more likely that more aphids were consumed due to the searching behaviour of the coccinellids. Wratten (1976) showed that the coccinellid *A. bipunctata* needed to contact its prey before perceiving it. Unlike the Petri dish trial on neonates, it may be that due to the searching behaviour of the coccinellid in this experiment they encountered more aphids than eggs as the aphids tended to settle along the veins of the leaves. Coccinellids are known to search along the edge of leaves and raised veins (Dixon 1959). As aphids were not counted in the second experiment it is not known if more were eaten than the heliothis eggs. However, the data suggest a similar trend occurred in that heliothis were still consumed in spite of the presence of aphids. At the densities of prey provided in this study, *C. transversalis* obviously eats suitable prey it encounters but encounter of prey is probably influenced by the searching behaviour of the predator. Therefore, this predator species appears to be opportunistic.

Opportunistic predators are considered more effective against target prey. Collops beetles (*Collops vittatus* (Say) and *C. bipunctatus* (Say)) consumed more target prey when alternative prey were present (Quayogode and Davis 1981). They also found that although predators preferred aphids they still ate considerable amounts of the target prey. Tamaki and Weeks (1972) showed that while geocorids and nabids were less effective in controlling aphids when other prey were present coccinellids were just as good at controlling both prey.

Coccinellids may prefer and eat more aphids than heliothis eggs as a result of their searching behaviour. However, given they are opportunistic feeders they appear to eat heliothis in spite of the presence of aphids at different densities. This indicates they do not switch to aphids. Murdoch and Marks (1973) suggest that although *C. septempunctata* showed a slight preference for particular aphid species they were most likely random (opportunistic) feeders consuming whatever prey they encountered. Stanley (1997) showed *C. transversalis* contributed to mortality of aphids in the field but also fed on heliothis larvae. Although there appears to be a relationship between coccinellid numbers and aphid abundance in Australia cotton (Stanley 1997, Bishop and Blood 1978, Room and Wardhaugh 1977) the effect of coccinellids on consumption of heliothis eggs with prey other than aphids present needs to be investigated. Also, Hassell and Southwood (1978) suggest that alternative prey in laboratory experiments does not limit coccinellids but that their preference in the field for patch type does produce preferences in which prey is consumed. This needs to be investigated for *C. transversalis*.

Nabis kinbergii feed on both aphids and heliothis eggs. In all cases the number of heliothis consumed was not affected by the presence of aphids. Stanley (1997) found *N. kinbergii* did not contribute to aphid mortality in the field. This may have been because he used heliothis larvae as the target prey. As this study showed *N. kinbergii* preferred heliothis larvae to eggs. They may also prefer heliothis larvae to aphids. Nordlund and Morrison (1990) showed *C. rufilabris* took less time to find cotton aphids than *H. virescens* eggs, but not *H. virescens* larvae. *N. kinbergii* did not take different times to encounter eggs or neonates so numbers consumed were not affected by searching time. Including the preferred prey (*Frankliniella occidentalis* (Pergande) by *Orius tristicolor* (Say) did not reduce predation on other prey present (Cloutier and Johnson 1993). Samson and Blood (1979) reared *N. kinbergii* on *H. punctigera larvae*. Siddique and Chapman (1987a) reared *N. kinbergii* on pea aphids (*A. pisum*). In both cases the development rate of *N. kinbergii* was similar. Therefore, the nutritional need for particular types of prey may not be important to *N. kinbergii*. Conversely, studies using serological techniques found *N. alternatus* did not often feed on whitefly (*B. tabaci*) or pink bollworm eggs (*P. gossypiella*) (Hagler and Naranjo 1994a). They suggest the nabids may have been preferentially feeding on other insect species or life stages. Whilst there may not be differences between consumption of aphids and heliothis larvae (Stanley 1997) or aphids and heliothis eggs (this study) the results indicate nabids prefer larvae to eggs (this

study). As aphid abundance did not affect the number of eggs consumed by *N. kinbergii* but did in *D. bellulus*, one of these species must be exhibiting a preference for either aphids or eggs. Further study would be required to identify which of the two predator species has a preference for heliothis.

It is not unusual for there to be differences in prey preferences between predator species. Ables *et al.* (1978) showed that the consumption *of H. virescens* eggs was reduced when *A. gossypii* was present on cotton except for the hemipteran *G. punctipes*. *Geocoris punctipes* consumed more heliothis eggs in the presence of *A. gossypii* at low densities than when aphids were absent. In their study coccinellid adults (*Hippodamia convergens* (Guerin-Meneville)) were only slightly affected by the presence of aphids at different densities. Larvae of *H. convergens* were most affected when aphid densities were 90 per plant. They suggest that the presence of low to medium aphid populations in the field may aid predation on heliothis eggs.

5.4.2 Conclusion

The results of this study suggest that when low numbers of heliothis occur, having alternative prey present (also in low numbers) may be an advantage as predators are attracted to the prey and (whether accidentally or not) eat heliothis as a consequence. Given both heliothis neonates and aphids are pests in cotton and all three predator species ate them, conservation of these predators is warranted.

Each species appears to respond to the presence of alternative prey differently. This has important implications for the efficacy of each species on heliothis eggs. Even though they are a good predator on heliothis eggs, *N. kinbergii* appear to prefer small heliothis larvae and their use as biological control agents on these should be further investigated. Nabids are also likely to feed on other stages of larvae (Sloderbeck and Yeargan 1983). The fact that they consume neonates is an advantage to management particularly as the current economic threshold is now two small larvae per metre as it allows them to have an impact for a longer time frame. Nabids also eat eggs when aphids are present. This is also an advantage to their use in management of cotton pests. However, their effectiveness needs to be investigated with other prey types that are available in the early cotton growing season.

Although they do not appear to prefer a prey type, consumption of heliothis eggs by *D. bellulus* appears to be influenced by aphid abundance. This requires further investigation but current indications are, that in the presence of aphids, *D. bellulus* should not be considered as efficient a predator on heliothis eggs as *N. kinbergii* or *C. transversalis*. The results also indicate that *C. transversalis* eat more aphids than eggs but interestingly this does not appear to hamper their feeding on heliothis eggs. In all other studies in this chapter they don't appear to prefer a one prey type to another.

These experiments provide an indication that predators are affected by the type of prey available and, in some cases fewer heliothis are consumed when alternative prey are provided. The next obvious step is to test the effect of alternative prey type on the functional response of the predator at similar heliothis prey densities to those in the previous chapter. Unfortunately time did not permit this. As laboratory trials tend to produce higher consumption rates than field trials, and other prey occur in the field, the effects of alternative prey on the consumption of heliothis in the field also need to be verified. The next chapter (chapter 6) looks at ways to do this directly without the use of field cages.

Chapter 6

6. The potential for using a polyclonal antibody to detect heliothis in the guts of predators.

6.1 Introduction

Serological methods can be used to detect target prey antigen in the guts of predators thereby quantifying predation in the field (Greenstone 1996, Sunderland 1988). They work by raising antibodies against a particular prey type (the target prey) and using the antibodies in a biochemical assay to test whether any individuals of a predator population have fed on the target prey. Given the problems of artificial conditions associated with laboratory, glasshouse and field cage trials, serological methods are certainly worth investigating. They allow direct sampling from the field without disturbance to the micro-environment of the insects. Another advantage is they provide a means of rapidly screening an array of predators as potential biological control agents without lengthy cage trials or field observations having to be done on each species. Useful information can also be gathered on the effects of seasonal change, daily change within a season and even changes with time of day, on the predation of a given species. In some cases it may also provide comparisons between gender and life stage feeding habits of the predator species.

6.1.1 A brief history on the development of serological assays

Serological assay has been used for over 50 years to evaluate the frequency of predation on invertebrate pests by predators through gut content analysis. Insect antigens cause an immunological response in vertebrates which produces antibodies. Over recent years there has been great advancement in the type of antibodies produced. Production of antibodies has progressed from early insensitive and non-specific polyclonal antibodies to currently producing species and even stage-specific monoclonal antibodies (Greenstone and Morgan 1989, Hagler and Naranjo 1994a, Hagler *et al.* 1992). The development of monoclonal antibodies has improved the accuracy of assessments on predation. Similarly there has been progression in the type of serological assay methods available. Dempster (1960), Boreham and Ohiagu (1978), Sunderland (1988) and Greenstone (1996) all provide comprehensive reviews

on the type and use of serological methods used in predation studies. The following highlight some of the main developments in serological assay methods.

The first serological methods to be used were precipitation tube tests (Dempster 1960). These were based on mixing antigen with a fixed amount of antibody in a tube and allowing a precipitate to form. The precipitate could then be quantified providing a means of measuring antigen quantity. The method could be carried out in liquid or gel form. This method had problems in that it was slow, insensitive (causing low detectability), and the antibodies used were generally not specific enough, thus causing reactions to non-target prey. Variations on this test were the capillary ring which uses a capillary tube in which a ring of precipitate forms (Nakamura and Nakamura 1977). This test is more sensitive but very time consuming (Greenstone 1977). Another form of the precipitation test is single radial immunodiffusion (SRI) (Hagler and Cohen 1990, McIver 1981). SRI consists of precipitation reactions between an antibody and an antigen using a medium made from agarose. This technique was considered reliable and inexpensive by Hagler and Cohen (1990). The Ouchterlony (Stuart and Greenstone 1990, Lund and Turpin 1977) and immunoelectroosmorphoresis methods both utilise gels. The Ouchterlony test uses holes into which antigen and antibody are placed separately. These spread towards each other forming a line of precipitate. These tests still have problems of low sensitivity, non-specificity and are time consuming. Immunoelectroosmohoresis utilises electrophoresis to speed up the precipitation process. There are many variations of this methodology. These along with their application are described in detail in Sunderland (1988). Another test used in predation studies is passive haemagglutination. Ohiagu and Boreham (1978) modified a latex agglutination test in order to provide an assay that could be used in field situations. This was comparable to the precipitin test but still had similar limitations due to quantification and sensitivity. There are variations of agglutination tests. These are described in Sunderland (1988).

Electrophoresis in predation studies

Electrophoresis works by separating prey proteins in support matricies such as paper, cellulose acetate, starch gel, agarose or polyacrylamide gel using charged molecules in response to an electric field. Bands of a chosen enzyme such as esterase are then revealed using a specific stain. It is a simple rapid and highly sensitive tool for determining if a target prey has been consumed by a predator (Murray *et al* 1989). However, there is potential for confusion from predator esterases (Sunderland 1988). Therefore, the method is often used on predators guts only. This can be a problem with very small predators where gut dissection is difficult. Interpretation of bands may also be confused if there are esterases common to both the target

prey , alternative prey and parasites within the prey (Sunderland 1988). The system is best used on predator/prey systems that are relatively simple.

Since the precipitation methods the indirect enzyme-linked immunosorbent assay (ELISA), often used in clinical immunology, has been adapted to study predator prey systems. These tests are based on prey antigen being detected by labelled antibodies. Using labelled antibodies is an advantage as they amplify the results providing a very sensitive test. ELISA is now the most commonly used assay type for predation studies (Stuart and Greenstone 1990, Greenstone 1996). Several types of ELISA have been developed. These may be either direct, where antigen is bound to a solid phase and reacted to an enzyme labelled antibody, indirect, in which the antigen is bound to the solid phase and a specific antibody is used, and then a labelled antibody attached, or double sandwich, where the antibody is attached to the solid phase then the antigen attached followed by an enzyme labelled antibody. The double sandwich is of advantage as it reduces binding to non-specific proteins. Greenstone (1996) provides an thorough review on the different ELISA techniques available.

One problem with ELISA is that the negative controls (predators starved of target prey) still show some colour therefore plates must be read (usually by an absorbence reading) in order to differentiate between positives (fed predators) and negatives (starved predators). The plate readers have a high cost making ELISA inaccessible to many researchers. More recently, dot blots have become a common method in serological studies. These were developed by Stuart and Greenstone (1990) and have great potential as they do not require a plate reader. The original dot blot used a Bio Dot microfiltration apparatus. Since then the assay method has been advanced to require no more than a membrane in a Petri dish (Greenstone and Trowell 1994). Stuart and Greenstone (1990) compared immunodot assay with ELISA and found immunodot assay to be as sensitive and specific as ELISA. Hagler (1998) compared the efficiency and accuracy of these methods on whole body coccinellid *Hippodamia convergens* Guerin-Meneville that had been fed *P. gossypiella* eggs. Dot blot, along with sandwich ELISA showed the highest frequency of positive reactions therefore, is more accurate. The dot blots provided the most accurate method overall. The dot blot is more sensitive due to greater number of binding sites on the membrane compared with a microplate (Hagler 1998). Western blots were also considered to show promise as a technique for analysis method (Hagler 1998).

Quantitative measurement

The quantification of predation by serological assay has been and remains a limitation to its use. Lack of quantification is largely due to abiotic factors such as temperature and humidity

(Hagler *et al.* 1992), digestion rates and starvation (Lovei *et al.* 1985) variable meal size and variation in accuracy of assays as a result of predator size (Hagler *et al.* 1997, McIver 1981). However, using some assumptions on predation behaviour there have been attempts to quantify results of serological studies (Greenstone 1996, Sunderland 1988).

For example, to estimate predation rate (k) Dempster (1967) used the model k=(pP)/D where p = the proportion of predators that score positive, P = predator density and D = the detection time. This model assumes that the predators only eat one prey within the detection time. Sigsgaard (1996) used the Poisson model of predation to estimate the percentage of eggs consumed by predators of *H. armigera* in pigeon pea in South India. This model is considered appropriate when predation is based on random encounter rather than deliberate feeding (influenced by prior feeding or searching behaviour). The mean number of items per gut is given by -ln (1-p) where p is the proportion of positives in the ELISA. The number of eggs consumed by a particular species is given by P[- ln (1-p)]/D where P is the predator density and D is the detection period after ingestion of prey. This is divided by the field egg abundance to provide the proportion of eggs predated. Sigsgaard (1996) then divided by the number of days it takes eggs to hatch in order to provide an estimates of predation over one night. Greenstone (1979) used the same model to estimate the number of prey species in the diet of spiders. This model assumes that prior feeding and feeding behaviour do not change predation by encounter. Ragsdale *et al.* (1981) quantified predator efficiency by using the predator species density and the number of positives obtained from ELISA. These were used in the following equation PE = (D)x(E)/sigma (DE))x100 where D is predator density and E is the number of positives. The model does not allow for the effects of the number of prey consumed, digestion time and pools results for the whole season. Whilst these models are useful there are many assumptions that need further testing. At the moment they do not offer sufficient information to be able to apply them to management decisions.

Studies on heliothis

Lesiewicz *et al.* (1982) studied predation by carabids on *H. zea*. Greenstone and Morgan (1989) used a MAb specific to *H. zea* fifth instars and studied their predation by *P. maculiventris*. Stuart and Greenstone (1990) used a MAb developed by Lenz and Greenstone (1988) to study predation of *H. zea* by *Phidippus audaz* (Hentz) and *P. maculiventris*, Greenstone and Trowell (1994) studied predation on *H. virescens* and *H. zea* by *P. maculiventris*. Greenstone (1995) looked a method to distinguish between *H. zea* and *H. virescens*. Trowell *et al.* (1994) used monoclonal antibodies to develop the Lep Ton™ kit to test for presence of *H. armigera* in the field. Sigsgaard (1996) used the MAb developed by Greenstone and Trowell (1994) to study predation of *H. armigera* eggs on pigeon pea in South India.

6.1.2 Using polyclonal antibodies

There are advantages in using polyclonal antibodies as they are highly sensitive and reasonably inexpensive to produce (Symondson and Liddell 1996). They do, however, have some limitations in that they are not as specific as monoclonal antibodies (MAbs) because of the way they are produced. Put simply, antigen is extracted from the required prey species. This antigen has a unique characteristic which will differentiate it from similar antigens. The antigen is injected into a vertebrate host (for example a rabbit or mouse) which is given several booster injections of target antigen over time until the vertebrate has produced antibodies to the antigen. The host is then bled and the blood allowed to clot. The serum is removed at which stage it may be stored until required. The resulting antibodies have combining sites that are complementary to the target antigen. As the antigen usually has two or more determinants (epitopes) antibodies are produced to all determinants. As these are naturally mixed in the serum they may also combine with another species of prey antigen that has one or more of these determinants. This is known as cross reactivity and is what makes polyclonal antibodies non species specific. When using a polyclonal antibody it is therefore important to check specificity with any other prey available to the predators in the field. Monoclonal antibodies are produced in a different way. Their production is outlined in Chapter 7. The production of antibodies is clearly described by Greenstone (1996).

6.1.3 Purpose of this study

This study looks at the potential of two different types of antibodies for use in detecting heliothis in the guts of predators commonly found in Australian cotton. If the antibodies have potential this work will provide a serological method that can show a distinction between predators that have fed on the target prey *H. armigera* and *H. punctigera* (positives) and individuals that have not fed on heliothis (negatives). The first method tested a polyclonal antibody. Its potential is discussed in this chapter. The second method investigated the use of a monoclonal antibody and is discussed in Chapter 7.

The two antibodies (one monoclonal and one polyclonal) that were available for testing were produced and supplied by Dr. S. Trowell CSIRO Entomology, Australia. Their use was authorised through an agreement with Abbott Australasia Pty. Ltd., P.O. Box 304 North Ryde NSW 2113. Both antibodies had been developed as part of research into the establishment of the Lep Ton™ kit (Trowell *et al.* 1994). The polyclonal antibody was tested first as it was likely to be more sensitive to prey antigen and, not being species specific, it was likely it would detect both *H. armigera* and *H. punctigera* antigen (but not be able to distinguish between the two), there-by allowing total predation on both species to be assessed. Provided both antibodies worked the homogenates of each predator would be tested twice: first with the

polyclonal antibody on *H. punctigera* and *H. armigera* and second with a species specific MAb to check how many positive reactions were to *H. armigera.*

Apart from some preliminary trials by Stanley (1997) that tested the same polyclonal antibody as a means of detecting *Helicoverpa* spp. consumed by *D. bellulus,* this antibody had not been used to detect prey in the guts of predators prior to this study. An assay method therefore needed to be established and tested against each predator species and prey type.

6.2 Methods

The type of assay selected for testing the polycolonal antibody was a simplified immunodot assay first established by Greenstone and Trowell (1994). Immunodot or dot blot assays utilise nitro-cellulose membrane as a solid phase template. Figure 6.1 shows the steps involved in an immunodot assay. Put simply, a microlitre (1 µl) of supernatant is dotted onto the membrane. The membrane is blocked to eliminate non-specific binding sites; the primary antibody is then bound to the target antigen by incubation. Next a biotinylated antibody is bound to the primary antibody, a streptavidin peroxidase bound to the biotinylated antibody (the biotin/avidin link), and finally a colour detection substrate added. Excess of each reagent is washed out between each step to avoid non-specific binding. If there is prey antigen present the final result will be a pink dot. Immunodot assays allow detection of antigen to be visually assessed. Details of the reagents and process used are given in Appendix 4a.

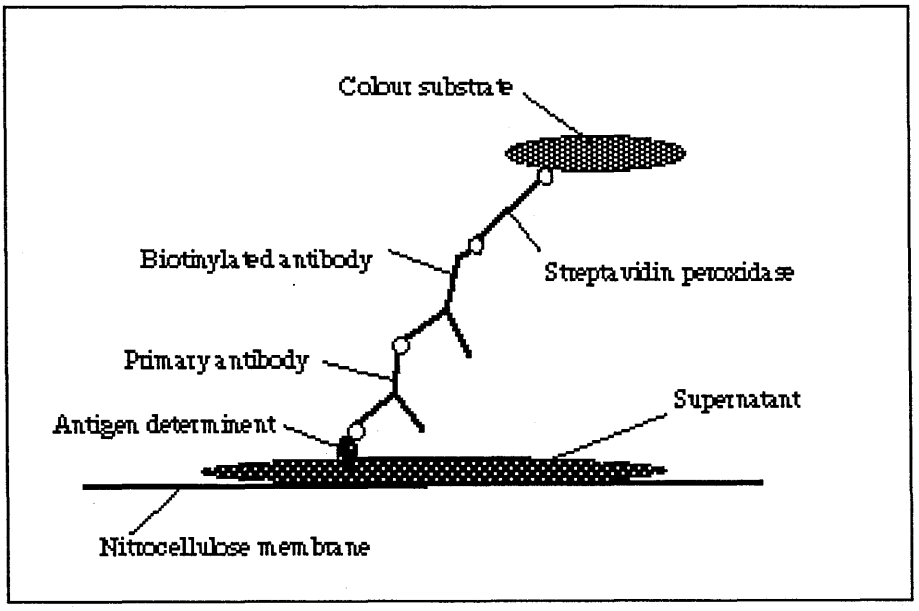

Figure 6.1. The steps involved in an immunodot assay. **O** represent binding sites.

This immunodot method was chosen for its accuracy of results and as a cheaper alternative that the ELISA plate reader which was unavailable. It also has potential to be used *in situ* in a

field laboratory in a similar manner to the Lep Ton kit and, as discussed above, dot blots can be more accurate and sensitive than ELISA methods (Hagler *et al.* 1997). Stuart and Greenstone (1990) showed dot blots to be up to eight times more sensitive than ELISA. Dot blots do have limitations in that they are time consuming which limits the number of predators that can be processed. Further limitations and advantages are discussed below (section 6.4). The following section outlines the methods used to run immunodot assays and the components used to test the potential of the polyclonal antibody.

Membranes

Nitro-cellulose membrane (Hybond C super, Amersham) were cut to 6 x 7 cm rectangles. For each assay the membranes to be used were placed into individual Petri dishes (8.5 cm in diameter). The membrane remained in the dish for all steps of the assay. Each membrane held a maximum of 5 rows of 6 columns of 1 µl dots and included positive controls of heliothis eggs (*H. armigera* or *H. punctigera*), and negative controls of homogenising solution [phosphate-buffered saline with 20% Tween and 5% Bovine Serum Albimum (PBST + BSA)]. A typical membrane layout is shown in Figure 6.2. In some trials a further negative control of predators starved of heliothis prey was included. The controls used for each trial are detailed in each experiment. When preparing a membrane at least two replicates were made for each trial in case the test was needed for future assay.

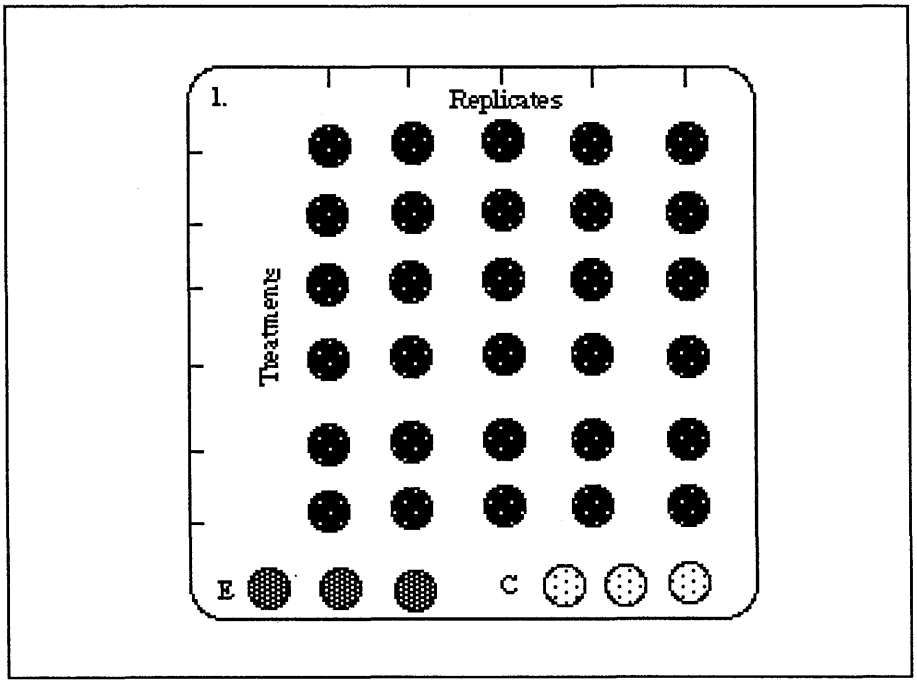

Figure 6.2 A schematic diagram of a typical membrane showing treatments for each row and replicates in each column. At the base of the membrane are replicates of positive (E) and negative (C) controls.

Feeding trials

As the methods for feeding predators have been outlined in detail in chapter 2 only a brief summary is given here. In general, each test used in the establishment of an immunodot assay method requires the following:

- the target prey (in this case eggs of *H. armigera* or *H. punctigera*),
- starvation of predators so that no ingested target prey can cause false positives (a positive reaction where there should not be one),
- feeding one target prey to each predator and freezing the predator immediately (Stuart and Greenstone 1990) and finally,
- feeding one target prey to each predator and allowing the predator to digest for pre-determined times immediately after ingestion of the prey (Sopp and Sunderland 1989).

The prepared prey and predators were stored in liquid nitrogen until required for assays.

Factors such as temperature are important considerations in feeding trials as they influence digestion time (Hagler and Naranjo 1997, Hagler and Cohen 1990, Sopp and Sunderland 1989). The temperature used for feeding trials was 27 °C as this reflected average daily temperatures found in cotton fields in Narrabri during the cotton growing season (ACRI meteorological data) and was likely to give slightly higher digestion rates than normally expected therefore providing a conservative estimate of digestion times in field collected material. It is also important to test different species of predators as there can be variation in the efficacy of assays among different predator species (Hagler and Naranjo 1997, Sopp and Sunderland 1989). Consequently as many predator species as possible were collected and used in feeding trials. In general tests, the species most commonly used were *N. kinbergii*, *D. bellulus* and *C. transversalis.*

The following sections outline the methods used to establish a final protocol and optimise it on both heliothis eggs and predators. Digestion times are addressed in chapter 7. For ease of explanation negatives are predators that have been starved of heliothis prey but still allowed to feed on non-target prey such as aphids or pollen. These are referred to as **unfed.** Positives are predators that have been fed one heliothis egg. These are referred to as **fed.** Controls are referred to as **negative** for PBST+BSA only and **positive** for heliothis eggs. All assays contained controls of negatives and positives. In some tests unfeds are also included as controls.

6.2.1 Assay method establishment

The original immunodot assay protocol used was established by Greenstone and Trowell (1994) and is outlined in Appendix 4a. As there are differences in sensitivity of antibodies and variation in laboratory reagents and the size of membranes it is important to check that the protocol works effectively in each new situation. This was done and slight modifications were made to obtain optimal signal to noise ratios, which provided nil to minimal reaction to negatives and unfeds and strong clear reactions to positives and feds without enhancing background absorption of immunological reagents. Supply details of specific reagents and materials are provided in Appendix 4b.

Change of reagents

The first step in the assay protocol is designed to block out all non-specific binding sites on the membrane by adding extraneous proteins. If this is not done background noise may occur. Background noise is a colouring of the membrane due to absorption of immunological reagents. The colouring is similar to the colouring of positives (in this case pink). This makes interpretation of results difficult. Background noise is usually caused by binding of reagents to non-specific proteins (endogenous peroxidase) on the membranes through poor blocking or washing. The blocking step includes phenolhydrazine and hydrogen peroxide (H_2O_2) with skim milk powder to inhibit endogenous peroxidases. The exclusion of phenolhydrazine and H_2O_2 was tested to see if they helped decrease background noise. The inclusion of both can contribute to loss of sensitivity (Greenstone and Trowell 1994, Villanueva and Malek-Hedayat 1987) so it was desirable to exclude them.

Two membranes were used. The two assay treatments consisted of a membrane assayed with phenolhydrazine (0.1%) and H_2O_2 (0.04%) and secondly a membrane assayed without including phenolhydrazine (0.1%) and H_2O_2 (0.04%). Each membrane contained three *H. armigera* and *H. punctigera* eggs squashed directly on to the membrane, five *H. armigera* and *H. punctigera* eggs individually macerated in 150 µl of homogenising solution (PBST + BSA), one fed *D. bellulus* and one unfed *D. bellulus*. The latter two were dotted onto the membrane five times. The preparation of specimens and assay followed the protocol outlined in Appendix 4a. Controls included three 1 µl dot negatives.

As well as checking the effects of phenolhydrazine and H_2O_2 the blocking step (dry skim milk powder in PBS - BLOTTO) was tested to make sure it was effective. At the same time a test was also done leaving out each step of the assay out. This would ascertain whether binding was occurring somewhere else in the assay and whether the blocking step was effective. Six

membranes were used, each with negative and positive controls and six fed and unfed *N. kinbergii*. Each membrane was assayed in the same way except that a different step was left out when assaying each one.

Washes

Background noise can also occur from poor washing of reagents between incubations. Two washes were compared in order to reduce background noise on the membrane. This trial was done twice in order to check that the rinsing method was effective. In the first test each membrane contained five replicates of 1, 2, 3, 4, 5, and 10 eggs homogenised together and included a negative control and a fresh egg squashed onto the membrane (a positive). The second test included 1, 2, 3 and 4 day old eggs with the same controls as above. Both tests formed part of another experiment discussed below (6.2.2). 50 mM Tris(hydroxymethyl)-aminomethane hydrochloride pH 8.0 (Tris) Fluka and distilled water were compared as washes on individual membranes. Tris was used in the following sequence. Tris 2 minutes, 2 M NaCl for 2 minutes and Tris 2 minutes. In the last step a final rinse was done with distilled H_20 to remove any traces of Tris prior to the substrate being added.

Biotin/avidin steps

Non-specific binding can also occur to proteins within the samples themselves. This can cause false positives (i.e. a reaction to something other than the prey antigen). It was suggested by Stanley (1997) that non-specific binding was occurring to biotins inherent in *D. bellulus*. The secondary antibody detects the binding of the primary antibody so any binding of it to inherent biotins would give a false positive. In order to test whether such binding was occurring with the predators used here, the biotin/avidin steps were substituted with a peroxidase-linked whole antibody. This means the biotin/avidin steps are conjugated thus avoiding non-specific binding. The use of a conjugated secondary antibody was tested with both the polyclonal and monoclonal primary antibodies. Each membrane contained 12 individuals of *N. kinbergii, D. bellulus*, and *C. transversalis*. Six of each were fed and six were unfed. Three of each fed and unfed pre-frozen predators were macerated and three fed and unfed predators were macerated live. The membrane also contained three positive and three negative controls. Individual membranes were tested using the original protocol and the adjusted protocol of the whole linked antibody at a ratio of 1:1000. This was done with the MAb as well. Results of the MAb test are discussed in chapter 7. Results of the polyclonal antibody tests are discussed below in section 6.3.

Storage of membranes

As the protocol was being established replicate membranes for all trials were made for future testing with different reagents and the same tests also needed to be repeated with the MAb. As it was not known how long these replicates would be required, the time that pre-prepared membranes could be stored at 4 °C was tested. Replicates of tests done at 1, 6, 9 and 14 months old were available. Each had been kept in small unsealed plastic bags at 4 °C in darkness. The bags were not sealed to avoid condensation occurring. All the membranes contained *H. armigera* eggs, fed and unfed insects and negative controls. The results were then scanned and compared with results from the original tests which had been stored as Tagged Image Format (.tif) files using Abode Photoshop Version 3 and a UMAX Vista-S8 flatbed scanner (UMAX Data Systems Inc., Hsinchu, Taiwan).

6.2.2 Eggs

Once the protocol was established the method was tested on treatments containing heliothis eggs. As using the polyclonal antibody was required to detect both *H. armigera* and *H. punctigera*, eggs of the two species were tested for any differences. As ultimately any insect specimens collected from the field would be frozen, it was also important to check that freezing and thawing did not alter results. Five fresh and frozen *H. armigera* and *H. punctigera* eggs were individually macerated in 150 µl PBST-BSA, spun and 1 µl of supernatant added to the membranes. A fresh *H. armigera* and *H. punctigera* egg was included and squashed directly on to the membranes. Three pre-prepared homogenates of each species were frozen to see if storage of specimens could be optimised. Time could be saved if homogenates such as controls could be stored and reused. These were thawed and 1 µl dotted onto the membrane. Three previously frozen eggs of both heliothis species from the ACRI culture were also included to check other sources of eggs provided the same results. Four replicate membranes were set up. These were tested using different ratios of primary and secondary antibody to test that the amount of reagent used did not affect results. The ratios used were 1:1000 and 1:5000 for the primary antibody and 1:300, 1:1000 for the secondary (whole linked) antibody. Tests that vary the ratios of antibodies were also done in more detail in section 6.2.4.

Since the number of prey consumed can also affect the strength of the positive reaction, 1, 2, 3, 4, 5, and 10 eggs were homogenised together to see if a different intensity in colour reaction occurred. Each combination was replicated five times. A negative control and a frozen egg squashed onto the membrane were also included. Likewise the age of eggs can change delectability. Eggs from 1, 2, 3 and 4 days of age were tested. This covered white, brown and

black stages of eggs. There were five replicates of each age. A negative control was also included.

6.2.3 Predators

The next step was to test the method on predators. As Stanley (1997) had suggested freezing predators was producing false positives, a test was done to make sure freezing did not alter results as, like the eggs, this was the storage method used for predators. Three species were tested. Six unfed *N. kinbergii, D. bellulus and C. transversalis* were used. Three of each were frozen in liquid nitrogen and three were macerated live. The membrane also contained three negative and positive controls. The ratios of primary and secondary antibody used were 1:1000 and 1:300 respectively.

The method was then trialed on predators that had been fed heliothis prey (fed) and ones that had been starved of prey (unfed). In most cases the predators were observed to consume or suck out the whole egg. Even when disturbed they tended to return to the same egg and resume feeding. On this basis one egg was used as a minimum detection quantity needed to assess the predators. Predators were tested on a basis of availability rather than in particular order. This maximised the number of species that could be tested. The species used were chosen largely on their abundance and because they represented both chewing and sucking predators. They were small, medium and large in size, and one was a soil dweller, one a aphidophagous feeder and one an ambush predator. This gave a good cross section of types of predators for comparison. Predators were kept and treated as outlined in chapter 2 - feeding trials.

The species tested were *D. bellulus*, *C. transversalis*, *H. octomaculata*, *N. kinbergii* and *D. signatus*. Tests were done as each species became available. There were 5, 10, 10, 5 and 8 replicates of both fed and unfed species respectively, and each trial contained positive and negative controls. Several of these tests were repeated with new individuals to test that results were consistent. These tests contained a mixture of males and females. Another test was done to check that gender was not causing variation in results. Five unfed *D. bellulus* and *C. transversalis* males and females were prepared. Controls of negatives and positives were included.

6.2.4 Optimisation of reagents for both eggs and predators

In several tests the signals from unfed predators in the assay results were appearing as strong as the fed predators. Optimal quantities of each reagent were therefore tested in the form of checkerboard assays. This may reduce the signal to noise ratio causing a greater difference in

colour intensity between fed and unfed predators. A checkerboard assay consists of prey antigen (eggs or fed predators) macerated in different amounts of PBST-BSA and then assayed at different ratio combinations for each primary and secondary antibody. This was done with the polyclonal antibody on heliothis eggs as one needs to be careful not to lose detection of the prey, and on fed and unfed predators available at the time.

Eggs

Two egg checkerboards were tested first. Five frozen eggs were individually homogenised in 200, 400, 600, 800 and 1200 µl of homogenising solution. These were assayed using the primary antibody at 1:1000 and 1:5000 and the secondary antibody at 1:300.

Predators

Checkerboard assays that used the polyclonal antibody included *D. bellulus*, *C. transversalis*, *N. kinbergii* and *H. octomaculata.* All membranes were replicated and each dot on a membrane represented one individual. There were 10, 5, 5 and 5 replicates of predators used in each dilution on the membranes and each contained a row of fed and unfed. The *D. bellulus* trial contained two membranes of five individuals for each treatment. The rest consisted of one membrane with five individuals for each treatment. An example of a membrane is shown in Figure 6.3 below. All membranes contained negative and positive controls.

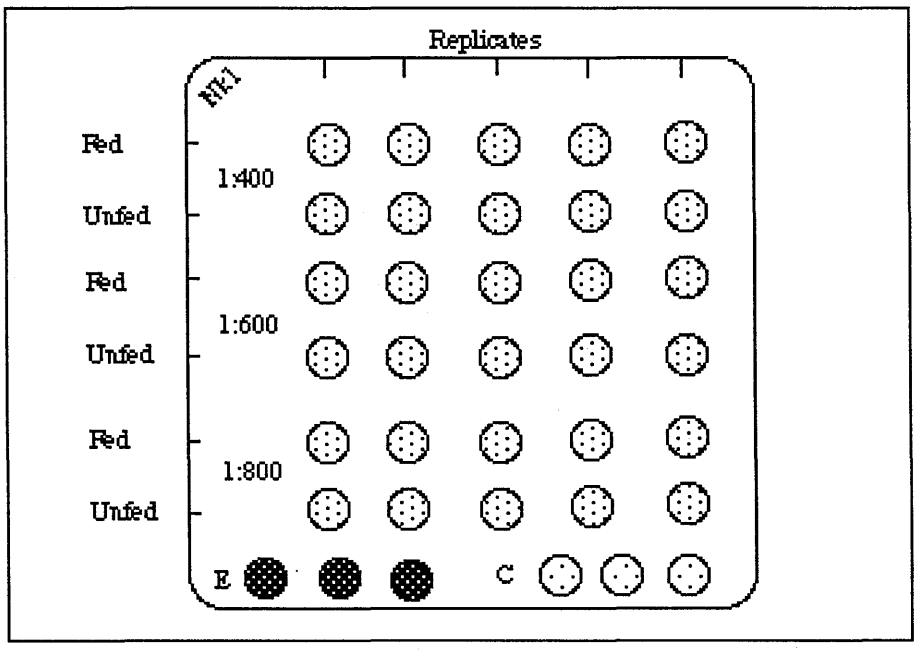

Figure 6.3 A schematic diagram of the layout of treatments and replicates on a membrane used in checkerboard assays. E = eggs and C = controls of unfed predators.

The ratios tested included individuals homogenised at 200, 400, 600 or 800 µl of PBST + BSA and assayed with 1:1000 and 1:5000 ratios of primary antibody and 1:300 of secondary antibody. The *N. kinbergii* trial also included 1:10000 for the primary antibody.

6.2.5 Cross reactivity

Alternative prey

Given that the polyclonal antibody is not likely to be species specific other prey commonly found in cotton needed to be tested in order to make sure no positives occurred from other sources. This is known as cross reactivity. Aphids (*A. gossypii*), thrips [*Frankliniella schultzei* (Trybom)], mites (*Tetranychus urticae* Koch), jassids (*A. viridgrisea*) and cotton pollen were the main source of alternative food found in cotton on sampling field trips. These prey were collected from the field by hand and frozen immediately in liquid nitrogen. Predator eggs may also be considered alternative prey therefore, *C. transversalis* eggs were tested as well. Five individuals of each prey type were prepared and negative and positive controls included. Another test included small medium and large heliothis larvae. These were replicated three times.

Predators

As the distinction between fed and unfed predators did not show up consistently, the predator body parts and haemolymph were tested. Haemolymph of three species (*N. kinbergii*, *D. bellulus* and *C. transversalis*) was extracted by piercing a hole with a syringe into the insect's abdomen, placing the individual into a small eppendorf tube (0.5 ml) that contained a glasswool plug and hole at the base, into another 1 ml tube containing 10 µl PBST + BSA and 0.005 grams of phenylthiocarbamide (PTU) to stop melanisation (blackening) of the haemolymph. The tube was spun at 14000g for three minutes to extract the haemolymph from the insect body and filter out any exoskeleton. A one microlitre dot was placed onto the membrane. On the same row in the next four replicates 200 µl of PBST + BSA was used instead of 10 µl. Another five replicates of haemolymph diluted in 600 µl was also included. In the larger coccinellids haemolymph (1 µl) was also extracted by using a 1 -10 µl capillary tube and then dotting straight onto the membrane.

Individual body parts of the coccinellid *C. transversalis* were also tested by sectioning with a scalpel and homogenising in PBST + BSA as per normal methods. The body parts included legs, head and prothorax, gut, and the rest of the body homogenised in 200 µl of PBST+BSA.

6. 3 Results

The results of each trial are given in the following sections. In all cases the positive and negative controls were used as a guide to show how well the assay worked. The large number of tests done in each section does not allow for full presentation of all the membranes using figures (colour scans) so results are only presented where they highlight important findings or provide examples of typical outcomes.

Changes of reagents

Inclusion of phenolhydrazine and hydrogen peroxide (H_2O_2) did not contribute to blocking background noise and, whilst their exclusion did not increase the sensitivity of the test, they were omitted from future assays. There was no discernible difference between *H. armigera* and *H. punctigera* or fed and unfed predators on the membrane and background noise remained a problem. Controls on the membranes were as expected, the negative showing no colouring and the eggs a clear pink dot. This showed the detection method was working.

The exclusion of the blocking step from the assay gave greater noise indicating that blocking using BLOTTO was important. Exclusion of the other steps failed to produce any background noise.

The test on different washes showed that using Tris did not decrease background noise, rather, it made it worse. On both occasions when this was tested the background was much pinker than before. Water was therefore considered to be the better washing medium. There was no discernible difference in results between treatment dots on the membranes with either washing method. However, it was noticed that greater care in washing did reduce background noise.

The use of a conjugated antibody reduced the strength of false positives from *N. kinbergii*, *D. bellulus* and *C. transversalis* but did not eliminate them. Interestingly, the signal from *C. transversalis* was much darker than the other two species. There was still little difference between most of the fed and unfed predators though the difference between fed and unfed was distinguishable in *N. kinbergii*. It was also noted that the signal from the control eggs was reduced when using the conjugated antibody, and eggs were lighter in colour than the predators. Controls continued to work indicating that the use of a conjugated antibody was acceptable. A final protocol is shown in Appendix 5.

Tests on the length of time that membranes could be stored showed that signals were the same for up to six months. Beyond this the strength of the reactions faded.

Eggs

In all tests done with the adjusted protocol, on both *H. armigera* and *H. punctigera,* there was no difference in signal between species when using the polyclonal antibody (Figure 6.4). Freezing of eggs or homogenates prior to use did not change the strength of the signals. No difference was found between eggs from UNE and ACRI cultures. Only the fresh eggs that were squashed directly onto membranes were much darker in colour intensity.

The fresh squashes also formed sprays of colour across the membrane so were discontinued due to the potential for contamination of other treatment dots.

The different egg numbers showed a slight gradient of strengthening colour as the number of eggs increased to five, and then a more prominent jump in intensity when ten eggs were used. All ages of eggs provided strong positives though two day old eggs appeared slightly lighter in signal. The pre-prepared eggs frozen as homogenates showed positives but the signal was much weaker when compared to fresh homogenates.

Predators

There were mixed results when testing for a difference between fed and unfed predators. Some variation in colour intensity was exhibited within replicates of predators whether fed or unfed. An unfed that shows a colour detection is called a false positive. There was variation in intensity of false positives within and between experiments.

All the fed *N. kinbergii* produced strong positives. There was a faint signal given for the unfed *N. kinbergii* although the difference between the two was notable (Figure 6.5). *D. bellulus* showed similar results with all five feds giving strong signals and all five unfeds slight colour reactions (Figure 6.6). This plate also highlights the type of background noise that occurred on some occasions. The positive controls were of similar signal to the fed predators and there was no detection of colour on the negative controls. Results for *H. octomaculata* showed a difference between fed and unfed predators in all ten replicates although like *N. kinbergii* and *D. bellulus* there was still a signal for unfeds.

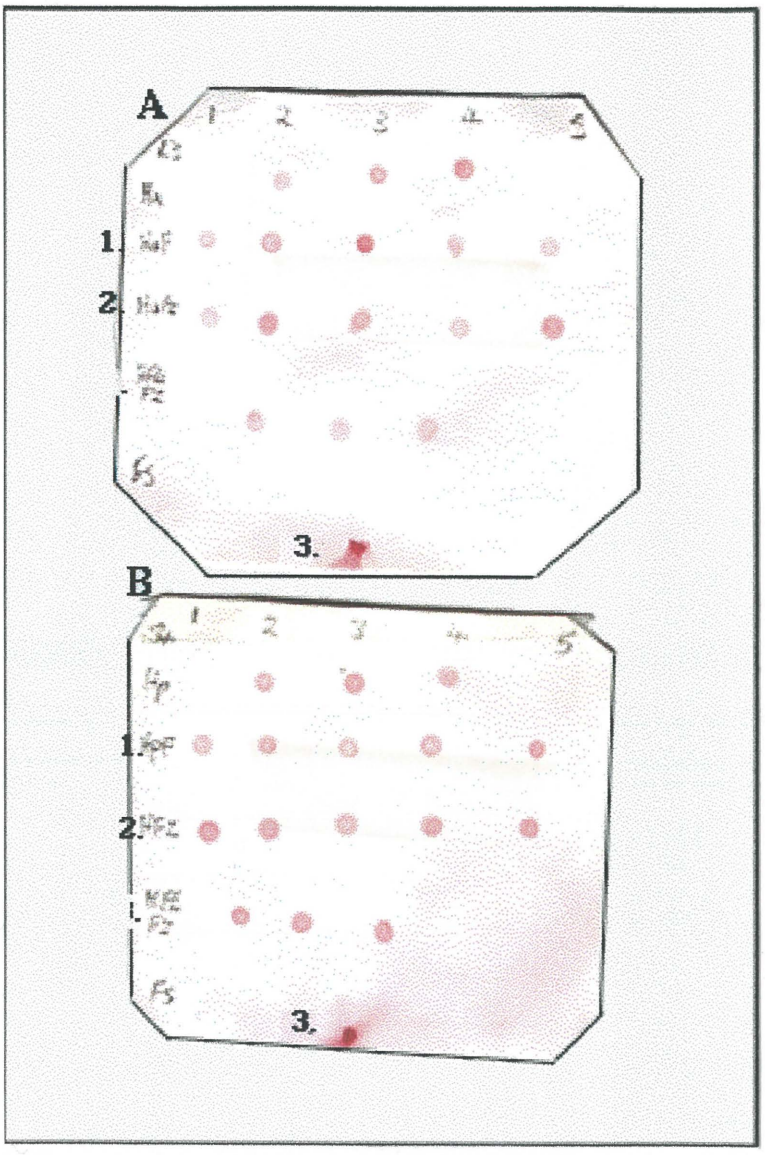

Figure 6.4 A comparison of the two species of heliothis used with testing of the polyclonal antibody. **A** = *H. armigera* and **B** = *H. punctigera*. **1** = fresh eggs, **2** = frozen eggs, **3** = frozen homogenates and **4** = a fresh egg squashed directly onto the membrane. The line of dots above number 1 are frozen eggs from ACRI.

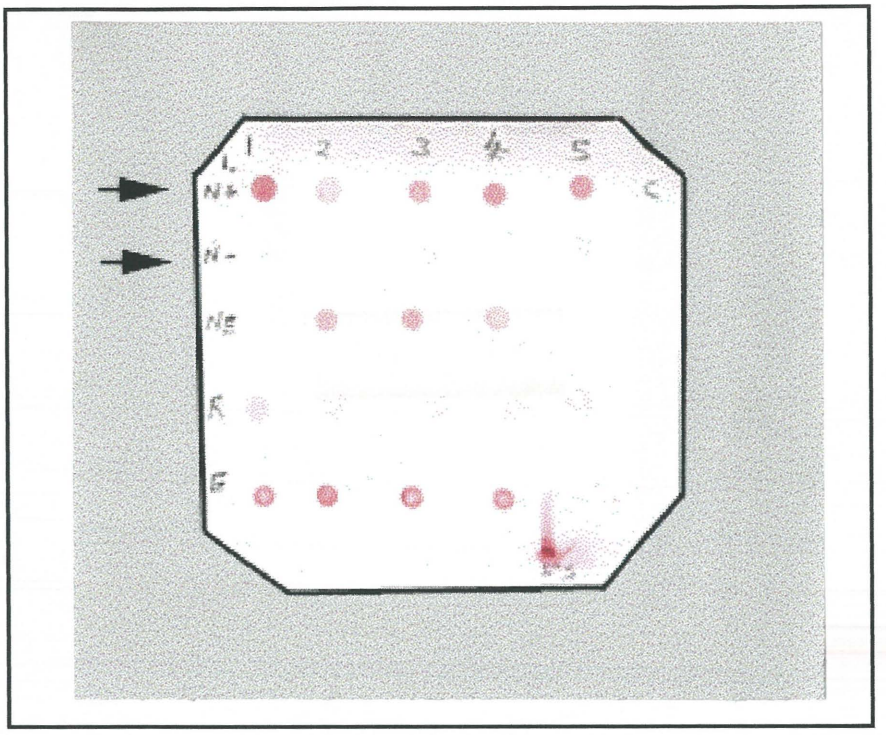

Figure 6.5 The distinction between fed and unfed *Nabis kinbergii*. Arrows indicate the two treatments (fed and unfed). Other treatments on the membrane not related to the results were NE = old samples of fed predators, R = random samples from an old field collection. E = heliothis eggs.

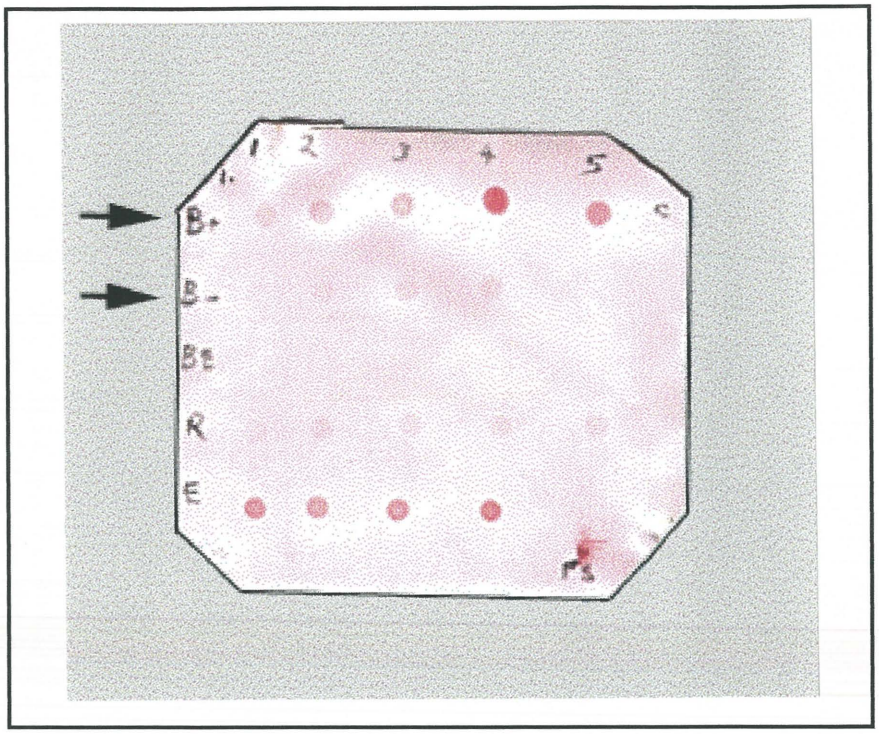

Figure 6.6 The distinction between fed and unfed *Dicranolaius bellulus*. Arrows indicate the two treatments (fed and unfed). Other treatments on the membrane not related to the results were BE = old samples of fed predators, R = random samples from an old field collection and E = heliothis eggs.

Coccinella transversalis showed a distinction between fed and unfed on ten replicates though the difference between positive and negative was not clear in every case. Some of the false positives (unfeds) were as strong as the weaker positives (feds). Egg controls for both coccinellid species were as strong as the fed predators and the negative controls were completely clear indicating the method had worked well. *D. signatus* were only available for limited tests, however the results were included as the species provided the clearest distinction between fed and unfed predators. All replicates were clear strong positives similar in intensity to eggs and all unfeds were very faint signals. Repeats of some of these tests did not provide consistent results. In two of the species (*D. bellulus* and *N. kinbergii)* unfeds in the repeated trials showed up just as strongly as the feds. Gender tests showed no distinction between unfed male and female *C. transversalis.* However, unfed female *D. bellulus* were slightly lighter in colour that the males.

Checkerboard assays

The heliothis egg checkerboards showed eggs could be best detected when homogenised at 1:200 µl or 1:400 µl and that 1:1000 and 1:300 were the best ratios for primary and secondary antibodies respectively. Beyond these ratios the signal for eggs was very faint. Positives were still discernible up to 800 µl using the optimal antibody ratios. Similar ratios were found to be optimal in the predator trials.

Adjusting the ratios of homogenising solution and antibodies did provide improved signal to noise ratios in some of the predator species tested. *D. bellulus* showed a distinction between fed and unfed which improved with each dilution up to 1:800 µl when assayed using the primary antibody at 1:1000 but did not entirely eliminate false positives. This was the case for replicated trials of each membrane. The false positives were eliminated at 1:400 µl, with the primary antibody used at a ratio of 1:5000 but beyond this the signal of the positives began to deteriorate. The egg signal too was fainter when using the primary antibody at 1:5000. This was consistent with the egg checkerboard results.

The results were similar for *N. kinbergii* although the signal when using 1:5000 was weaker than the *D. bellulus*. Using the primary antibody at a ratio of 1:10000 did not produce any signals. The best *N. kinbergii* signal to noise ratio was insects macerated at 1:800 µl using the primary antibody at 1:1000. Results of the *N. kinbergii* checkerboard assay can be seen in Figure 6.7A and B.

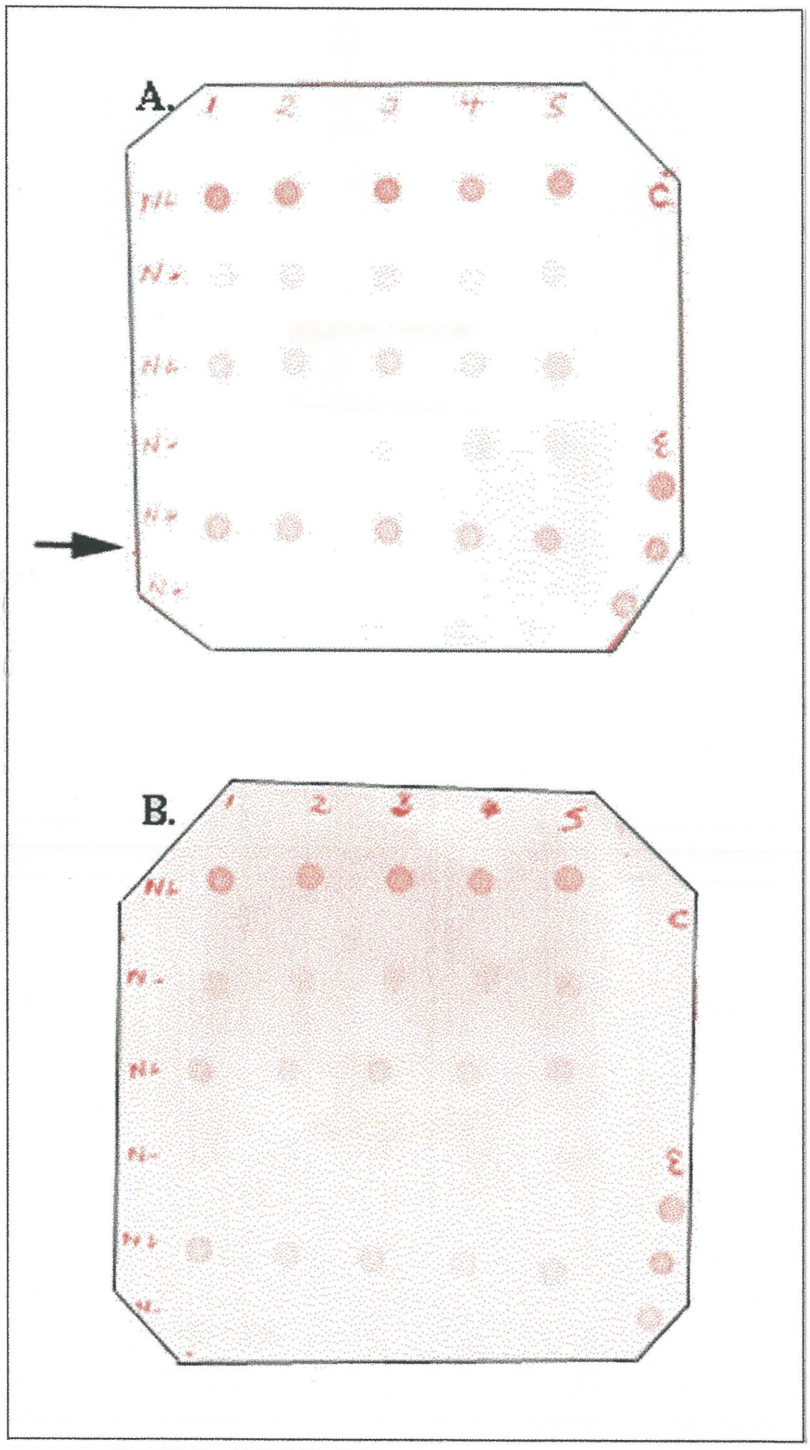

Figure 6.7A and B. Optimising the difference between fed and unfed *Nabis kinbergii*. **A** = 1:1000, **B** = 1:5000. The arrow indicates the best signal to noise ratio. Each membrane contained a row of fed predators and a row of unfed predators. The ratios used for homogenising individual fed and unfed predators were 1:200, 1:400 and 1:800 µl of PBS + tween respectively.

The checkerboard assay using *H. octomaculata* also produced improved signal to noise ratios with a distinction between fed and unfed best at 1:400 µl and the primary antibody at 1:5000. However, once again there was a reduction in strength of the positives when using the antibody at this strength. In *H. octomaculata* the difference between fed and unfed was not as distinct as the previous two species and false positives did not completely disappear. *Coccinella transversalis* was again different from the other species in that a clear distinction between fed and unfed did not appear at any of the ratios tested. Eggs gave the same signal strength as the unfeds and feds. The negative controls were clear indicating the method had worked properly.

Alternative prey

The polyclonal antibody did not react with any of the alternative prey that were tested. Nor did it react to the coccinellid eggs. Negative and positive controls showed up clearly indicating the test had worked well. Heliothis larvae of all sizes showed positive reactions implying the antibody would detect larvae as well. The larger the larvae the greater the intensity of the colour reaction. The small larvae (neonates) were of the same intensity as the egg controls.

Haemolymph

In all three predator species tested results on the haemolymph were similar (Figure 6.8). Haemolymph of each species produced false positives. The haemolymph diluted at 1:10 gave a stronger signal (Figure 6.8 – first colunm) than the higher dilutions of homogenate but false positives were still visible at 1 µl in 600 µl of homogenising solution. The haemolymph dots were all as strong in colour intensity as the positive control eggs, although in a few cases the 1:200 and 1:600 dilution of haemolymph was fainter than the control eggs. All were considered false positives indicating that cross reactivity was occurring to the predators' haemolymph. The intensity of the reactions did appear to be greater in *C. transversalis*. The body parts of unfed *C. transversalis* all showed up as strong false positives with the exception of the gut which was somewhat lighter in colour intensity than the other body parts.

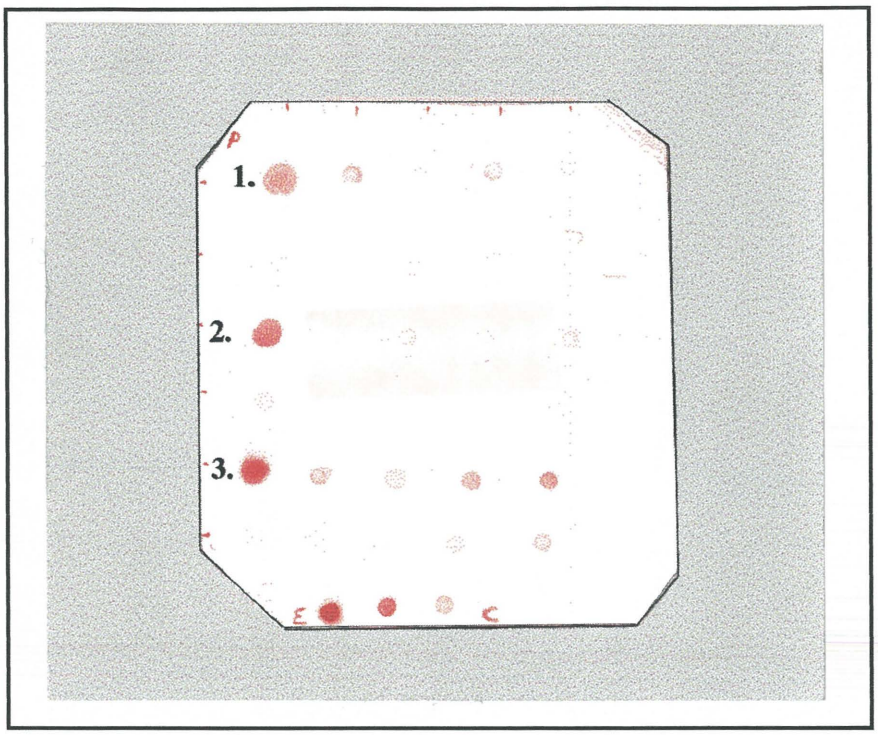

Figure 6.8 Haemolymph tests on three species of predator. **1** = *Nabis kinbergii*, **2** = *Dicranolaius bellulus* and **3** = *Coccinella transversalis*. E = Heliothis eggs and C = negative controls.

6.4 Discussion

The polyclonal antibody initially showed promise in detecting a difference between fed and unfed predators. Although unfed predators produced some colour reaction it was possible to optimise reagent concentrations and homogenate solutions to obtain a detectable difference between fed and unfed predators by visual assessment. It was not, however, possible to do this consistently. The initial tests on checking the protocol provided some useful adjustments and were subsequently used in the testing of the MAb discussed in chapter 7.

Membranes could be kept in a fridge for up to six months without loss of sensitivity. Stuart and Greenstone (1990) kept membranes for up to two months at 4 °C. Similarly Ohiagu and Boreham (1978) tested storage methods for latex, sensitised with anti-locust IgG. They found sensitivity and activity were retained after storage at 4 °C for up to three months. Alternatively, other researchers macerated their insects and then stored the homogenate (Greenstone *et al.* 1991, Hagler *et al.* 1992). However, the results from these tests showed a slight decline in sensitivity so this method was not practised here. Storage time of membranes is likely to vary with the type of preparation methods, and the type solid phase utilised, so it needs to be tested with each project.

The two species of heliothis egg tested here produced similar colour intensity and freezing them proved suitable as a storage method. This makes either species adequate for laboratory trials using the polyclonal antibody. It is possible to determine the minimum antigen level the assay can detect (Stuart and Greenstone 1990). However, as the predators were observed to feed on a whole egg, even with disturbance, a minimum detection level of one egg was considered acceptable as a minimum detection amount.

Sources of variation

Throughout the trials on the polyclonal antibody there was some variation in the intensity of the colour substrate from trials on both eggs and fed predators. There was an increase in colour intensity with an increase in egg numbers so, the number of eggs consumed by a predator could cause variation in results. As some of the predator species consume eggs so quickly it is possible to miss a feeding event when doing feeding trials. Some predators may therefore exhibit a stronger colour reaction than others. Although there was a gradient in colour intensity seen with an increase egg number it is unlikely that this could be quantifiable when applying the assay to eggs consumed by predators in the field. This is due to factors such as the consumption of alternative prey and therefore, meal size, as well as digestion times (Hagler and Naranjo 1997, Hagler and Naranjo 1996).

Egg age may help to account for some variation within and between tests. Two day old eggs were slightly lighter in intensity. Hagler *et al.* (1992) showed a strong negative linear relationship between egg age and decline in delectability in *Lygus hesperus* Knight eggs. If the target antigen was linked to vitellin (unknown in this study) the decline may be due to consumption of vitellin as the embryo develops (Hagler *et al.* 1992). The eggs in this study were largely one or two days old and therefore may account for some variation in the fed predator results. Gender of the predators may also cause variation in the colour intensity of the dots.

In the test specifically on gender differences, *D. bellulus* females were lighter in colour intensity than the males. This did not occur with *C. transversalis* and no pattern emerged between male and female predators in any of the other trials. The results for *D. bellulus* on this occasion were therefore an exception. The reasons for this are unknown. However, there may be detection differences due to reproductive status within females and variation may also occur from body size (Hagler *et al.* 1997) and the size of the target prey ingested.

Despite numerous tests that compared insects frozen and stored in liquid nitrogen, with fresh insects, a difference between the two was not found. This is contrary to the suggestions made by Stanley (1997) that *D. bellulus* could not be stored frozen as it altered results by producing false negatives. Stanley (1997) did not state how many replicates were used in his study and what method of thawing occurred. If only one replicate was used the difference may have been due to inherent variation within the species. The tests were also done on different days. Given that there was variation in whether false positives occurred or not, on a regular basis in this study, the different experimental times may be the reason for the discrepancy. Certainly the most common form of storage of insect specimens in serological studies is freezing at temperatures below minus 20 °C (Greenstone and Trowell 1994, Stuart and Greenstone 1990, Lovei *et al.* 1985, Boreham and Ohiagu 1978).

Background noise

Whilst the method was working on eggs and predators two problems continued to occur. These were background noise and false positives. Early tests done on blocking and washing in order to reduce background noise did not provide significant improvements and the problem was found to be inconsistent. The most likely reasons for inconsistent blocking of background noise on the membrane were possible variation in reagent or membrane quality and inconsistent washing technique. Several other types of washes are available (Harlow and Lane 1988) and, Stanley (1997) also tried a salt wash to reduce background noise. However, it was noted that the background noise problems here seemed to dissipate with greater care in washing. The washing technique was therefore considered to be the main source of the

problem. The use of the wholelinked antibody also seemed to reduce background noise. This may be due to the reduced sensitivity of the antibody. The biotin/avidin test results on positive control (eggs) indicated that there was a reduction in sensitivity and therefore, colour intensity of the dots, when using the conjugated antibody.

The use of the conjugated antibody also reduced the second problem of false positives occurring. However, it also appeared that binding to non-specific biotins was not solely responsible for false positives. It was hoped that optimising the ratios of reagents would alleviate this problem. Dilution of the homogenate and primary antibody did produce distinctions between fed and unfed predators but once again the results were not consistent. Greenstone and Hunt (1993) indicated that background noise (false positives) can be eliminated through utilising different membranes and or substrates. Other membrane types were not available for this study. Substrates were tested with the use of the MAb and are discussed in chapter 7.

Cross reactivity

Although a distinction between fed and unfed predators was achieved with the polyclonal antibody on nitro-cellulose membrane the difference was not consistent within and between species. The source of false positives was therefore investigated further by looking at cross reactivity. At the same time, sources of cross reactivity from the field such as alternative prey, were considered, as this would hinder the eventual use of the antibody in field trials. Ohiagu and Boreham (1978) suggest all antisera be tested for cross reactivity particularly with closely related species. Hagler and Naranjo (1996) viewed cross reactivity as unacceptable in American cotton where there is such a diverse species complex. Given the low incidence of other *Helicoverpa* spp. in Australian cotton and that the tests showed cross reactivity did not occur with alternative prey, the use of the polyclonal antibody was thought to be acceptable in this study. Stanley (1997) found the polyclonal antibody cross reacted with *Agrotis infusa* (Boisduval) but considered this to be inconsequential due to the rarity of this species in the field. Had there been no cross reactivity to the predators themselves, it would have been desirable to test other noctuids found in cotton as knowing sources of cross reactivity would aid in interpretation of results. In addition, unless the cross reactivity occurs on co-existing species it is not a problem. For example, Hagler *et al.* (1993) found cross reactivity to *Trialeurodes vaporariorum* (Westwood) was not a problem as this species did not co-exist with the target prey *B. tabaci.*

Cross reactivity to other life stages of heliothis (especially the destructive larval stage) was considered an advantage, since it would give a more complete picture of total predator impact. Naranjo and Hagler (1997) did not view cross reactivity to life stage a limitation as the size of

prey type and the feeding behaviour of predator allowed for interpretation of results by concluding that a smaller predator was not likely to attack a larger adult prey type. In some cases cross reactivity can be seen as an advantage as it may allow the study of population dynamics of several species (Hagler *et al.* 1993).

It is possible to use polyclonal antibodies as long as they are checked for cross reactivity and if cross reactivity does not interfere with interpretation of results. Cherrill and Begon (1989) used polyclonal antisera that did not cross react with other prey available to spiders in sand dunes and detected predation on two species of grasshoppers, *Chorthippus brunneus* (Thunberg) and *Myrmeleotettix maculatus* (Thunberg). Predation was recorded as the percent positives feeding on one and or the other species. Given the low numbers of other *Helicoverpa* species and no cross reactivity to other prey in Australian cotton this type of study would be acceptable. The cross reactivity to the predators (haemolymph) themselves was considered to make future results using this antibody uninterpretable.

6.4.1 Conclusion

Using the immunodot assay technique with some modifications showed it to be a suitable method for use in the laboratory situation provided. Tests on the method found that freezing both prey and predators was acceptable for future tests and the signal to noise ratio could be improved with testing various ratios of reagents and homogenates. A predator that had fed on a minimum of one egg was able to be detected and eggs of all ages could be used. A range of predators provided similar results and male and female predators could be used in the samples indicating that using this method would be reliable on a study of a predator complex from the field. The polyclonal antibody used in this study provided strong signals for both heliothis species and did not cross react with other prey types.

Whilst cross reactivity was expected to occur with close relatives of the prey antigen (Stanley 1997) the possibility of it occurring with the predators was not expected. The use of the polyclonal antibody is not acceptable when it is cross reacting with the predators themselves. The reason for the cross reactivity not occurring at all times and therefore providing good results on some occasions was unknown. It is possible to analyse the protein complex to find the major source of cross reactivity (Hagler *et al.* 1993). It was beyond the limits of this study to do this. Gut dissection as shown in the *C. transversalis* body parts trial may improve results but may be difficult with such small insects (Hagler and Naranjo 1997) and too time consuming. It was therefore concluded that this polyclonal antibody would not be acceptable for use in detecting *H. armigera* and *H. punctigera* predation by predators in Australian cotton. This left the potential of the MAb to be studied.

Chapter 7

7. The potential of a monoclonal antibody for detecting *Helicoverpa armigera* in the guts of predators.

7.1 Introduction

As *H. armigera* is the heliothis species resistant to most pesticides, the impact of predators is important in the management of this species. A monoclonal antibody (MAb) (Trowell *et al.* 1992) was available to test for *H. armigera* antigen in the guts of predators. The advantage of this antibody was its potential specificity to *H. armigera* thus avoiding the problem of cross reactivity discussed in the previous Chapter. Specificity in MAbs occurs as a result of the way they are produced.

Monoclonal antibody technology was first developed by Kohler and Milstein (1975). The technology is designed to produce antibodies which identify single antigen determinants. Greenstone (1996) describes in detail the way MAbs are produced. Put simply, MAbs are derived from the fusion of myeloma cells with healthy lymphocyte cells in mice spleens. The new cells are called hybridomas. The hybridomas are isolated and refined so that an antibody of single specificity is produced, that is, an antibody with combining sites that only recognise one determinant. The antibody is then cloned. Once this clone has been created a large supply of uniform antibody can be produced and stored in liquid nitrogen. This is an advantage as the same antibody can be used for prolonged periods.

Using MAbs and immunodot assay for predation studies offers similar advantages to the polyclonal antibody (Chapter 6) in that it allows a method of studying predation without the use of cumbersome cage trials. Also, the same information on predation such as seasonal changes and species comparisons can be collected. There are only a few studies on helionthine species using MAbs. The first species, stage and instar specific MAb was produced by Lenz

and Greenstone (1988) against *H. zea* arylphorin. This MAb was used to compare immunodot assay and ELISA techniques using the predators *P. maculiventris* and *P. audax* fed with *H. zea* fifth instar (Stuart and Greenstone 1990) and again by Greenstone *et al.* (1991) for phylogenetic studies. Greenstone and Trowell (1994) developed a simplified immunodot assay using three MAbs to test their sensitivity on *P. maculiventris* eggs, larvae and *H. virescens* eggs. A MAb was also developed and used by Greenstone and Morgan (1989) to determine the effectiveness of a MAb-based ELISA on studying predation of fifth instar *H. zea* by *P. maculiventris.*

Studies directly investigating natural enemies include Stuart and Greenstone (1996) who used a MAb to determine levels of parasitism by *Microplistis croceipes* (Cresson) in *H. zea* and Sigsgaard (1996) who looked at predation of *H. armigera* in India using a MAb specific to helionthine eggs. Studies that utilise antibodies raised against other target pests are tabled in Greenstone (1996). Whilst useful, limitations to these types of studies remain the same as those discussed in Chapter 6 in that they are not able to accurately quantify predation (Hagler and Naranjo 1996).

There are advantages in using MAbs. Not only can they be employed in studies of predation, they can also be of benefit in phylogenetic studies by using the antigen determinants as character states (Greenstone *et al.* 1991). Stage specific antibodies have been developed (Greenstone and Morgan 1989) that can be used to determine predation within a species. Sigsgaard (1996) did this when measuring *H. armigera* egg predation by *H. armigera* larvae. Furthermore kits can be used to detect the presence of resistant species. The Lep Ton Test Kit (Abbott Laboratories) developed by Trowell *et al.* (1992) allows pest managers to make better use of insecticides for the purpose of resistance management (Daly and Trowell 1996). Further studies include identification of active microsporidian infection as opposed to presence in target hosts (Ragsdale and Oien 1996). Consequently, although the field is only relatively young the development of MAb technology has already provided a diverse range of applications in areas that affect pest management.

7.1.1 Purpose of this study

The aim of this chapter was to test a MAb using the immunodot assay methods developed in the previous chapter (see also Appendix 5) and, provided there was no cross reactivity, add another series of experiments to test how long the prey antigen could be detected in the predator gut after ingestion. This is called the antigen half life (Greenstone and Hunt 1993). The antigen half life is an important parameter as it helps to determine the time that sampling should occur and interpret assay results (Sunderland *et al.* 1987). There are several factors that influence antigen half lives. These include temperature, size of meal, species and size of predators, differences in prey type and whether predators are allowed to feed on non- target prey prior to and after feeding on the target prey (Hagler and Naranjo 1997, Symondson and Liddell 1996, Greenstone and Hunt 1993, Symondson and Liddell 1993, Hagler and Cohen 1990, Lovei *et al.* 1990, Sopp and Sunderland 1989, Sunderland *et al.* 1987, Lovei *et al.* 1985). In general, 24 to 72 hours is the maximum detection interval for prey antigen (Sigsgaard 1996, Hagler and Naranjo 1994a, Hagler *et al.* 1994).

The following section describes the methods used to evaluate the immunodot assay protocol and determine antigen half lives for each predator species. The species tested in each experiment are provided in the description of each experiment.

7. 2 Methods

Due to the cross reactivity to predator haemolymph exhibited when using the polyclonal antibody (Chapter 6) the first trial done on the MAb was testing predator haemolymph. As well as testing for cross reactivity, the sensitivity of the monoclonal antibody was checked as, preliminary testing showed it was likely to be less sensitive than the polyclonal antibody. Some of the trials done in the polyclonal tests were still applicable in establishing changes to the protocol. Therefore, the protocol used in this study was similar to the immunodot assay using the polyclonal antibody (Appendix 5) except the HRP linked secondary antibody used was anti-mouse Ig. As in Chapter 6, details of products specific to the immunodot assay technique are listed in Appendix 4b. Unless specified, the preparation of specimens followed those outlined in Appendix 5. The MAb was originally produced *in vivo* (ascites fluid). The

use of ascites fluid was substituted for hybridoma cell culture supernatant (*in vitro* culturing) early in the testing of the MAb due to time constraints involved in producing ascites fluid.

Controls on each membrane were the same as those outlined in Chapter 6 when testing the polyclonal antibody. 'Fed' and 'unfed' refer to predators fed one target prey (*H. armigera*) or not and 'positives' and 'negatives' refer to heliothis eggs and homogenising solution without target protein, respectively. Unless specified in the methods section in this chapter all heliothis eggs were *H. armigera*. In the protein concentration tests mentioned below another control was included in the chemiluminescence trials (section 7.2.2). This was a control using the protein concentration step (described in Appendix 6) without any predator or prey protein added and was used as a substitute for the usual negative control.

Feeding trials

The same methods were used for feeding trials as described in Chapter 2 (2.4.1 - Feeding trials) where each predator was fed one target prey. The exception to this method was 'spiked' trials where an egg (target prey) was included with a whole body predator and macerated together. This was done instead of lengthy feeding trials in order to simulate a fed predator.

As well as fed and unfed predator testing, another type of feeding trial was done in the testing of the MAb. The time that a heliothis egg could be detected after ingestion by the predator was tested (antigen half life trials). A predator was allowed to feed on one egg as in the other feeding trials but, after feeding, it was allowed to digest for a pre-determined time (usually times between one and 24 hours). Upon feeding on one egg the predator was placed in a small plastic container with a moist dental wick for supply of water and a source of alternate prey such as aphids or pollen for continuous feeding. Photoperiod was 14:10 light/dark hours, similar to that of the field at the time of feeding. Temperature was 27 ^{o}C. At the end of the pre-determined time the predator was frozen in liquid nitrogen and stored until required for assaying. The specific times used are outlined in section 7.2.2.

7.2.1 Protocol establishment

Cross reactivity

The haemolymph trial used the same membrane set up as the polyclonal antibody described in Chapter 6 (6.2.5 - Predators) except that the predator haemolymph was only used at a ratio of 1:10 µl (one egg in 10µl). This was replicated five times for each species. The predator species used were *N. kinbergii, D. bellulus* and *C. transversalis.* The membrane was assayed using the protocol described in Appendix 5. Alternate prey consisted of other small prey found in cotton throughout the growing season. These were tested using a different assay method later in this study. The species and methods used are shown in section 7.2.2 - *Alternative prey.*

Protocol changes

As mentioned in Chapter 6 (6.2.1. - Biotin/avidin steps) the binding of the streptavidin to non-specific biotins in the predators was tested using both the polyclonal and monoclonal antibodies. The use of a conjugated secondary antibody (anti-mouse Ig HRP linked whole antibody) was tested with the monoclonal primary antibody. Each membrane contained 12 individuals of *N. kinbergii, D. bellulus*, and *C. transversalis*. Six of each were fed and six were unfed. Three of each fed and unfed pre-frozen predators were macerated and three fed and unfed predators were macerated live. The membrane also contained three positive and three negative controls. Individual membranes were tested using the original protocol and the adjusted protocol of the whole linked antibody at a ratio of 1:1000. The membranes were assayed using the same protocol as the polyclonal antibody tests (Appendix 5) using MAb ascites fluid.

Eggs tests

In order to ensure cross reactivity did not occur with other heliothis, *H. armigera* and *H. punctigera* were compared using the monoclonal antibody. Ten *H. armigera* and *H. punctigera* frozen eggs were prepared in 200 µl of PBST + BSA and each species dotted onto a separate membrane. Another five fresh *H. armigera* eggs were prepared and dotted onto each membrane. This provided a comparison between *H. armigera* and *H. punctigera* species

on a membrane and between membranes. Each membrane also contained three negative controls.

The sensitivity of the antibody was tested by doing checkerboard assays on *H. armigera* eggs. As the use of the conjugated secondary antibody in the biotin/avidin test in this study had produced only a faint signal on heliothis eggs the ratios selected were much lower than those of the polyclonal trials. Two membranes were made up: one for frozen eggs and one for fresh eggs. Five eggs were individually homogenised at 1:10, 1:20, 1:40, 1:80 and 1:160 µl of PBST + BSA. One µl of each was dotted onto the membrane for each dilution and treatment. There were three negative controls on each membrane. These membranes were assayed using the primary antibody at a ratio of 1:1000 and 1:500, and the secondary antibody at 1:300.

A continuous supply of MAb ascites fluid was not available so hybridoma cell culture supernatant was produced from stock cell cultures at CSIRO - Entomology, Canberra. The strength of the supernatant was compared to ascites to ensure no changes in sensitivity occurred between experiments. This was done using the membranes prepared for the heliothis egg checkerboard tests. Replicates of both fresh and frozen egg membranes were tested with the supernatant used neat (10 ml per Petri dish). This assay was compared with the results of the egg checkerboard assays that used ascites fluid as the primary antibody (the previous experiment).

Predator tests

Fed and unfed *D. bellulus*, *H. octomaculata* and *C. transversalis* were tested with the MAb. These trials were carried out prior to testing the sensitivity of the antibody. Therefore, all predators were homogenised in 200 µl of PBST+BSA. Five fed and unfed *D. bellulus* were prepared and one µl from each aliquot dotted onto the membrane. These were included on the same membranes used in the tests on *H. armigera* versus *H. punctigera* in the egg trials. Three negative controls were included. For *H. octomaculata* and *C. transversalis* there were ten fed and unfed predators and controls of three positives (eggs) and three negatives.

As with the polyclonal antibody the difference between fresh and frozen predators was again checked. As the MAb was less sensitive than the polyclonal antibody unfed predators were homogenised at a ratio of 1:20 µl. A test was done to check if differences occurred between

unfed fresh and frozen *N. kinbergii*, *D. bellulus* and *C. transversalis*. There were three replicates of each species for each treatment. Positive controls were three frozen eggs macerated at 1:10 µl and three at 1:20 µl, and three negative controls.

Macerating predators in 20 µl of homogenising solution caused problems due to excess lipids and pigmentation in the homogenate. Four approaches can be used to overcome problems associated with such low volumes of homogenate solution. Firstly, the antibody could be concentrated by using a higher volume of antibody in PBS. This was not possible here due to the use of supernatant neat. Secondly, the sample protein could be diluted and reconcentrated to allow extraction of predator lipids and pigmentation; thirdly, the substrate could be changed to enhance colour of the positives; and finally, the membrane could be washed to bleach non-positive results. One month was spent at CSIRO - Entomology (Canberra, Australia) working with Dr S.Trowell in order the test the latter three of these methods.

Protein concentration

Two types of protein concentration methods were tested. One used acetone the other ammonium sulphate $(NH_4)_2SO_4$. Three fed and unfed *N. kinbergii*, *D. bellulus* and *C. transversalis* were used. In both methods the insects in each treatment were macerated into 200 µl of PBST+BSA. 200 µl was extracted and added to 600 µl of either saturated $(NH_4)_2SO_4$ or acetone. These were set on ice for 30 minutes and then spun at 14000g for 20 minutes to form a pellet. The excess liquid was removed with a pasture pipette and 20 µl of 0.1% SDS (ionic detergent) added to the acetone treatment or 20 µl of H_2O_2 added to the $(NH_4)_2SO_4$ treatment. These were vortexed to dilute the protein and 1 µl dotted onto the membrane. Respinning the diluted protein produced false negative results so it was not done. 3-AEC was used as a substrate in these tests. Three positive and negative controls were included. Another control was added using the precipitation treatment without a predator or heliothis egg added. This control was included in all experiments that used protein concentration.

Next the $(NH_4)_2SO_4$ method was used at different ratios to see if it made dispersal of the dots onto the membranes easier. Two fed and unfed *D. bellulus* and *C. transversalis* were used for each treatment. Three dilutions of 75%, 50% and 30% were tried. 200 µl of homogenate was

added to 600 µl of $(NH_4)_2SO_4$, 200 µl to 400 µl $(NH_4)_2SO_4$ + 200 µl H_2O and 200 µl to 240 µl $(NH_4)_2SO_4$ + 360 µl H_2O respectively to obtain each dilution. Controls included six positives and three negatives.

Substrates

Another substrate (4-chloro-1-napthanol) (CN) was compared against 3-AEC. CN was made up to 30 mg/ml. 200 µl was added to 10 ml of 50 mM TRIS + 20 µl of H_2O_2. The substrate was added to the membrane for three minutes, rinsed for two minutes in H_2O and two minutes in H_2O with rotation. 3-AEC was used as per the current protocol (Appendix 5). The two substrates were compared twice. Firstly, it was used on eggs to check the CN was working well. Heliothis eggs were both fresh and frozen and a negative control included. Secondly, it was used on fed and unfed *D. bellulus*. There were five replicates of each treatment. Five *H. armigera* eggs using the protein concentration method were included with five eggs homogenised in 10 µl of PBST + BSA. Three negative controls were included.

Bleaching

The latter trial of fed and unfed *D. bellulus* was also tested using two post assay washes, ethanol and acetone. Each wash was tried on membranes using both substrates. Ethanol was used at 100% and 30% and acetone at 50% and 30%. After assaying, the membranes were submerged into one of the dilutions for ten seconds, with rotation, taken out and checked to see if any noticeable difference in signals between the fed and unfeds had appeared.

7.2.2 Chemiluminescence trials

The colour detection method was still causing problems when used on fed and unfed predators therefore another detection method was tested. This used a chemiluminescence blotting substrate (POD) kit as a detection method. Prior to the last step in the protocol the substrate was mixed with starting solution at a ratio of 100:1 and incubated at room temperature for 30 minutes before being added to the membrane. The amount used was 125 µl/cm^2. The substrate was allowed to cover the membrane for 60 seconds. The membrane was then blotted on tissue to remove excess substrate, and exposed to x-ray film in darkroom conditions. Exposure and development times were set at 20 seconds for the film exposure, 60 seconds for the film

developer, 30 seconds wash and 60 seconds in the fixer. These times were adjusted in later tests to gain optimal signal to noise ratios.

To check that the method worked the first test using chemiluminescence was on *H. armigera* eggs and fed and unfed *D. bellulus* and *C. transversalis*. Six fed and unfed *D. bellulus* and *C. transversalis* were prepared using the precipitation method in 20 µl of homogenising solution and dotted onto the membrane. Three fresh *H. armigera* eggs were also prepared in the same way and another three homogenised in 10µl of PBST + BSA without the protein concentration method. These acted as a control. The membrane was assayed in the same way as the protocol in Appendix 5 but using the chemiluminescent substrate instead of 3AEC. X-ray results were both scanned and stored as a permanent record of results.

As it was advantageous to leave out the protein concentration step in order to decrease preparation time and the strength of the reaction appeared to be much stronger with chemiluminescence, the method was tested on heliothis eggs using four treatments. These were eggs directly squashed onto the membrane, and eggs prepared at 1:10 µl of PBST + BSA, 1:100 µl of PBST + BSA and, 75% $(NH_4)_2SO_4$. There were three fresh and frozen eggs for the squashes and $(NH_4)_2SO_4$ treatments and three fresh eggs for the others. Controls included three negatives.

'Spiked' predators

The method was then tested on predators that had been 'spiked' with an egg. Again three methods of preparation were tested. 1:20 µl, 1:100 µl and 75% $(NH_4)_2SO_4$. There were three species tested (*N. kinbergii*, *D. bellulus* and *C. transversalis*) and each trial had three 'fed' and three unfed frozen predators. The second treatment (1:100 µl) was not included for *N. kinbergii* as there were not enough specimens available. Positive controls were two eggs homogenised at 1:20 µl of PBST + BSA, 1:100 µl of PBST + BSA and, 75% $(NH_4)_2SO_4$. Both negative controls were also included.

Fed and unfed predators were tested using the protein concentration preparation method with chemiluminescence substrate. *N. kinbergii*, *D. bellulus* and *C. transversalis*, *D. notescens*, *M. frenata*, *H. conformis* and *H. octomaculata* were tested. Tests for all species contained five

replicates of fed and unfed predators, each dotted twice on the membrane to form two rows of fed and unfed. Controls included three positives and two negatives of each type. Exposure times were tested with each species from between 20 seconds and four minutes until false positives began to appear or background noise obscured results. Some of the species were tested twice to confirm results. These were *M. frenata*, *H. octomaculata* and *D. notescens*. Two tests using one aliquot of a previously prepared *D. bellulus* known to show a positive result were also tested. The entire aliquot was dotted onto the membrane in 1 µl dots. This was to test whether the extracted 1 µl was providing false negatives.

Alternative prey

Several types of potential prey of the generalist predators were tested. Three replicates of eggs laid by *C. transversalis*, *N. kinbergii* and *M. frenata* in the laboratory were prepared. Three unfed small and medium *C. transversalis* larvae and, three small *C. transversalis* larvae fed one *H. armigera* egg, were prepared using the protein concentration method. Also included were three whitefly (*T. vaporariorum*), aphids (*A. gossypii*) and cotton pollen samples. There were three negative controls and three positive controls. These were also prepared using the protein concentration method.

Antigen half lives

Predators were fed and allowed to digest for pre-determined times. It is important to test individual species as digestion times can vary even in similar species (Symondson and Liddell 1993). For this reason several species were tested. These were *D. bellulus*, *C. transversalis*, *N. kinbergii* and *D. notescens*. Two trials were conducted. Firstly, in order to check how long ingested heliothis eggs could detected, the times 0, 8, 24, 48 and 72 hours were used. Five predators were used for each time. There were also five unfed controls included in each test as well as three positive and negative controls. Secondly, a series of trials were done testing ingestion between 0 and 24 hours. The times used for *C. transversalis* were 0, 1, 2, 4, 6, 8 and 24 hours. For *D. bellulus* 0, 0.5, 1, 2, 4, 6 and 8 hours and for *N. kinbergii* and *D. notescens* 0, 4, 6, 7 and 8 hours. There were three replicates of each time for each species. Controls included three or five unfed predators and three positives and negatives.

Blocking and predator weights

Three final tests were done on blocking methods to try and improve signal to noise ratios (background noise). A check was done using the blocking solution provided in the POD kit instead of BLOTTO, and leaving all steps of the assay out. The length of time BLOTTO was used was also tested (overnight) to see if this improved the results. The POD blocking solution was used at 1% for one hour at 37 °C. All tests were done on eggs. Finally, the weight of each predator species was determined by freezing each individual then weighing each using a Mettler AE 260 Deltarange balance.

7. 3 Results

All tests were recorded as successful when negative controls provided no false positives and positive controls provided a strong signal. Due to the large number of results (colour scans) figures are only provided to illustrate key changes in the protocol and to illustrate final results. The tests and species used when establishing the protocol are shown in Table 7.1. The results that instigated changes to the protocol are highlighted in the table. A final protocol is provided in Appendix 6.

Table 7.1 The trials done in establishing the monoclonal antibody protocol. * highlights where results changed the protocol.

Species Treatment	*H.armigera*	*N. kinbergii*	*D. bellulus*	*C.transversalis*	*H. octomaculata*
Haemolymph		✓	✓	✓	
Biotin *	✓	✓	✓	✓	
Fresh/frozen	✓	✓	✓	✓	
Fed/unfed			✓	✓	✓
Checkerboards *	✓				
Protien concentration *		✓	✓	✓	
Bleaching			✓	✓	
Substrates	✓		✓		
Supernatant	✓				
1:20 macerating * (fed and unfed)		✓	✓	✓	
H. punctigera	✓				

7.3.1 Protocol establishment

Cross reactivity

Predator haemolymph tests showed that no cross reactivity occurred with the MAb on *N. kinbergii*, *D. bellulus* and *C. transversalis* when haemolymph was extracted and used dilute in 10 µl PBST + BSA. The positive controls provided strong signals and there were no false positives on negative controls indicating the assay worked well.

Biotin/avidin test

Results of the biotin avidin test were similar to those of the polyclonal antibody. Non- specific binding to predator biotin was occurring. The conjugated antibody eliminated non- specific binding to biotins inherent in the predators but also reduced the sensitivity of the assay to a point where positive results were very faint. As a result heliothis egg checkerboard assays were redone using much lower ratios of antigen to homogenising solution.

Egg tests

The heliothis egg checkerboard tests showed that a single egg could be detected when homogenised in 10 µl up to 80 µl but the best signals were at 1:10 µl and 1:20 µl. A signal could not be detected at 160 µl (Figure 7.1). There was no discernible difference between fresh and frozen eggs in any of the tests on heliothis eggs. Using the ascites fluid at a ratio of 1:500 did not improve the strength of the signal. In the test on different heliothis species there was no reaction to *H. punctigera* indicating no cross reactivity would occur to this species. Despite the high dilution rate (1:200 µl) there was a faint signal for *H. armigera* eggs in the species test and no difference between the membranes in the intensity of the positive signals. There was no signal on the negative controls indicating the assay had worked. There was also no visual difference in signal strength between assays that used ascites and supernatant.

Predators

Early trials where predators were macerated at higher dilutions showed a distinction between fed and unfed *C. transversalis*, *H. octomaculata* and *D. bellulus*. However, these signals were very faint. Using a ratio of 1:20 µl to increase the sensitivity of the assay provided much stronger signals when tested on *N. kinbergii*, *D. bellulus* and *C. transversalis* and no difference occurred between fresh and frozen predators. However, the distinction between fed and unfed predators at this low dilution was hampered by a yellowing of the dots due to the excess lipid and melanization from the predators.

The final result showed a row of pink/yellow dots for fed and yellow dots for unfed. This made the distinction between the two treatments very subjective. Figure 7.2 highlights this problem.

Testing other substrates did not improve the distinction between the two treatments (fed and unfed predators). The signal from CN was also much fainter which, given the low sensitivity of the antibody, was likely to contribute to further problems. Precipitation of the protein did not provide a means of eliminating the problem of colouration of dots in any of the species tested, nor did bleaching with acetone or ethanol.

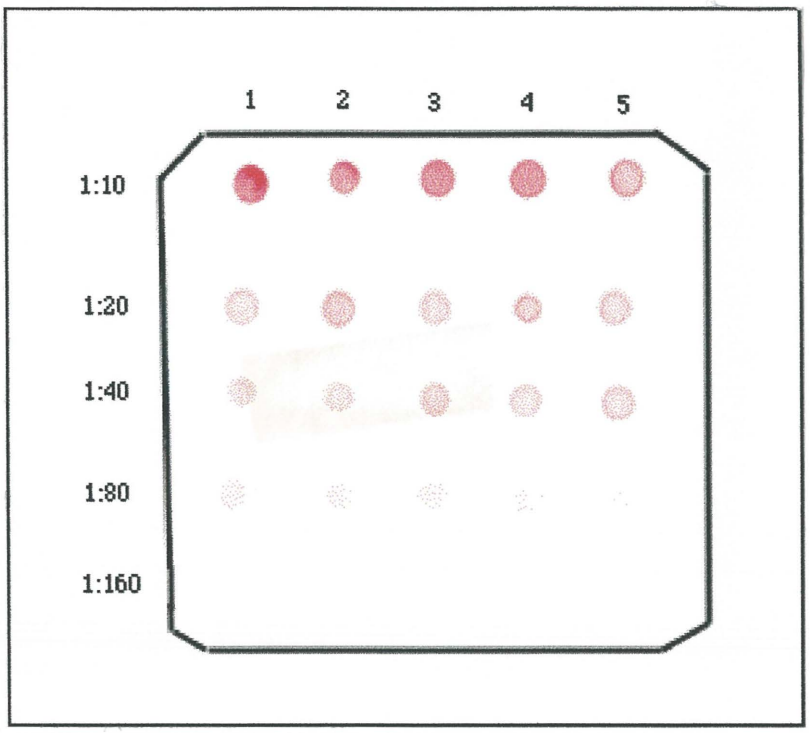

Figure 7.1 The heliothis egg checkerboard assay using the primary antibody at a ratio of 1:1000. Each number along a row represents an individual replicate and each row lists the dilution of a single egg to buffer (µl) that was used.

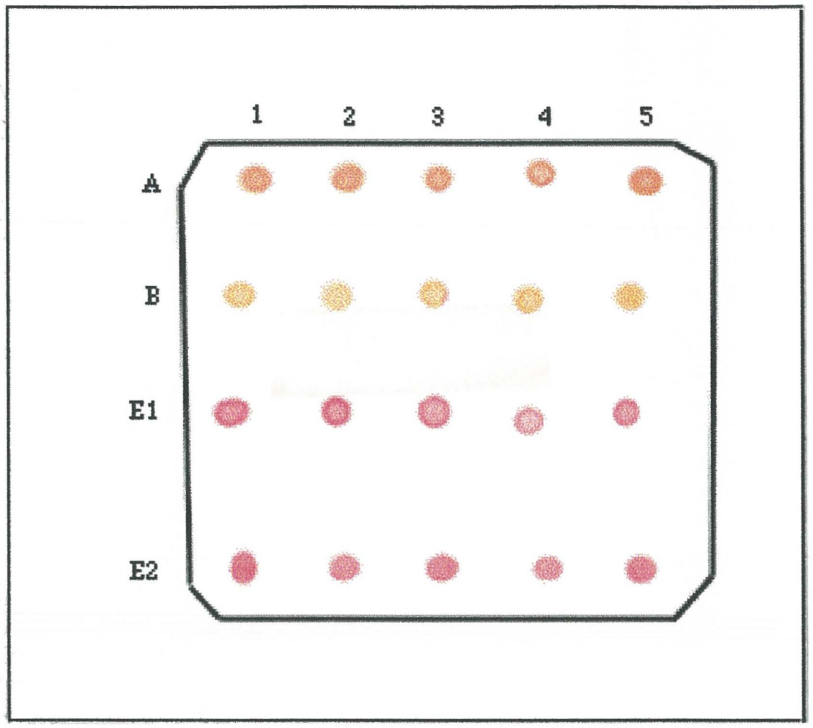

Figure 7.2 The effect of melanin on distinguishing between fed and unfed *D. bellulus*. A = fed, B = unfed, E1 = *H. armigera* eggs homogenised at 1:20 and E2 = *H. armigera* eggs prepared using protein concentration. Numbers across the top show replicates.

7.3.2 Chemiluminescence trials

Results from using the chemiluminescence substrate provided a complete distinction between fed and unfed predators (Figure 7.3). There was no signal for unfed predators and very dark signal for the fed predators. Controls showed the strength of the reactions were similar for positive and negative signals as those of fed and unfed predators (Figure 7.3).

Tests carried out using the chemiluminescence substrate are summarised in Table 7.2. All chemiluminescent trials except *M. frenata* produced a conclusive outcome. These tests were done using the final protocol outlined in Appendix 6.

Table 7.2 The trials done using the chemiluminescence substrate. + highlights tests that produced a conclusive outcome.

Species Treatment	*H.armigera*	*N.kinbergii*	*D.bellulus*	*C.transversalis*	*D. notescens*	*M. frenata*	*H. octomaculata* and *H. conformis*
Fresh/frozen	✓+	✓+	✓+	✓+			
Spiked fed		✓+	✓+	✓+			
Fed and unfed		✓+	✓+	✓+	✓+	✓	✓+
1/2 life 0-8 hours		✓+	✓+	✓+	✓+		
1/2 life 0-24 hours		✓+	✓+	✓+	✓+		
Alternate prey	Aphids, whitefly and cotton pollen+. Predator eggs +. *C. transversalis* larvae. +						

'Spiked' predators

Spiked predators showed that using the protein concentration method provided the strongest and most consistent signal of the three preparation treatments tested. Signals varied for the treatments without protein concentration. In *D. bellulus* two of the three signals at 1:10 µl were faint and all three at 1:100 were barely visible. In *C. transversalis* two of the signals at 1:100 µl were barely visible and in *N. kinbergii* one signal at 1:10 µl was faint. All species and treatments provided no signal for unfeds. Figure 7.4. shows a typical result of these trials.

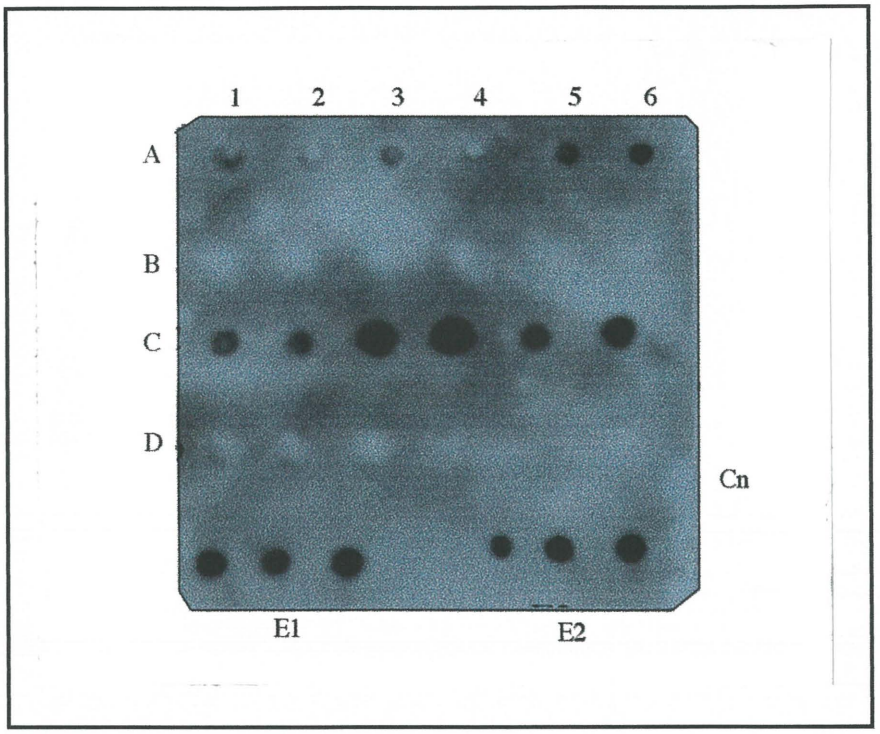

Figure 7.3 The first trial done using chemiluminescence on fed and unfed predators. A = fed *D. bellulus*, B = unfed *D. bellulus*, C = fed *C. transversalis* and D = unfed *C. transversalis*. Cn = three negative controls. E1 are three *H. armigera* eggs prepared using protein concentration and E2 = three eggs homogenised at 1:20. Numbers across the top indicate replicates.

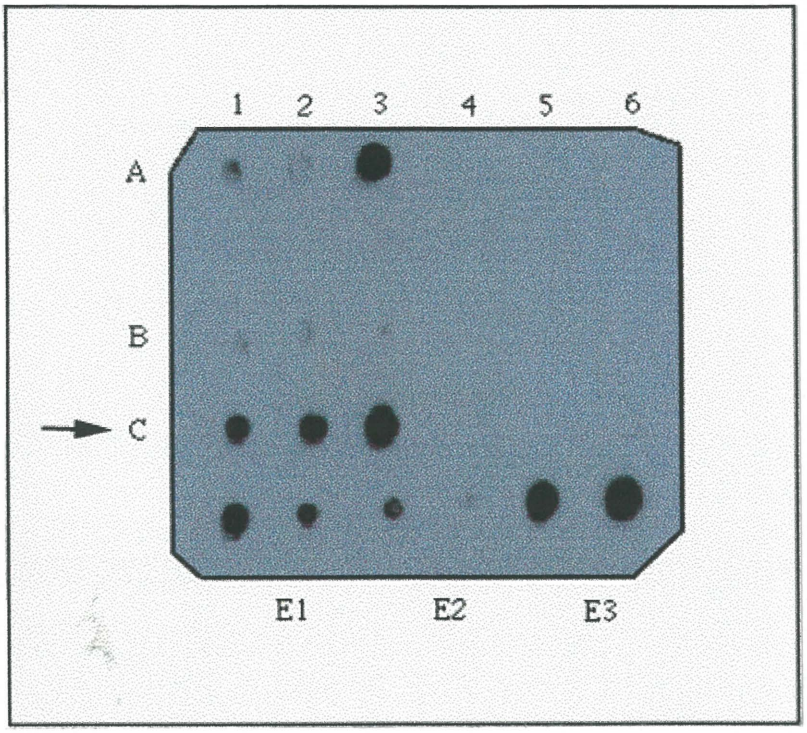

Figure 7.4 The difference between three preparation treatments on fed and unfed *D. bellulus*. A = homogenised at 1:20, B = homogenised at 1:100 and C = prepared with protein concentration. Numbers 4, 5 and 6 along the rows show where controls of unfed predator treatments were dotted onto the membrane. E1, 2 and 3 use heliothis eggs only but follow the same treatments as A, B and C respectively.

The fed and unfed trials worked well on all species tested with the exception of *M. frenata* which failed to provide a distinction between fed and unfed on two occasions. The majority of signals were positive for fed predators. However, in each species there were some instances where false negatives occurred. This is where a fed predator did not provide a signal. Figure 7.5 shows the results of fed and unfed *D. bellulus*. Note the faint signal (top arrow) for the fourth replicate. The optimal exposure times varied for each species and differed within some species due to background noise problems. The times tested and the optimal exposure time are shown in Table 7.3. The exposure times varied depending on the results from the 20 seconds exposure.

Table 7.3 Times tested and optimal exposure (**o**) times for each species when using the chemiluminescence substrate.

Exposure times (minutes) Species	0.3	1	2	4	5	6	8
N. kinbergii	✓**o**	✓		✓	✓		
D. bellulus	✓**o**	✓	✓				
C. transversalis	✓	✓	✓	✓**o**		✓	
D. notescens	✓**o**	✓			✓		
M. frenata	✓			✓			✓
H. conformis	✓**o**			✓			
H. octomaculata	✓**o**		✓		✓		

Alternative prey

Tests on alternative prey showed that there was no cross reactivity to aphids, whitefly and pollen. The tests on *C. transversalis* and *N. kinbergii* eggs were negative. The *C. transversalis* larvae that were unfed did not provide any signal, showing that no cross reactivity occurred, and the three fed *C. transversalis* larvae provided positive signals.

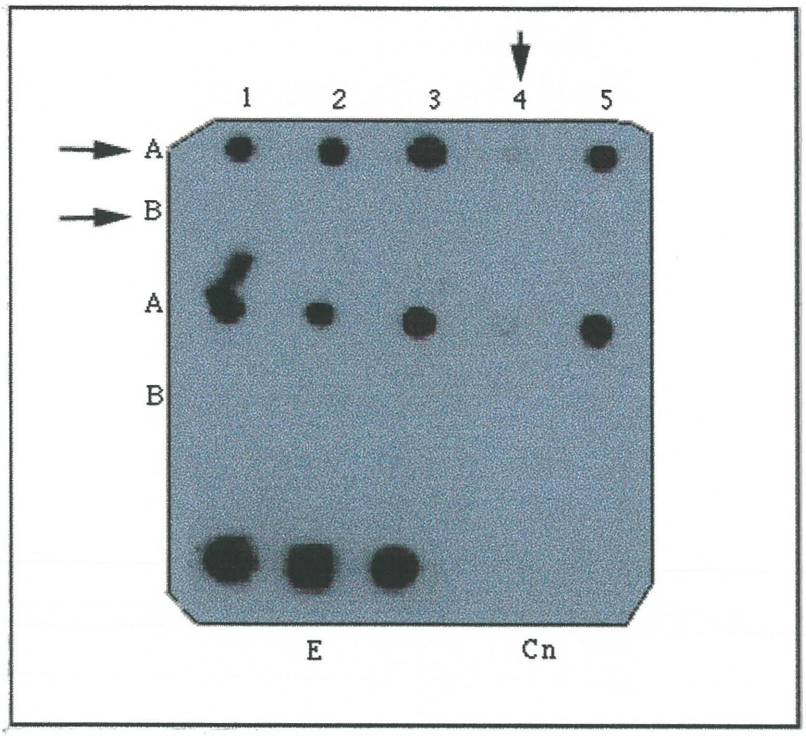

Figure 7.5 The distinction between fed and unfed *D. bellulus* using protein concentration and a chemiluminescent substrate. Side arrows show rows A = fed *D. bellulus* and B = unfed *D. bellulus*. E = *H. armigera* eggs and Cn = three negative controls. Numbers across the top indicate replicates.

Antigen half lives

The first antigen half life trials (0 – 72 hours) did not provide positive signals for any of the fed predators beyond eight hours. There were some signals present in *N. kinbergii* at zero and eight hours. In *D. notescens* all replicates at zero hours and one replicate at eight hours showed positive signals. In *D. bellulus* six zeros were positive and possibly some up to 24 hours. However, the background noise made results somewhat subjective. In *C. transversalis* only one zero showed positive. The controls provided correct signals (positive and negative) indicating the assay method had worked properly.

In the second series of half life trials (zero to eight hours) the majority of fed predators showed positive. *C. transversalis* showed positives up to eight hours and none for 24 hours. *D. bellulus* produced a similar result with most of the predators showing a signal up to and including eight hours after ingestion of an egg (Figure 7.6).

Nabis kinbergii showed positives from zero to eight hours. However, the number of false negatives was higher than the other species.

Blocking and false negative checks

Only the test with BLOTTO left out caused background noise. The use of BLOTTO overnight failed to improve background noise and the POD kit blocking solution made it worse. The check on the entire contents of one sample (aliquot) showed a positive signal for all dots from the sample indicating there was no variation as a result of the sub sample (1 µl).

Finally, in order to assess predator protein content the means and standard errors were calculated from each predator species' weights and plotted (Figure 7.7).

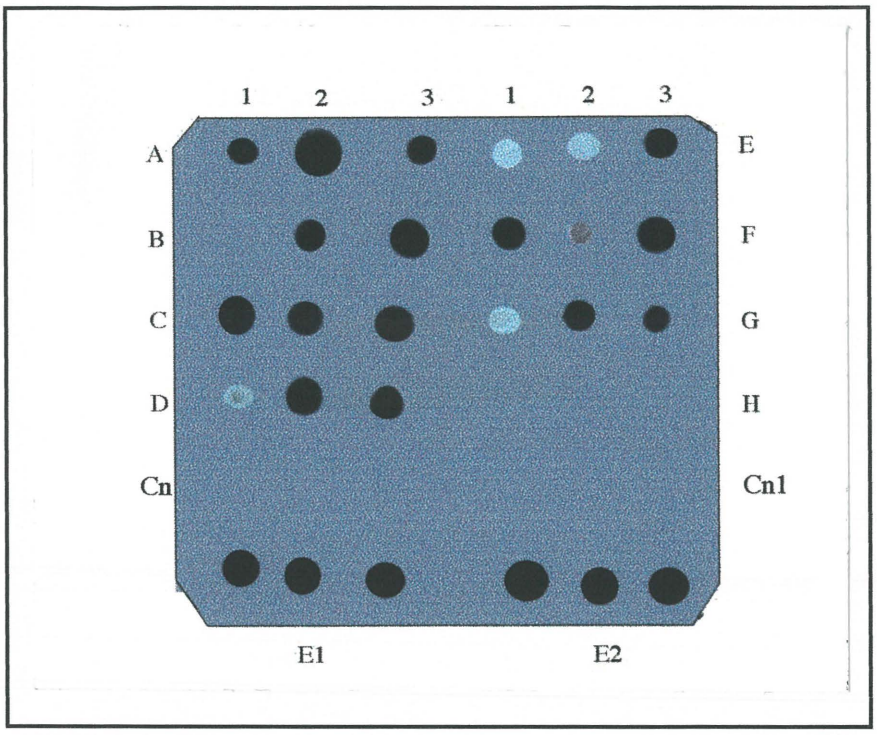

Figure 7.6 The antigen half life trial for *D. bellulus*. A = 0 hours, B = 0.5 hours, C = 1 hour, D = 2 hours, E = 4 hours, F = 6 hours and G = 8 hours. H = unfed *D. bellulus*. Pale dots indicate weak signals on the membrane. E1 = *H. armigera* eggs homogenised at 1:20 and E2 = eggs prepared with protein concentration. Cn and Cn1 are the same treatment as E1 and 2 respectively but without eggs.

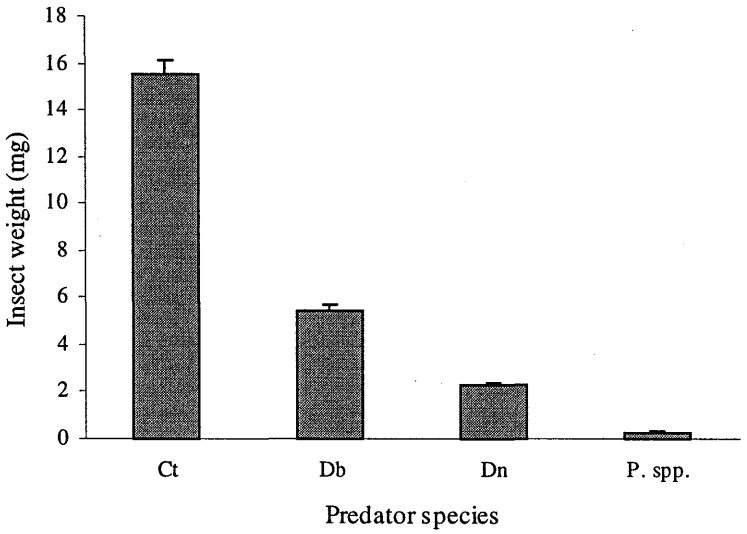

Figure 7.7 Mean weights of predators used in assays. Bars indicate standard errors. Ct = *C. transversalis* (n = 34), Db = *D. bellulus* (n = 14), Dn = *D. notescens* (n = 25) and P. spp = *Pheidole sp.* (n = 8).

7.4 Discussion

7.4.1 Protocol establishment

The tests run to establishment of the protocol showed that the MAb was suitable for use in detection of *H. armigera* in the guts of most predator species. There was no cross reactivity to predator haemolymph nor to alternative prey sources (including *H. punctigera*) indicating that the antibody was likely to be species specific. The main changes to the protocol were as a result of the strength of the antibody. Consequently the reagent ratios had to be adjusted and the approach to preparation of specimens was changed.

Sensitivity

Whilst the conjugated antibody eliminated the source of non-specific binding to biotins inherent in the predators it also revealed the antibody was far less sensitive than the polyclonal antibody. MAbs differ in their levels of sensitivity. The antibody used in this study had low sensitivity. It is possible that the sensitivity problem here could be overcome by testing other MAbs such as those used by Sigsgaard (1996) in India on *H. armigera* and others produced as

part of the development of the Lep Ton Test Kit (Trowell *et al* 1992). Provided these could be accessed, the MAbs could be rapidly screened for sensitivity using immunodot assays (Smith *et al.* 1984).

Substrates

The comparison of substrates in these trials showed chemiluminescence provided the best signal to noise ratio and was more sensitive than the other two substrates (3-AEC and CN). This partly agrees with the findings of Stuart and Greenstone (1996) who compared three substrates (CN/3,3'-diamino-benzidine, 3-AEC and chemiluminescence) on nitro-cellulose membranes. They found chemiluminescence was more sensitive than the other two substrates. They also found a problem distinguishing between coloration from melanin and substrates, although they suggested PTU or dilution of supernatant could alleviate this problem. They did suggest that the colour of 3-AEC would provide the best contrast if coloration by negatives could not be avoided. This was not the case in this study although a different antibody and insect complex was used. There are other detection methods available such as radioisotopes (Stott 1989). However, these were not available for this study and have problems associated with handling and exposure times.

7.4.2 Chemiluminescence trials

Using a chemiluminescence substrate provided a clear distinction between fed and unfed for all predator species with the exception *of M. frenata.* This highlights the importance of screening each species prior to field studies. The reason for the negative result in *M. frenata* is not known and it was beyond the limits of this study to investigate this further. All the other species tested worked well regardless of whether they were fresh or frozen, indicating they would be good candidates for field experiments.

Sources of variation - false negatives

In some of the tests false negatives occurred. False negatives are not uncommon in testing of predators and the number of occurrences can vary with predator species (Hagler *et al.* 1994). They can be attributed to several factors. High levels of false negatives in hemipteran may in part be attributed to what looks to be feeding but is only probing. Sigsgaard (1996) noted this with *Orius tantillus* (Motschulsky). To some extent this was overcome in this study by continuous observation under the microscope. The eggs shrivel upon sucking up of internal fluid leaving the chorion intact. Likewise the other species were watched continuously until an entire egg disappeared. There may also be some vomiting when predators are handled after feeding but again this was not observed. Consequently, non-feeding was not considered the main reason for the occurrence of false negatives in this study.

The only step in the preparation of samples that may have caused false negatives was the protein concentration method. Formation of pellets in the protein concentration was not always consistent. Whether this affects results is not known. Certainly the sub-sample taken from the homogenate did not affect the occurrence of a positive nor the strength of the positive. There are some methods that may help overcome or improve the concentration step. Gut dissection can improve signals by decreasing protein content. However, as mentioned in Chapter 6, when considering large numbers of predators for screening it is not recommended as it would be too time consuming (Hagler *et al.* 1997). Another way of decreasing unwanted protein content is by adjusting the volume of PBS (Stuart and Greenstone 1990). However, the sensitivity of the antibody needs to be considered as dilution will decrease sensitivity. In this case sensitivity and coloration was a major problem making the protein concentration step necessary.

Predator size

Another cause of false negatives may be the size of the predators (Hagler *et al.* 1997). Larger predators provide more false negatives than smaller ones. This is attributed to protein content of the predators (Hagler and Naranjo 1997). If samples exceeded 125 μg of protein consistent negative results were obtained (Hagler *et al.* 1997). The weights of the predators used in this study were similar to those in Hagler *et al.* (1997). The larger of the predators (the

coccinellids) weights fell in the same range as those of *H. convergens* whose protein content was well above 125 µg. This is likely to be the reason for false negatives occurring in the larger predators. However, false negatives also occurred in the smaller predators. Another source of false negatives may be attributed to interference from background noise. This is discussed below in the section on exposure times and background noise.

Sources of variation - signal strength

There was also some variation in the strength of the signals from fed predators. Variation in intensity of dots is not uncommon. The source of variation in strength of signal can be partly attributed to using mixed predator populations. Sopp and Sunderland (1989) attributed some of the variation in ELISA results on predators fed on aphids to the age, sex and development state of the predators they used. The predators in this study were all adults but their age and sex were mixed and their previous feeding history was unknown. Consequently, the size of each individual was likely to be variable. One source of variation may be the gut content prior to feeding. Sopp and Sunderland (1989) starved their insects up to ten days to eliminate all food in the predator guts. This would not be possible for all species. Mortality of *N. kinbergii* was high in the first few days after they were collected from the field so they needed to be fed as soon as possible after capture, allowing only a minimum starvation time. The amount consumed can also cause variance in results (Sigsgaard 1996) as can egg age (Hagler *et al.* 1992). The amount of prey did not vary in this study and eggs were always one day old so these are unlikely to be sources of variation. It is also unlikely that variation occurs as a result of variation on the membrane. Stuart and Greenstone (1990) noted an absence of membrane to membrane variation and edge effect in immunodot assay in contrast to ELISA.

7.4.3 Antigen half lives

Short detection times

As most studies assume a maximum 24 - 72 hour detection time for the prey antigen the reason that this study could detect prey for only eight hours needs to be considered. There are certainly species whose detection time seems to be within eight hours but there are also huge differences between species. Hagler and Cohen (1990) detected antigen (rabbit IgG) in *G.*

punctipes for up to 48 hours after ingestion when predators were held at 27 °C. Conversely, Hagler and Naranjo (1997) found 12 hours was the maximum detection time for *G. punctipes* when fed low numbers of *P. gossypiella* eggs. They also showed that detection of *P. gossypiella* eggs was negative after two hours for *H. convergens* and up to 15 hours for *O. insidiosus*. Symondson and Liddell (1993) showed detection times were vastly different between two carabid species feeding on the same prey in the laboratory. Although eight hours may be reasonable as a detection time it is difficult to compare these results as the antibodies may have different sensitivity than the one used here, the amount and type of prey varied and the assay methods differed.

Apart from rate of digestion, species differences and meal size there are some other factors that can affect the detection of antigen in the gut. Temperature plays an important role in antigen half life studies. Ideally it would be best to allow digestion to occur at fluctuating temperatures that reflect those found in the field. If this is not possible Sopp and Sunderland (1989) showed little difference occurred in detection time when predators were allowed to digest at fluctuating and constant temperatures. However, they did show a decline in prey antigen detection with increased temperature when using ELISA on aphids in guts of predators. Another factor affecting antigen half life is the feeding of predators prior to and after ingestion of the target prey. The rate of antigen decay was faster in the beetle *Pterostichus melanarius* (Illiger) if alternative prey was not provided (Symondson and Liddell 1996). Alternative prey was provided after ingestion of target prey by predators in this study and the detection time of nabids was similar to the other non-ambush species.

Insect type and digestion

As mentioned above there is variation between species of predators but there may also be variation in the feeding type and gender of the predators. The digestion in sucking insects is different to those with chewing mouthparts as digestion may start through enzymes injected into the food. This means that antigens may be broken down much sooner than insects with chewing mouth parts, resulting in a lower antigen detection time. Hagler and Cohen (1990) thought this might occur with *G. punctipes*. However, they found that the digestion time was sufficient to justify further studies on predation. In this study there was no apparent difference in detectability after ingestion between *N. kinbergii* when compared with the chewing predator species. Behavioural strategies may also affect digestion times. Ambush predators (such as

nabids and spiders) may slow their digestion when prey are in low numbers as a survival strategy (Symondson and Liddell 1996, Sopp and Sunderland 1989). This will prolong antigen detection time. Gender may also make a difference to antigen half lives. Symondson and Liddell (1993) compared male and female antigen decay rates in crops of carabids. They suggested female carabids ate more due to their larger size (sexual dimorphism), resulting in a longer detection time.

The factors mentioned above, such as insect type and temperature, were not noted as sources of inaccuracy in this study. However, when species and size (protein content) are considered with antigen half life and development of assays it may help to explain the short detection time and occurrence of false negatives in this study.

False negatives in digestion times

Ohiagu and Boreham (1978) were able to detect aphid antigen in the guts of *C. septempunctata* for up to 48 hours. The number of positives declined with time from 88% in the first hour to 25% at 48 hours. They attribute the lack of 100% positives to not all the coccinellids feeding. However, Hagler and Naranjo (1997) showed that the number of false negatives increased not only with predator size but also with time. With low replication this can mean that no positives may occur as a result of inaccuracy rather than complete digestion of the antigen.

Exposure times and background noise as a source of variation

Due to the variation in optimal exposure time between species and within species the best method in further studies would be to expose the membranes for 20 seconds and then increase the exposure time by one minute intervals until false positives appear. This needs to be done in future studies until the background noise problems can be rectified. In some cases background noise occurred before false positives did. The detection of positives may have been hidden by background noise. This may partly explain the lower detection times of antigen half life, as greater exposure was required to obtain a signal for dots of low sensitivity. Although some attempt was made to reduce background noise by improving blocking it still remained a problem.

7.4.4 Conclusion

This study provided a immunodot assay protocol that enables a researcher to detect whether predators have fed on a minimum of one *H. armigera* egg within an eight hour time frame. The best method for detection of the prey antigen was by concentrating the sample protein and using chemiluminescence as a substrate detection system.

Many of the factors such as temperature, prey and predator type, that influence antigen detection time were accounted for in preparation of predators for this study. It is also difficult to compare digestion times of similar species when the antibodies and assay techniques are different between studies, so whether eight hours is truly the digestion time of these predators is not known. The reason for the short detection time is more likely to be a combination of low sensitivity of the MAb and an increase in the number of false negatives with digestion time. Either way the eight hour detection time is sufficient to allow testing of field collected predators.

Whilst the method detects fed predators for up to eight hours after feeding, there are some limitations that could be improved upon, thus increasing the efficiency and accuracy of the assays. Preparation of homogenates was slow and could be improved with better equipment such as multi-pipette dispensers and further work may enable the exclusion of the protein concentration step. Feeding trials are also time consuming. Sigsgaard (1996) used excessive individuals to start with in order to obtain enough for assay work. This can only occur when there are enough predators available. The protein concentration step solved the problem of homogenising at small ratios but the accuracy of this step was not known. This warrants further investigation as does the use of other antibodies if available. At times background noise (in the developing step) obscured results. The cause of background noise needs to be further investigated and reduced. Despite these limitations the method was working sufficiently well to enable testing in the field to go ahead. The following chapter is a preliminary study on using this method on predators collected directly from the field.

Chapter 8

8. Investigating immunodot assay as a means of detecting predation by predators of *Helicoverpa armigera* in cotton.

8.1 Introduction

Using immunodot assay to detect *H. armigera* eggs in the guts of predators found in cotton showed promise in the laboratory (Chapter 7). However, it is important to test the usefulness of the method in the field. Doing a field test provides information on the practicality of the method (collecting and sorting methods), the type of data that can be obtained (insect populations, stage and gender) and which species of predator are feeding at the time collections are made (predation levels). The only other study of this type on predation of *H. armigera* was done by Sigsgaard (1996) on pigeon pea in India. To date no serological study has been conducted using immunodot assay on predators collected directly from crops in Australia.

The types of data obtained in other studies that investigate predation by using serological assay include not only differences between species but also differences between gender and stage. Ragsdale *et al.* (1981) included the distinction between coccinellid larvae and adults in their studies on predation of the green vegetable bug *Nezara viridula* (L.) in soybeans. Hagler and Naranjo (1996) included a comparison between nymphs and adults of the predator *L. hesperus* as part of the predator complex they assessed in cotton. Symondson and Liddell (1993) compared male and female carabid (*Abax parallelepipedus* Piller and Mitterpacher) predation levels on Mollusca (slugs). Information on gender and stage differences in predation can provide useful additional information in assessing different predator species. Obtaining such information is dependent on being able to quickly identify gender and stage differences with external morphological characteristics; in some cases immature species may not be available or easily identified (Stanley 1997).

Collecting and processing methods depend on the abundance of the predator population and the processing resources available, especially if field sites are distant. In general a suction sampler (D-vac) is used for sampling of predators in cotton (Stanley 1997). This is suitable as a collection method for most predators but can cause contamination of predator samples if high target prey numbers are present. Washing the predators in phosphate buffered saline (PBS) can eliminate contamination (Hagler and Naranjo 1994b).

Hagler and Naranjo (1996) sampled 120 metres / 2 hectares of cotton in the USA. In India, Sigsgaard (1996) sampled 71 metres of pigeon pea per sample date, in a crop 72 hectares in size. In both studies many more predators were found per sample than would be expected in Australian cotton. In both studies collections provided around 15 predators per suction sample. Numbers of predators per metre are generally much lower in Australia particularly when pyrethroid spraying occurs (Stanley 1997) and one is only assessing particular species of predator rather than the total predator complex. Any sampling strategy therefore, will need adjustment to enable enough predators to be collected.

The method of predator storage and sorting is also an important consideration in field studies that utilise serological assays. Insects need to be frozen immediately after collection to stop further digestion of target prey occurring and avoid denaturing of any target protein in dead insects. Sigsgaard (1996) transported field samples in an ice box at about 11 °C, transferred them to a 2 °C refrigerator and then to a –80 °C freezer. Hagler and Naranjo (1996) placed predators on dry ice in the field, immediately after collection, and then stored them in a laboratory freezer at –80 °C.

8.1.1 Purpose of this study

The purpose of this study was to determine the best sampling strategy for using immunodot assay on predators in Australian cotton. This had to account for a 300 km distance from the field site to the laboratory. Consequently, storage of specimens in the field was an important consideration. As well as the best sampling strategy, the method would help determine which predator species were available at the time and how many samples were needed to collect enough of each species. It would also provide an indication of which predator species feed on

H. armigera thereby enabling an assessment of the role of individual predator species in cotton.

8.2 Methods

To obtain the equivalent predator numbers of the overseas studies shown earlier in this chapter, each site in Australian cotton should be sampled at a rate of at least 200 metres / 2 hectares (about 10 x 20 metre suction samples per site). This provides a good coverage of the site and obtains a good sample population of predators.

Data collection - Wheat Research Station

Two sampling strategies were tested on unsprayed cotton sites. Firstly in the 1996/7 season unsprayed cotton was sampled at the Wheat Research Station (WRS), near Narrabri (see Figure 2.4 in Chapter 2 for location details). Four trips were made during the season when cotton was at early pre-squaring to early boll maturation stages. On each occasion a Homelite® D-vac suction sampler was used. Twenty x 20 metre rows of cotton were sampled in order to collect predators for future assay. At the time of these trips the sampling and sorting method was based on being able to detect prey antigen of both *H. armigera* and *H. punctigera* and assumed an antigen detection time of up to 24 hours after consumption; an assumption that is made by other researchers (Chapter 7). On the morning of each sampling occasion a bug check was done to ensure heliothis were present in the field. This was done using the protocol from the entomoLOGIC 1996 manual (Anon 1996a). A total of five metres of cotton were checked over the experimental site. Although the polyclonal antibody (tested in Chapter 6) could not be used for detection of predation on *H. punctigera* and the 24 hour antigen detection time was not accurate (Chapter 7), samples were still assayed as *H. armigera* were present in low numbers during the season. Consequently, any predators that had eaten in the eight hour detection time (determined in Chapter 7) would provide positive signals when assayed with the monoclonal antibody.

Sampling for predators at the WRS was done between 9:00 and 12:00 am on each occasion. Samples were then stored in an ice filled cooler (12 - 14 ^{o}C) and taken to a field laboratory

where they were sorted, placed into 2 ml eppendorf tubes, labelled and stored in liquid nitrogen for future assay. Sorting was required to avoid contamination from squashed target larvae and egg prey as a result of suction sampling and to enable counts of populations of predators and alternate prey. Storing predators individually is also recommended as it is unwise to thaw whole samples of insects at once as lengthy thawing may denature the egg protein required for assay (C. Fernon pers. comm). The total number of predator species and any prey other than heliothis (alternate prey) in each sample were also recorded to indicate the abundance of each in the field. Similar collections were made from conventionally sprayed cotton crops. However, these samples did not yield enough predators to enable useful information to be obtained so they were not assayed.

As the results in Chapter 4 indicated that plant size affected predation levels, the plant height and leaf area were also recorded on each field trip. If the abundance of alternative prey on pre-squaring plants does not differ with plant size and the detected predation rates vary collecting this information will enable more accurate interpretation of results.

Data collection - Australian Cotton Research Institute

In the following season (1997/1998), towards the end of this study, and after more laboratory feeding trials to test prey detection time, another field trip was completed. This study was conducted at the Australian Cotton Research Institute (ACRI) (see Figure 2.4 in Chapter 2 for location details) and ran over one week from the 14th – 21st of December 1997. The aim of this trip was to test the immunodot method using the monoclonal antibody on predation of *H. armigera*. As *H. armigera* are scarce at this time of year and, to ensure that the method could be thoroughly tested, *H. armigera* eggs were 'seeded' into the field on every second day during the week. To do this, adult moths reared in culture (UNE) were transported in a laying cage to the field site. Eggs laid on paper towel each night were collected in the early afternoon, cut into squares so that an average of 20 eggs were on each piece of paper towel. In the early evening each piece of paper towel was stapled on to the second open leaf of a cotton plant at one metre intervals along randomly pre-selected rows of unsprayed cotton. Different rows and plots were randomly selected on each day.

The next morning between 9:00 and 11:00 am the paper towel pieces were removed from the plants and stored in a field freezer. At the same time any predators feeding on eggs on the papers were collected and frozen, prior to suction sampling. As in the previous season, a preliminary bug check (Anon 1996a) was done to test for species of heliothis present in the field. Any naturally laid heliothis eggs found during the bug check were collected and frozen for future assay. Finally fifteen x 20 metre rows of cotton were suction sampled. Samples were frozen immediately in a portable car freezer, taken back to a field laboratory, sorted whilst still frozen and snap frozen in liquid nitrogen. All other invertebrates were stored in 70% ethanol for future sorting. This gave an estimation of what other prey was available at the time of sampling.

Preparation and assaying of field collected insects

After the field trip, samples were prepared as outlined in Chapter 7. The gender (if distinguishable) was recorded for each individual and all predators were assayed using the final protocol outlined in Appendix 6. Each assay included a positive control of *H. armigera* eggs, negative controls of field collected predators fed on aphids and pollen but starved of heliothis for at least one week. A negative control of homogenising solution without predator or prey was also included. Each membrane held 35 predators and two were prepared each day. Where possible the same species were put on the same membrane. The membranes were allowed to dry and assayed the following day. In the chemiluminescence step (Appendix 6) the membranes were exposed from 20 seconds until false negatives appeared. The x-ray that showed no false positives on the controls and the most positives was used for evaluation of predation. The number of positive reactions on each membrane were recorded.

8.3 Results

Wheat Research Station

It was expected that due to the low numbers of *H. armigera* in the field and the assumption of a 24 hour sampling strategy that very few positive signals would occur from the sampling at the WRS. On the first trip no *H. armigera* were present. Consequently no positive signals to

predation on *H. armigera* were to be expected. These samples were not assayed. The bug checks showed that on the second trip only low numbers of *H. armigera* eggs were present (three out of 20 eggs collected). There were more eggs present in the field in the third and fourth trip but few of these were *H. armigera* (Table 8.1).

The abundance of predator species varied with each sampling date at the WRS (Table 8.1). The major species of predators present in the second trip (15/12/96) at the WRS were *D. bellulus, D. notescens, C. transversalis* and *Chrysopa* spp. In the third trip (14/01/97) *D. bellulus* was the most common species and in the fourth trip (24/02/97) *D. bellulus* and *Chrysopa* spp. The only species that provided positive signals (indicating predators that had fed on *H. armigera* eggs) were six *D. notescens* in the second trip and one *Chrysopa* larvae in the third trip. Assay controls showed that there were strong signals for positive controls (eggs) and no signal for negative controls. This indicated that there was nothing wrong with the assay process. With so few positives to work with plant height and gender data were not analysed.

Table 8.1 The abundance of predators found in unsprayed cotton (400m) at the Wheat Research Station in the 1996/7 growing season. Numbers in brackets indicate the percent of predators scoring positive to *H. armigera* antigen. The last row provides the number of heliothis egg samples collected in the bug check and the number (in bold) of those that were *H. armigera.*

Species / Date	**2 -15/12/96**	**3 -14/01/97**	**4- 24/02/97**
N. kinbergii	2	14	1
D. bellulus	18	113	15
D. notescens	**6**/15 (40%)	7	-
C. transversalis	14	3	-
Chrysopa spp. larvae	11	**1**/28 (3.5%)	11
Creontiades dilutus	-	-	5
C. transversalis larvae	-	-	1
Geocoris lubra	-	-	2
Egg species	**3**/20	**7** /30	**5** /30

Australian Cotton Research Institute

In contrast, predators sampled in the following season at the ACRI provided a much higher proportion of positives for each species. Table 8.2 outlines the abundance of each species and the number of positives from each collection assay. *Harmonia octomaculata* and *D. notescens* were the most abundant species found during this field trip. The gender of these two species was not easily distinguished without a microscope and therefore not recorded. The gender of *C. transversalis* and *D. bellulus* were recorded. However, no differences occurred in the number of positives for gender in each species.

Table 8.2 The abundance of predators and *H. armigera* eggs in cotton (300m) each day at ACRI on the 1997/8 field trip. **A** = the number of predators that scored positive to *H. armigera* antigen out of the total number collected each day. **B** = the percent of each population scoring positive in the immunodot assays. **C** = indicates the number of predators which were taken directly from the pieces of paper towel; all of these scored positive. Numbers in the bottom row indicate the number of naturally occuring eggs collected during the preliminary bug check that were *H. armigera* (bold).

Sample Day	**15/12/97**			**17/12/97**			**20/12/97**		
Species	A	B	C	A	B	C	A	B	C
H. octomaculata	11/28	39%	2	14/43	32%	1	19/35	54%	3
C. transversalis	3/7	43%	1	2/8	25%	-	0/6	0%	-
D. bellulus	0/9	0%	-	2/7	28%	2	0/8	0%	-
N. kinbergii	0/1	0%	-	-	-	-	-	-	-
D. notescens	42/49	86%	2	114/132	86%	2	6/72	8%	-
Iridomymex vicinus	1/2	50%	1	5/5	100%	3	3/3	100%	3
Pheidole sp.	5/5	100%	5	2/2	100%	2	-	-	-
Geocoris lubra	1/3	33%	-	1/2	50%	-	0/3	0%	-
H. armigera	**0**/3			**2**/4			**1**/2		

The eggs tested from the bug checks in the 1997/8 season showed that as well as on the papers, *H. armigera* were also present in a natural population on two of the three days. All of the predators collected directly from the paper containing eggs showed strong positive reaction to *H. armigera* antigen (Table 8.2, C). The exception to this was one ant (*I. vicinus*). No negatives were provided in these trials for ants although a later test showed no false positives occurred on unfed ants. Other individuals that showed positives were a mantid, flower beetle and rutherglen bug. No negative controls were provided for these.

All the paper towel pieces recovered after the experiment on each day were categorised as the percent of eggs missing per paper (0, 25, 50, 75, 100%). Figure 8.1 shows the percentage of papers that fell into each category on each sampling date. For example on the first sampling date 46% of papers had 100% of the eggs missing after being in the field c.17 hours.

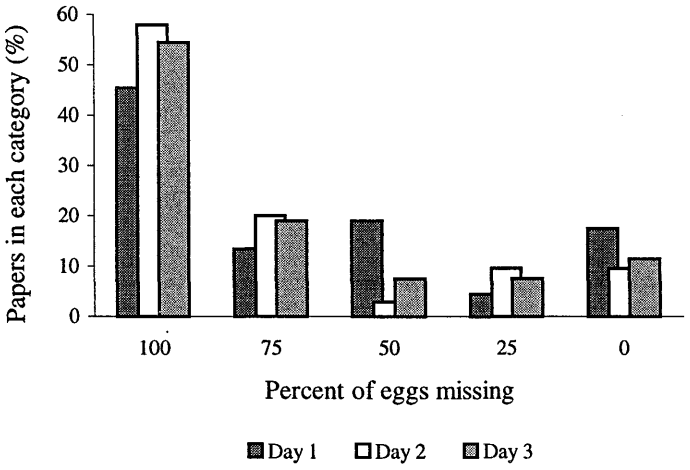

Figure 8.1 The percentage of papers that had 100, 75, 50, 25 and 0 percent of *H. armigera* eggs missing on each sampling day.

Alternative prey available at each site

The number and species of alternate prey on each trip to the WRS are outlined in Table 8.3. In all cases, green and brown jassids (*A. viridigriesea* and *Amrasca terraereginae* Paoli respectively) were the most abundant species of alternative prey present. No attempt was made to distinguish species of larvae, leaf hoppers and small flies as it was their grouped abundance that was important. Other prey included immature hemipteran and small coleopterans.

Table 8.3 The abundance and types of alternate prey on each trip in the 1996/7 season. Numbers in brackets indicate the mean per D-vac suction sample (n = 20, 1 D-vac sample = 20 metres of cotton)

Prey / Date	2 - 14/12/96	3 - 14/01/97	4 - 24/02/97
Green jassids	487 (24.35)	110 (5.5)	467 (23.35)
Brown jassids	636 (31.8)	51 (2.55)	270 (13.5)
Small larvae	3 (0.15)	13 (0.65)	12 (0.6)
Leaf hoppers	26 (1.3)	20 (1)	29 (1.45)
Small flies	30 (1.5)	1564 (78.2)	520 (26)
Others	11 small beetles, 4 immature bugs	3 small beetles	8 immature bugs

At the ACRI jassids were again the most abundant alternative prey available. Leafhoppers, small larvae and dipterans were also present. The main difference between the two seasons was the presence of whitefly in low numbers at the ACRI (Table 8.4).

Table 8.4 The total abundance and types of alternative prey per sample date in the 1997\8 season field trip. Numbers in brackets indicate the mean per D-vac suction sample (n = 15, 1 D-Vac sample = 20 metres of cotton).

Species / Sample date	15/12/97	17/12/97	20/12/97
Green jassids	114 (11.4)	85 (8.5)	170 (17.0)
Brown jassids	182 (18.2)	145 (14.5)	136 (13.6)
Leaf hoppers	5 (0.5)	4 (0.4)	8 (0.8)
White fly	32 (3.2)	19 (1.9)	-
Small dipterans	402 (40.2)	345 (34.5)	468 (46.8)
Small larvae	6 (0.6)	2 (0.2)	5 (0.5)
Others	9 immature bugs, 1 flea beetle	15 immature bugs	8 immature bugs

8.4 Discussion

The immunodot method established to detect *H. armigera* antigen in the guts of predators was able to detect predation in the field when *H. armigera* eggs were both in naturally occurring low numbers and when they were 'seeded' into the cotton crop. Data from the ACRI field trip provided some useful preliminary information on differences between the number of each predator species that were consuming *H. armigera*. Testing the sampling method over the two seasons provided some useful information on ways in which the method could be improved to obtain better information on predation. As the accuracy of the method at the WRS was limited by the sampling strategy used (sample size and limited numbers of field trips) and the sensitivity of the test (eight hours detection time) the majority of these improvements are based on the field trip in December 1997. The improvements are discussed below.

8.4.1 Practical application of the method

In the study on the 1997/8 trip two species were available in sufficient numbers to make comparisons between days. The species were *H. octomaculata* and *D. notescens.* Predation was also detected in other predator species although the proportion of positives seems high in these species when one considers the number collected in each day (Table 8.2). The role of each of these species is discussed in section 8.4.4. The low sample size of predators made interpretation of the results difficult for some species and trips. This is not unusual. In their study of predation of *C. brunneus* and *M. maculatus* by spiders Cherrill and Begon (1989) found that only one species was captured in enough numbers to make comparisons of predation between sites. To aid in interpretation of data in future collections the results may be improved by making comparisons on predator species that naturally occur in large numbers or increase sample size and dates to obtain larger numbers of predators. As this sampling was early in the season before any pyrethroid sprays had been used numbers of naturally occurring predators were higher than would be found as the season progressed. Consequently, a greater increase in sample size would be the better method of increasing the number of predators collected.

Other studies have also been hampered by pesticide application in cotton and small numbers of predators were overcome by pooling data from sites (Hagler and Naranjo 1994b). They conducted weekly samples until pesticides destroyed the predator complex. Their study still provided useful information on predation. The range of predation positives was highly variable from 1.6% to 68% from week to week (Hagler and Naranjo 1994b). This highlights the importance of continuous assessment of predators throughout the season. Although an increase in sample size or dates was not possible at the time of this study it would be feasible to do this in future studies.

Better equipment would also enable greater numbers of samples to be processed. Hagler and Naranjo (1994b) could assay up to 1000 predators per day. This is of course is also dependant on the quality of equipment and available labour. These two factors account for the lower numbers of predators that were able to be processed here. Also, as mentioned in Chapter 7 the extra protein concentration step slowed the procedure down. The distinction between genders was easy on *D. bellulus* and *N. kinbergii* and these could easily be recorded as each individual was being assayed. However, although the species of coccinellids in this study can be sexed by external features (Leeper 1976 & Pope 1988), doing so was much slower as a light microscope was required to see each diagnostic feature. A source of dry ice would allow sexing to be done without fear of lengthy thawing and storage of specimens in a –80 °C freezer would cut costs of liquid nitrogen.

Given an eight hour detection time for the prey antigen, 15 suction samples could be sorted, labelled and stored within two hours after collecting. If dry ice were available this would be the most efficient way of handling field material as greater numbers of samples could be sorted without fear of the predators thawing. Another way of collecting some predator species may be with the use of liquid nitrogen traps (Lund and Turpin 1977). This is particularly useful for ground dwelling predators such as *D. bellulus*. However, it is necessary to replenish the liquid nitrogen every 12 hours so a supply at close proximity to the field site is required. Storage space is also an important consideration as only one large liquid nitrogen container was available for use. Again this depends on the locality of field sites and the availability of storage facilities. In this case the field site was 300 km away so liquid nitrogen was the most suitable storage method available.

8.4.2 Assay sensitivity

There are several factors that can and did affect the results of the assays. Field temperature can affect the detection time of antigen as digestion rates vary with temperature. However, even at 40 °C for 24 hours Hagler and Cohen (1990) were still able to detect antigen in the guts of *G. punctipes* despite a marked decline in detectability above 25 °C. The field temperature is therefore an important consideration for determining the time of day that sampling is done. As the sampling in this study was done in the morning it was not expected that field temperatures would decrease the detection time of the antigen. The low detection time of the antibody (eight hours) was important in interpreting the results from the WRS and in determining when sampling should be done.

As mentioned earlier, the trips at the WRS were based on a 24 hour sampling time for the antigen half life detection. The predators were therefore not frozen immediately in the field and more time was taken to collect the predators. This, and the low prey numbers, probably accounts for the very low numbers of positives found here. As discussed in Chapter 7 improved sensitivity of the antibody to allow for a 24 hour sampling strategy would be of advantage as it would enable more meaningful data on predation to be obtained and allow a more flexible sampling time. In particular, if some predator species prefer to feed between dusk and early morning they would not show up within an eight hour detection time. The time that predators fed was not known in this study although searching activity throughout the day and night was noted on one occasion for *N. kinbergii, D. bellulus and C. transversalis* (personal observation). Increasing the strength of the antibody and improving signal to noise ratios would aid in lengthening the prey detection time. Investigation of other antibodies that provide stronger signals would also be useful. Nevertheless, given a low detection time this antibody was still able to detect predation. It was not able to provide enough comparative information on predation at the WRS. Further discussion on predation at the WRS is outlined below in section 8.4.4. The use of eggs in the next season at the ACRI provided much more information on predation.

8.4.3 Egg usage

The immunodot method did provide positives in both sampling seasons. The small number of positives in the first year can be attributed to the 24 hour sampling strategy and low to nil *H. armigera* numbers at the time of sampling. More intensive sampling when *H. armigera* egg lays occur would yield more useful information on predation. Sigsgaard (1996) began sampling at the onset of oviposition on pigeon pea in India and continued every three days for one month when there were up to eight eggs per plant. Such sampling is only feasible when the field sites are in close proximity to the laboratory.

Alternatively, if access to the field is limited, 'seeding' the crop with heliothis can provide useful information. This also allows for prey density studies to be done directly in the field. If the number of positives from assay results are higher when alternative or target prey is more abundant it implies that prey abundance is the factor influencing predation. In this study, in the second season, placing 20 eggs per metre increased the chance of predators feeding. Also there was a background *H. armigera* population (although it was low in number) meaning that predators could have been feeding on the target prey in rows other than those where eggs had been placed. A better way of 'seeding' eggs to reflect normal oviposition sites would be of advantage.

Although the ACRI trial did not include different densities of target prey it did provide a means of determining which predators were feeding at the time samples were taken. The results were further enhanced as insects observed feeding on the paper and collected, acted as positive controls. The fact that other predators from the suction samples were also positive shows the method will work on predators that have fed some time in the eight hours before collection.

8.4.4 Predation on *H. armigera* eggs

Little information can be gained on predation from the WRS results other than the fact that *D. notescens* and *Chrysopa* larvae feed on *H. armigera* when they occur naturally in low numbers in the field. It is important to evaluate prey density as it aids in interpretation of results. In their studies on predation of whitefly and pink bollworm, Hagler and Naranjo

(1996) put low predation of pink bollworm down to low egg densities in the field. The comparison of alternative prey between sites in this study does not suggest that it was abundance of other sources of prey that may have caused such low predation detection, rather that the target prey was in very low numbers.

At the ACRI the initial trial showed the predators *H. octomaculata* and *D. notescens* were feeding on *H. armigera* eggs. The proportion that fed was high but varied with the sample day. This was particularly noticeable in *D. notescens*. The reason for this difference was not known. However, the weather may have played a role here as high winds and rain occurred in the two days before the last sample date. *D. notescens* numbers decline with heavy rain and high wind (personal observation). This may have caused the low predation rate on the third day as they may take time to recover from poor weather conditions. Sigsgaard's (1996) results on predation of *H. armigera* also showed variation in the percentage of predators of one species scoring positive every three to five days. In spite of the difference exhibited on the third day of sampling the data does show that *D. notescens* has potential as a predator of *H. armigera.*

Another reason that can cause variation in results from day to day is the prey complex. The high rate of predation by *C. imornatum* on *H. armigera* on one occasion was attributed to the lack of alternate prey (Sigsgaard 1996). This highlights the necessity of knowing the abundance of alternate prey to aid in interpreting predation results. Ragsdale *et al.* (1981) also considered the presence of alternative prey to play a role in the variation in predation levels. Hagler *et al.* (1992) looked at predation on *L. hesperus* by several predators. The percentage positives from a sample of 143 was 35% for *N. alternatus* down to 0% (n = 18) for *Sinea confusa* Caudell. Their findings support the notion that some predator species are generalist and therefore unlikely to feed solely on the target prey (unless switching occurs). There is no clear evidence of switching with the species used in this study (Chapter 5) and the results from the alternative prey samples (Table 8.4) indicate that prey numbers and types did not differ dramatically from each date. It is therefore likely that the predators were feeding on *H. armigera* eggs on a particular day in spite of changes to other prey populations or types.

The high proportion of *H. octomaculata* scoring positive is also of interest as this species is generally considered an aphid predator (Pope 1988). Given the high number of individuals that were found to feed on *H. armigera* this species is worthy of further investigation.

Ants were observed feeding on the papers every day in the 1997/8 season trip. They certainly warrant further investigation as major predators in the field. The species tested here were *I. vicinus* and *Pheidole sp.* These are the more common ant species found in cotton fields and both are plant canopy climbers (J. Lytton-Hitchins pers. comm.). Although these species are thought to be reduced with irrigation, their ability to move nests to the upper plant canopy during these times has been observed in Australian cotton. McDaniel and Sterling (1982) suggested the fire ant *Solenopsis invicta* Buren contributes to maintenance of heliothis species below economic thresholds in the US. Sigsgaard (1996) also suggests ants may be important in small fields of pigeon pea against *H. armigera.*

The abundance of predator species is also an important consideration when interpreting results. Greenstone (1996) refers to several studies where the most abundant species of predators were not necessarily the main feeders on a target prey. As mentioned earlier a larger sample size may aid in obtaining larger numbers of predators. More intensive sampling to see if results are consistent would help to understand the role of less abundant species. Weekly or bi-weekly intervals seem to be the most common time frame for sampling (Sigsgaard 1996, Hagler and Naranjo 1994b & Sunderland *et al.* 1987). Given the variation on the third day an even more intensive sampling regime may be necessary in Australian cotton.

Predation from eggs on paper towel

The results from the papers that had eggs on them cannot be overlooked. The predators were certainly feeding on the eggs off the papers and the number of papers with 100% loss of eggs was consistent with each sample day despite variation in weather conditions. There may have been some loss due to abiotic factors such as wind and rain (Dillon *et al.* 1992, Titmarsh 1992 & Kyi *et al.* 1991). However, it is unlikely that all the eggs would have disappeared off the papers. It is not known if using paper in this way biases predation as the paper may attract predators to the eggs. Certainly having all the eggs in one place can alter predation levels. It is important to find a better method of spreading eggs out over the metre of cotton row as results

from Chapter 4 showed that if predators find one egg, searching intensifies and they will probably find them all.

Quantification

Given the variation in the accuracy of the assay results in the antigen half life trials (Chapter 7), and the fact that up to 20 eggs were available to one predator at a given time it would be difficult to quantify the number of prey consumed per predator from this study. However, if a prey resource is limited, as it was in *H. armigera* at the WRS, normally one can assume that one positive is one egg eaten (Sopp *et al.* 1992). This means the results have potential to be quantified although the accuracy of the assay must still be taken into consideration.

8.4.5 Conclusion

In general the immunodot method worked well at detecting predation on *H. armigera* in the species of predators present in the field at the time of sampling. There are two potential applications of the method. One is to utilise natural *H. armigera* populations on intensive sampling occasions (every three days) to provide an indication of whether predators feed directly after an egg lay the night before. Alternatively if eggs are 'seeded' into the crop it can provide useful information on whether prey density influences predation levels. This can provide obtaining the functional response of a predator without the limitation of field cages.

Quantifying the number of eggs consumed by each individual, given the variation in controlled laboratory experiments, is difficult to say the least. Hagler (1998) suggests quantification is impossible. The method described here is therefore more useful as an indicator of which beneficial insects are feeding and when, given the presence of other prey. Ragsdale *et al.* (1981) showed that *G. punctipes* were more prominent predators of *N. viridula* in the later part of the season in soybeans despite some low predation in the early part of the season. Likewise Cherill and Begon (1989) found that alternative prey may influence the rate of predation by lycosids. They found lycosids to be predators of nymphal grasshoppers only at certain times and sites. The immunodot method would therefore be of greater use if continuous studies were made throughout the cotton growing season. The results here are likely to be an under-estimation of predation when high egg numbers are available.

Overall this study can be seen as a first step towards improving the assessment of individual predator species impact on *H. armigera* and thus warrants further investigation as it provides a means of testing the frequency of predation on *H. armigera* by predators found in cotton and eventually in other crops. It also allows the assessment of predators when other prey are present and in field conditions without the use of field cages. The species tested by Sigsgaard (1996) were different to these as was the antibody and crop type. This makes comparison between the studies difficult. However, both studies show that predators do feed on *H. armigera* in the field and both show the potential of serological assay as a means of assessing predator species.

Chapter 9

9. Key findings and their implications for management.

9.1 Introduction

There is no doubt that the predators investigated in this study feed on heliothis eggs and larvae. To date, attempts to evaluate their direct impact in Australian cotton have been few. Initial work in the 1970's (Pyke 1980, Room 1979b & Twine 1973) has not been followed up until quite recently. Even in the more recent studies, including those of Mensah and Singleton (1998) and Stanley (1997), the question of whether predators are effective biological control agents has remained largely unanswered. In part, the potential of predators has not been realised due to early season crashes in their populations as a result of frequent broad spectrum pesticide usage and, by the low economic thresholds set for control of heliothis. Furthermore, there is a lack of understanding of the role of predators due to experimental difficulties caused by variation in consumption of prey within predator populations and the difficulty in extrapolating from laboratory data to field situations (O'Neil 1989, Frazer and Gilbert 1976). Very few studies have attempted to measure the direct impact of predators of heliothis in Australian cotton growing systems. Understanding the direct impact of predator species for use in management was thought important particularly in light of pesticide resistance problems in *H. armigera*, the likelihood of resistance developing to transgenic cotton and the growing public concern on toxicity and environmental impacts of pesticides. The key findings of this study and their contribution to knowledge of the effectiveness of predators in controlling heliothis in cotton in the field are summarised in this chapter. Furthermore, the implications for pest management in cotton are discussed.

9.1.1 Cage studies versus field studies (serological trials)

Studying predation through laboratory trials and serological field trials both provided useful information. The laboratory cage studies contributed towards understanding the parameters

that affected the predators ability to feed on heliothis and indicated their potential if no limitations such as competition and other biotic factors were placed on them. The parameters that influenced predation by *N. kinbergii*, *D. bellulus* and *C. transversalis* adults were temperature, position of heliothis eggs on the plants, size of the cotton plants, density of prey and preferences for other prey types. In all cases there was high variation in the number of prey consumed within each treatment and differences occurred between species. In spite of this some trends were apparent.

Predators were able to find and consume prey in positions on the plant where eggs would normally be oviposited in the field. These sites were the upper and under sides of leaves and the growing tips of the cotton plants. The preference for a particular site when no other prey are available is likely to be influenced by the searching behaviour of a particular predator species. For example, the coccinellids search along the main veins of the leaves where aphids would normally be found. The predators also consumed both species of heliothis eggs (*H. armigera* and *H. punctigera*) in either white or brown stages and in some instances showed a strong preference for neonates (newly hatched larvae) over eggs. In most cases temperature affected the number of prey consumed by the predators studied in this thesis. Lower temperatures appear to reduce the number of prey consumed and in some instances the highest temperature studied (36.8 °C) was found to reduce predation. A temperature of 30.2 °C was considered the most reliable for studying predation of all species when caged cotton plants were used in an environment cabinet.

In all predator species tested in this study, the proportion of prey consumed was not affected by prey density alone, when studied in the laboratory. This occurred when prey were offered in realistic numbers that reflected those found in the field and, at very high numbers not often seen in cotton crops. The number of prey consumed was affected by the size of the plant; the greater the size of the plant the less heliothis eggs consumed. In some instances, the presence of alternative prey influenced the number of heliothis consumed but, in general, the presence of alternative prey did not stop the predators feeding on heliothis eggs.

Whilst prey density did not affect the number of prey consumed by the predators it did have an effect on the number of predators that found prey. At lower prey densities fewer predators were able to find the prey. Prey density did not affect the number of heliothis eggs consumed

by each predator gender but, although variable, gender was considered important when assessing predation.

These findings show that there are parameters, such as temperature and size of plants, that influence the results of predation studies and that laboratory studies are useful in determining which parameters influence predation rates of a target prey. To use functional response experiments alone without considering the influence of the predators' search behaviour and the type of study arena being used, and the fact that these are generalist predators, provides a biased assessment of each predators' efficiency on a target prey.

All the laboratory studies provided useful information on the number of prey consumed by predators under different parameters. However, given differences found in consumption rates between field and laboratory studies (O'Neil 1989, Frazer and Gilbert 1976) the impact of predators is better assessed from direct field studies. The serological trials allow this to be done. Tests using the serological technique (immunodot assay) showed that this method provided a better picture of when and where predators are feeding in the field. This question is important if predators are to be used in IPM situations (Seymour and Jones 1991). Using serological techniques in conjunction with laboratory trials means the species of predator is much more effectively assessed. The serological technique is useful for providing information on the effects of the crop type and size, the time of year and predator species and stage, on consumption of the target prey, directly from the field. Its main limitation is quantifying the number of prey consumed. Given the low numbers of prey and the fact that eggs of heliothis are depsoited singly on cotton plants, quantification may be feasible (Sigsgaard 1996) but results may be confounded by the size of the predator, the size of the meal ingested and field temperatures (Hagler and Naranjo 1997, Hagler and Cohen 1990). Another limitation to this study is the time it takes to evaluate each primary antibody. Intensive preliminary trials were required to obtain feeding trials. This was time-consuming and labour intensive. Sigsgaard (1996) also found that to obtain accurate estimates of feeding, trials required a lot of time.

In this study, testing of the serological technique (immunodot assay) showed that it was best to use a monoclonal antibody to determine predation as the polyclonal antibody cross reacted with predator haemolymph, confounding results. Immunodot assay using a monoclonal antibody with a protein concentration step and chemiluminescence as a detection substrate provided a method that distinguished prey antigen from one ingested *H. armigera* egg by each

predator species for up to eight hours. The method worked best on small predators such as *D. notescens* but was able to detect prey antigen in larger predators up to and including those the size of *H. octomaculata*. Collecting predators from the field for assay using the immunodot method proved practical and produced important preliminary information on the differences between predator species in consuming *H. armigera,* early in the cotton growing season.

9.2 Which predator species were best at finding and consuming heliothis?

In laboratory studies the results examining the effects of prey density in Chapter 4 indicate that these predators show a linear response (Type I functional response) to prey density. This suggests that they are efficient predators as, when provided with a high population of heliothis eggs, the more prey available the more they consume. However, given the more realistic situation of a low number of target prey in the field and influences such as the role other prey take in the predators' ability to consume the target prey, the functional response of these predators is unlikely to be linear. Although predation rates would probably be lower in the field, the laboratory experiments did provide useful information on the differences between each predator species.

Nabis kinbergii was a consistently better predator in most of the laboratory studies as it often ate more heliothis eggs than the other two species. Given that the predators fed on high numbers of heliothis eggs and their obvious preference of neonates, this means they are potentially good predators of heliothis. The results for *D. bellulus* were not as conclusive. The numbers of heliothis eggs eaten by this species were sometimes as high as those for *N. kinbergii*, but more variable. The number of prey consumed differed between genders and the presence of alternative prey appeared to have an impact on this species' ability to consume heliothis eggs. With the exception of the gender experiment, the results from this study on the number of prey consumed by *D. bellulus* differ from results found by Stanley (1997). He found that *D. bellulus* consumed much lower numbers of heliothis than the numbers in this study. The reasons for this need further exploration but are probably due to the use of different cohorts of predators. *Coccinella transversalis* were the most interesting of the three predator species because, in the laboratory, their feeding on heliothis eggs produced very mixed results. In general, this predator species ate less than the other two predator species tested. *C. transversalis* was often parasitized which may have influenced predation rates. The

results from the serological trials showed that *C. transversalis* fed on heliothis eggs in the field which supports the findings of the laboratory trials showing that they fed on heliothis in spite of the presence of alternative prey. It is likely that coccinellids adults have a more complex series of behaviours that affect their feeding. For example, if the coccinellids migrate or enter temperature-related diapause feeding may temporarily cease (Anderson and Hales 1984). Consequently, their presence alone does not imply that they are feeding. They are certainly capable of feeding on heliothis eggs even in the presence of cotton aphids but relying on them to feed on heliothis at all times is not advisable.

The field study on direct predation (using immunodot assay) also produced interesting results. The species *D. notescens* and ants scored the highest percent of predation on heliothis eggs and are worth future consideration as important predators. Since the aphidophagous coccinellids (*H. octomaculata* and *C. transversalis*) also produced positive signals to *H. armigera* antigen, it would be of interest to see what proportion of positive signals to *H. armigera* antigen are obtained when cotton aphids (*A. gossypium*) are present in the field. The fact that all predators did not respond the same way to prey numbers under different conditions in the laboratory, coupled with the fact that prey numbers in the field are of considerable importance to management decisions, highlights the need to assess predator species individually.

9.3 Using predators for management of heliothis

Economic thresholds and current management

Both species of prey (heliothis eggs and aphids) and small heliothis larvae were consumed by *N. kinbergii*, *D. bellulus* and *C. transversalis*. However, as a sole replacement for chemical control, these predators are unlikely to maintain populations of heliothis eggs below economic thresholds. The predators could find and consume low numbers of prey and even single eggs on cotton plants but their effectiveness is likely to be lower due to low numbers of target prey naturally available in the field. Using them for management of a target species also assumes that they are specific predators that will seek out heliothis. The fact that these are generalist predators complicates their use as specific control agents. Under conventional cotton management practices it is unlikely that, even with increased predator numbers, the

population of heliothis in a cotton field would ever be reduced to below two heliothis eggs or larvae per metre of cotton. To expect predators to do this is unreasonable.

This conclusion does not rule out the use of predators in a cotton system. Under some conditions the predators were able to consume all the heliothis eggs provided to them. Given small cotton plants in the early season without the pressures of pesticides and when cotton plants are able to compensate for some damage (Ring and Benedict 1993, Brook *et al.* 1992) predators may be of use in conventional management programs. This was shown by Deutscher and McKewen (1996) who suggested the presence of predators in the early season aided in suppression of heliothis levels to a point where no chemical spray was needed. Also, as Hutchison and Pitre (1983) suggested, it may be possible to increase economic thresholds if it is known that predators can consume a certain amount of the target prey. Economic thresholds, as defined by Stern *et al.* (1959), are set lower than the economic injury level to allow farmers time to respond to increasing pest populations. If the rate of increase is reduced by predators then economic thresholds can be set higher.

Given the differences in consumption of prey exhibited in this study between predator species, it is also important to verify the role any other predator species have to play in predation on heliothis. The findings of this study suggest that similar work on some of the many other predator species found in Australian cotton fields will aid in reducing risks if predators are used in IPM programs.

Conserving and enhancing predators for use in IPM

Predators may not reduce heliothis numbers to below the current economic thresholds in conventional cotton. However, this is not a reason to dismiss them. With the more recent management tools such as transgenic cotton, low impact NPV sprays and other selective pesticides (reviewed in Chapter 1), predators will increase in numbers due to reduced pressure from broad spectrum pesticides. As a result the effectiveness of predators as biological control agents may be increased. Although anecdotal reports suggest that such increases in effectiveness are already being achieved by some cotton growers their value for management has yet to be quantified. Meanwhile as others have suggested (Chapter 1 – 1.2) conservation and enhancement of predators through habitat manipulation (Gurr *et al.* 1998) is important. It allows a greater supply of predators both in and outside cotton and other crops which aids in

reduction of populations of pests such as aphids, mites and heliothis, possibly providing some early season control. Conserving predators may also have an impact on a regional scale which is yet to be realised (Zalucki *et al.* 1998). They may contribute to prevention of early season outbreaks of polyphagous pests such as heliothis by reducing their numbers in non-crop vegetation.

In an IPM situation predators will be effective only if they are managed correctly. This may be through their enhancement by providing refuges and nursery vegetation as described for other crop/pest interactions by Wratten and van Emden (1995). Mensah (1999) has described preliminary research in this area for Australian cotton.

The use of food sprays such as Envirofeast (Mensah 1997) represents another way of enhancing predator numbers. Increases of several fold in the numbers of some species of predators have been achieved by these means. The information found on differences in consumption rates between predators in this thesis may aid these programs by adding to the usefulness of predator/prey ratios such as those used by Murray and Mensah (1996). The serological method will help us to better understand when different predators are feeding.

Predator abundance will also be important in an IPM situation. An increase in predator numbers will increase predation rates in some species. In general, the results of this thesis suggest that predators should not be considered as the centre of a control program. More realistically they should be viewed as being effective control agents in some instances i.e.: on small plants, with high heliothis numbers, at certain reproductive stages and possibly at certain temperatures or times of the season.

Transgenic cotton

Transgenic cotton may provide us with a means of better assessing predation in the field given that it required less pesticide applications. With lower impact of pesticides it will allow us to further assess the role of beneficial insects later in the season when their populations would have normally crashed due to conventional management practices.

9.4 Future considerations and conclusion

These studies suggest that if predators were to be used as a tool in IPM situations then they would provide useful control of heliothis on some occasions. Adult *N. kinbergii* and *D. notescens* in particular showed promise as biological control agents. However, future studies should also include the impact of predator nymphal stages. Whilst immature stages were not available for this study they are considered to feed on more prey, in some cases, than adults (Donahoe and Pitre 1977). This is particularly true for coccinellids although the efficiency of coccinellid larvae as predators changes with larval age (Dixon 1970). Also, as mentioned in Chapter 1, an increased understanding of the ecology of the predator species will aid in our understanding of their abundance, seasonality and feeding requirements.

Conclusion

The predators in this study are generalist feeders. Given that they are predators of both heliothis and other secondary pests such as aphids they are worth conserving and enhancing, particularly in light of reduced pesticide usage as a result of the introduction of transgenic cotton. There is still too little known about predators to either disregard them or to use them effectively. Until more is know it is unlikely that cotton growers and consultants will accept them as the centerpiece of their management programs, though they will become increasingly important as a compoinent of IP:M. This study adds to information that will aid in their future use and, through the establishment of a serological method, will significantly aid in the interpretation of predation efficiency. Given the high numbers of heliothis consumed by predators in the laboratory in this study and the results of the serological field trial predators in cotton are well worth further investigation.

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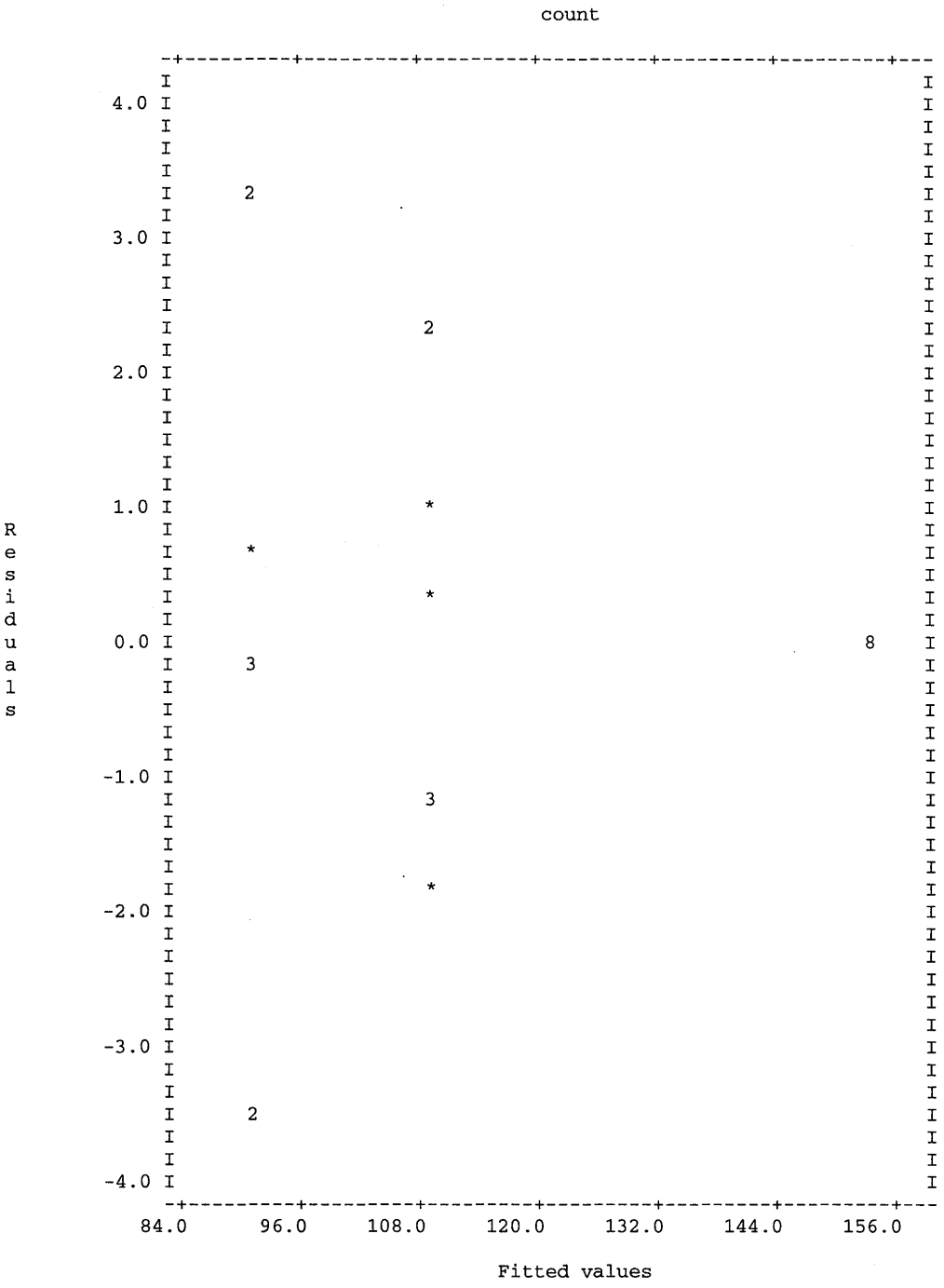
Appendix 1.

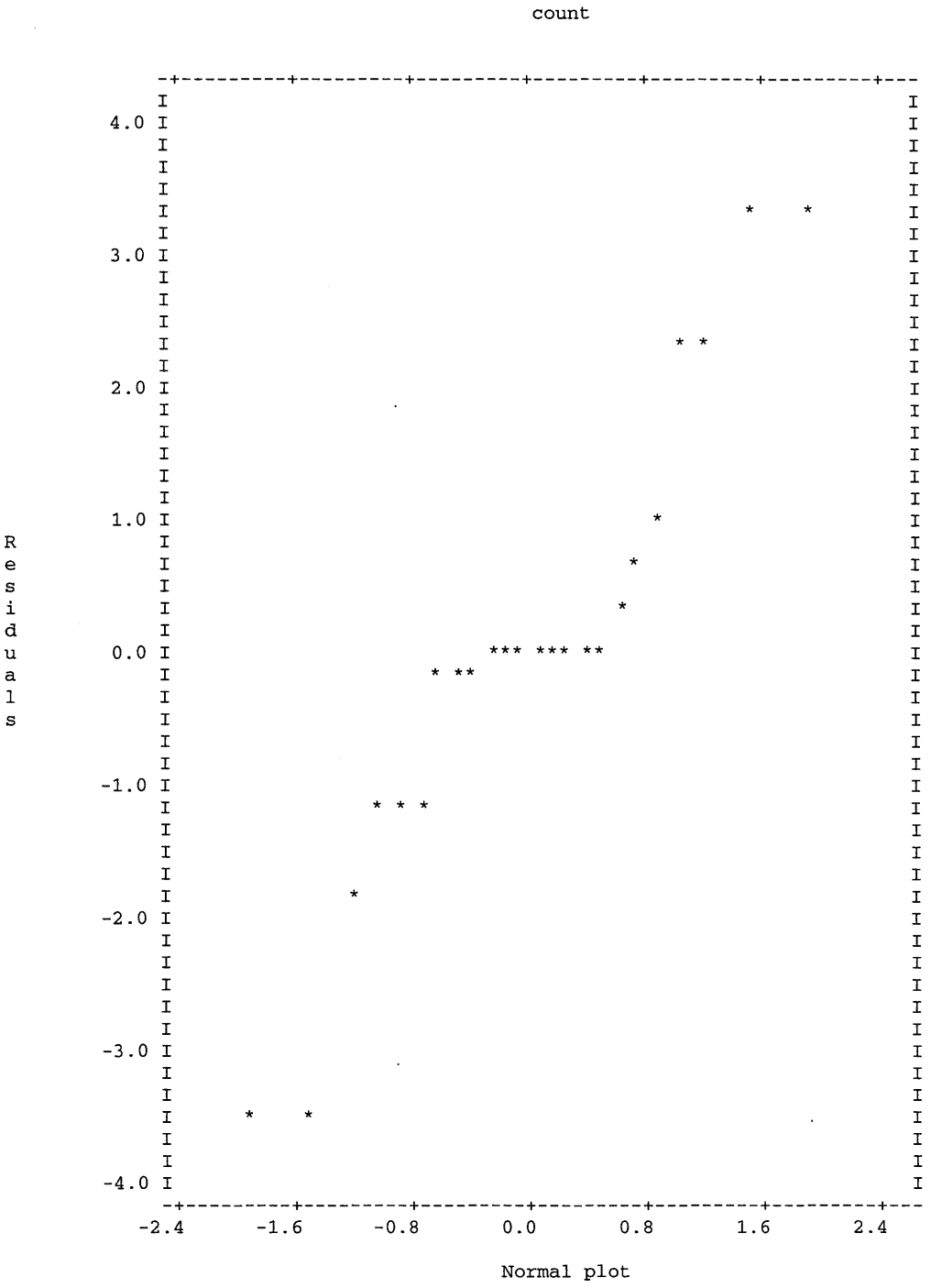
Generalised linear model with a single term.

NB: in this case there is only one term (treatment) so the saturated model is used.
The numbered lines indicate commands used in the analysis.

```
Genstat 5  Release 3.2  (IBM-PC 80386/DOS) Copyright 1995, Lawes
Agricultural Trust (Rothamsted Experimental Station)

   1  "uplow24 species 20 egg density only for C. transversalis"
   2
   3  unit[24]
   4  open'a:up24ct.prn';ch=2
   5  factor [level=3]treatment
   6        &[level=8]replicate
   7  read[ch=2]treatment,replicate,count

    Identifier    Minimum       Mean    Maximum     Values    Missing
         count      0.000      7.292     10.000         24          0

    Identifier     Values    Missing     Levels
         treat         24          0          3
           rep         24          0          8

   8  calc count=count-0.5*(count==10)+0.5*(count==0)
   9  variate total
  10  calc total=10
  11model[distribution=binomial;link=logit]count;nbin=total;res=res;fit=fit
  12  terms treatment
  13  fit[print=model,summary]treatment

***** Regression Analysis *****

 Response variate: count
  Binomial totals: total
     Distribution: Binomial
    Link function: Logit
     Fitted terms: Constant, treatment

*** Summary of analysis ***

                                          mean  deviance
              d.f.      deviance      deviance     ratio
Regression       2         44.83        22.416     22.42
Residual        21         57.01         2.715
Total           23        101.84         4.428

Change          -2        -44.83        22.416     22.42

  14  rcheck residual;xmeth=fit,normal
```



Note: the fitted values lie above and below 2. This indicates overdispersal.


count
Residuals
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-4.0
-2.4
-1.6
-0.8
0.0
0.8
1.6
2.4
Normal plot

```
  15  extrabin[print=acc;phi=phi;modify=yes;method=III]\
  16  terms=treatment

***** Regression Analysis *****

*** Accumulated analysis of deviance ***

Change                                          mean  deviance
                  d.f.      deviance      deviance      ratio
+ treat              2        28.775        14.388      14.39
Residual            21        25.077         1.194

Total               23        53.852         2.341
* MESSAGE: ratios are based on dispersion parameter with value 1
 *** WARNING in procedure EXTRABINOMIAL
 The MODEL statement is modified to include options WEIGHTS and
DISPERSION=1

  17  extrabin[print=m,s,e,c;phi=phi;modify=yes;method=III]\

  18  terms=treatment

***** Regression Analysis *****

 Response variate: count
  Binomial totals: total
     Distribution: Binomial
    Link function: Logit
   Weight variate: Following METHOD=III in procedure EXTRABINOMIAL
     Fitted terms: Constant, treatment

*** Summary of analysis ***

                                          mean  deviance
              d.f.      deviance      deviance      ratio
Regression       2         28.78        14.388      14.39
Residual        21         25.08         1.194
Total           23         53.85         2.341

Change          -2        -28.78        14.388      14.39

The residual degrees of freedom and deviance indicate the model is a good
fit.

*** Estimates of regression coefficients ***

                estimate          s.e.      t(*)
Constant           2.944         0.574      5.13
treatment 2       -2.270         0.671     -3.38
treatment 3       -2.894         0.667     -4.34
* MESSAGE: s.e.s are based on dispersion parameter with value 1

*** Correlations between parameter estimates ***

estimate      ref    correlations

Constant        1     1.000
treat 2         2    -0.854  1.000
treat 3         3    -0.860  0.735  1.000
                        1      2      3
```

Bonferoni adjustment for significance

p = 0.05 / 3
bf = 0.016 (used as the measure of significance), therefore if p < 0.016 there is a significant difference between treatments.

P value calculations

Treatment 3 from 1.

t = 3.38 df = 21, p = 0.0028

Treatment 2 from 1.

t = 4.34, df = 21, p = 0.0002

Treatment 3 from 2.

$(SE)^2 = \sqrt{var(1-2) + var(1-3) - 2cov(1-2,1-3)}$

$= \sqrt{0.671^2 + 0.667^2 - 2(0.671 * 0.677 * 0.735)}$
= 0.487

t =(1-3)- (1-2) / SE

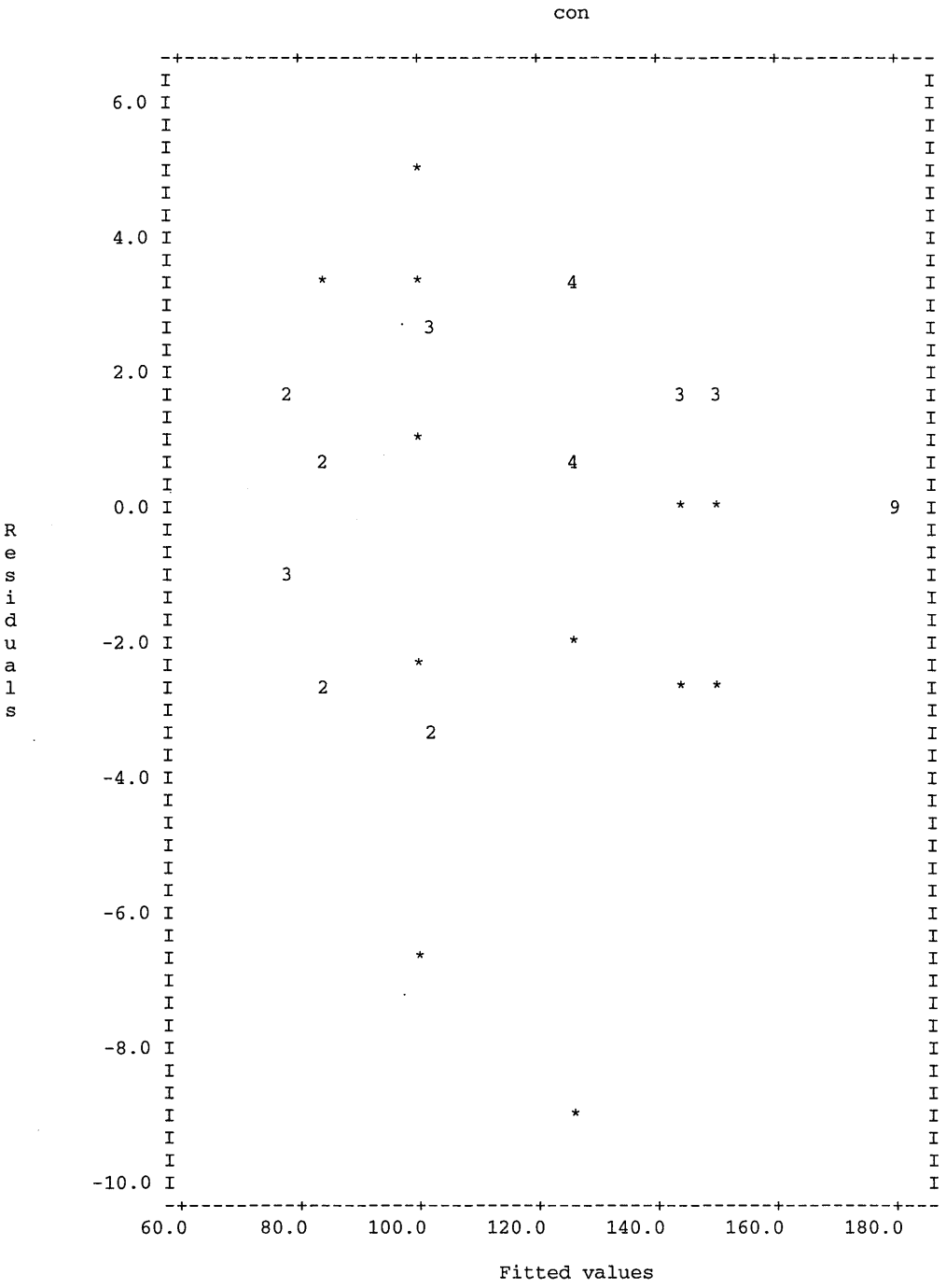
t = -2.894 + 2.27 / 0.487

t = 1.28, df = 21, p = 0.107

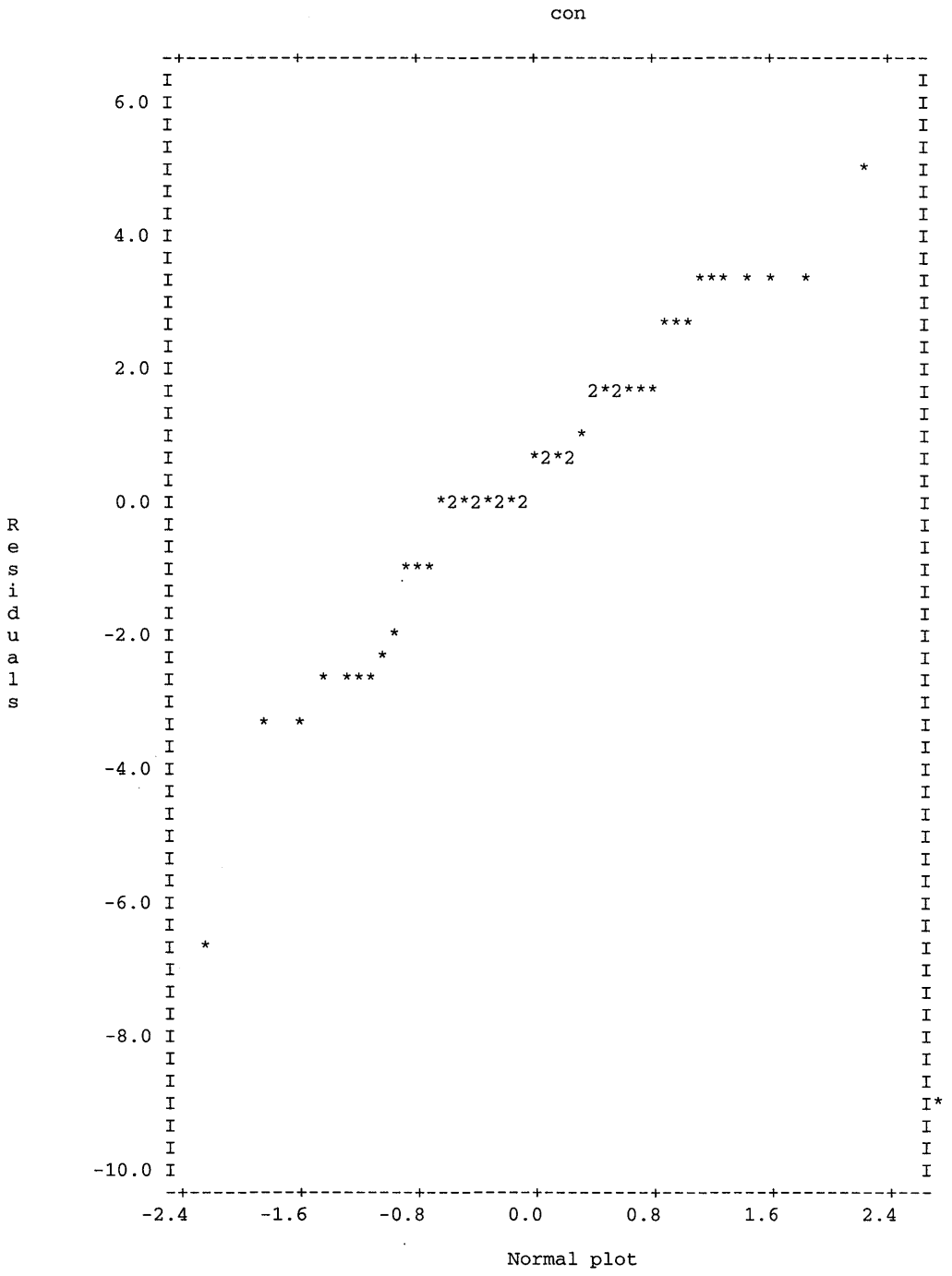
Appendix 2.

Generalised linear models with more than one term in the model.

```
Genstat 5  Release 3.2
Copyright 1995, Lawes Agricultural Trust (Rothamsted Experimental Station)

  1  "Gender differences for N. kinbergii"
  2
  3  unit[50]
  4  open'a:gennk.prn';ch=2
  5  factor [level=2]gender
  6        &[level=5]density
  7  read[ch=2]gender,density,replicate,con,n

 Identifier   Minimum      Mean   Maximum    Values   Missing
        rep     1.000     3.000     5.000        50         0
        con     0.000     8.220    20.000        50         0
          n      1.00     10.20     20.00        50         0

 Identifier    Values   Missing    Levels
     gender        50         0         2
       dens        50         0         5

  8  model[distribution=binomial;link=logit]con;nbin=n;res=res;fit=fit
  9  terms gender*density
 10  fit[print=model,summary]gender*density


***** Regression Analysis *****

 Response variate: count
  Binomial totals: n
     Distribution: Binomial
    Link function: Logit
     Fitted terms: Constant + gender + dens + gender.dens

The saturated model is used.

*** Summary of analysis ***

                                        mean  deviance
             d.f.     deviance      deviance     ratio
Regression      9        117.6        13.066     13.07
Residual       40        262.9         6.572
Total          49        380.5         7.765

Change         -9       -117.6        13.066     13.07


 11  rcheck residual;xmeth=fit,normal
```



The fitted values lie above and below two. This is an indication that over-dispersal is occurring.




```
 12  extrabin[print=acc;phi=phi;modify=yes;method=III]\
 13  terms=gender*density

***** Regression Analysis *****
*** Accumulated analysis of deviance ***
```

Change	d.f.	deviance	mean deviance	deviance ratio
+ gender				
+ density				
+ gender.dens	9	50.895	5.655	5.65
Residual	40	45.776	1.144	
Total	49	96.671	1.973	

Terms are dropped to measure their contribution to deviance in the model.

```
  14  drop[print=*]gender.dens
  15  drop[print=*]density
  16  drop[print=a]gender

***** Regression Analysis *****

*** Accumulated analysis of deviance ***
```

Change	d.f.	deviance	mean deviance	deviance ratio
+ gender				
+ density				
+ gender.dens	9	50.895	5.655	5.65
Residual	40	45.776	1.144	
- gender.dens	**-4**	**-4.070**	**1.017**	**1.02**
- density	**-4**	**-31.695**	**7.924**	**7.92**
- gender	**-1**	**-15.131**	**15.131**	**15.13**
Total	49	96.671	1.973	

A chi-square test is used to determine which terms are included in the amended model. Note that as 'density' is only just non significant it is kept in the model.

```
  17  extrabin[print=m,s,e,c;phi=phi;modify=yes;method=III]\
```

The amended model is used to determine significance between treatments.

```
  18  terms=gender+density

***** Regression Analysis *****

 Response variate: con
  Binomial totals: n
     Distribution: Binomial
    Link function: Logit
   Weight variate: Following METHOD=III in procedure EXTRABINOMIAL
     Fitted terms: Constant + gender + dens
```

The amended model is used.

*** Summary of analysis ***

	d.f.	deviance	mean deviance	deviance ratio
Regression	5	35.52	7.104	7.10
Residual	**44**	**44.04**	1.001	
Total	49	79.56	1.624	
Change	-5	-35.52	7.104	7.10

The residual degrees of freedom and deviance indicate the model is a good fit.

*** Estimates of regression coefficients ***

	estimate	s.e.	t(*)
Constant	1.300	0.805	1.62
gender 2	**-1.647**	**0.679**	**-2.42**
dens 2	-0.396	0.973	-0.41
dens 3	2.96	1.12	**2.65**
dens 4	3.40	1.12	**3.02**
dens 5	0.387	0.974	0.40

* MESSAGE: s.e.s are based on dispersion parameter with value 1

For the difference between genders:

t = 2.42, df = 44, bf = 0.025, p = 0.0198 therefore, the overall difference between gender in *N. kinbergii* is significant.

Note there are significant differences between densities therefore it was valid to retain this term in the model. As density is explored in detail in Chapter 4 these values were used only to indicate differences do occur as a result of density. They were not used as a measure of the effect of density on predator species.

*** Correlations between parameter estimates ***

estimate	ref	correlations					
Constant	1	1.000					
gender 2	2	-0.499	1.000				
dens 2	3	-0.652	0.061	1.000			
dens 3	4	-0.518	-0.047	0.445	1.000		
dens 4	5	-0.514	-0.048	0.442	0.390	1.000	
dens 5	6	-0.649	0.056	0.517	0.445	0.442	1.000
		1	2	3	4	5	6

Appendix 3.

Generalized Linear Mixed Models

```
Genstat 5 Release 3.2 Copyright 1995, Lawes Agricultural Trust (Rothamsted
Experimental Station)

1 unit[96]
2 open 'uplowc.dat';ch=2
3 factor [level=3]site
4 &[level=8]replicate
5 &[level=4]time
6 &[level=24]predator
7 read [ch=2]site, time, replicate, predator, count

Identifyer  Minimum     Mean  Maximum     Values      Missing
count       0.0000      4.562 10.000      96          0

Identifyer  Values      Missing     Levels
site        96          0           3
time        96          0           4
replicate   96          0           8
predator    96          0           24

8  variate t1,t2,t3
9  variate n
10 calc n=10 & t1=time &t2=t1**2 &t3=t1**3
11 glmm[distribution=binomial;link=logit;fmethod=fixed;\
12 print=*;random=site.rep;dispersion=*;\
13 const=0;fixed=site+t1;pterms=site;pse=all]\
14 cound;nbin=n
15 vdisplay[print=waldtest;pse=allest]

***Wald tests for fixed effects ***

Fixed term        Wald statistic    d.f.

site              14.1              3
t1                37.2              1

* All wald statistics are calculated ignoring terms fitted later in the
model

16 glmm[distribution=binomial;link=logit;fmethod=fixed;\
17 print=*;random=site.rep;dispersion=*;\
18 const=o;fixed=site+t1+site.t1+t2+site.t2;pterms=site;pse=all]\
19 count;nbin=n
20 vdisplay[print=waldtest;pse=allest]

*** Wald tests for fixed effects ***

Fixed term        Wald statistic    d.f.

site              12.6              3
t1                28.5              1
site.t1           0.1               2
t2                2.0               1
site.t2           3.2               2

Chi-square statistic used to test for significance.
```

Significant terms used as the amended model for the glmm analysis. Models were checked by fitting the amended model to the count data to see how well they fit.

****** Generalized Linear Mixed Model Analysis******

Method: cf Schall (1991) Biometrika
Response variate: count
Distribution: binomial
Link function: logit

Random model: site.rep
Fixed model: site + t1

*** Table of predicted means for site ***

Site	1	2	3
	3.179	-0.569	-3.120

Standard errors of differences between pairs

site 1	*		
site 2	1.195	*	
site 3	1.248	1.216	*
	site 1	site 2	site 3

standard error of differences: average 1.220

*** Back transformed means (on the original scale)

site	
1	0.9601
2	0.3614
3	0.0423

Means are probabilities not expected values

21 vdisplay[print=comp,effects,means,vcov,dev,s;pse=allest]

*** Estimated Variance Components ***

Random term	Component	s.e.
site.rep	5.086	1.798
units	1.189	0.199

*** Estimated Variance matrix for variance components ***

site.rep	3.231	
units	-0.023	0.040
	site.rep	*units*

*** Approximate stratum variances ***

		Effective d.f.
site.rep	10.139	**20.63**
units	1.189	71.37

* Matrix of coefficients of components for each stratum *

```
site.rep          1.76          1.00
*units*           0.00          1.00

*** Deviance: -2*Log-Likelihood ***

Deviance d.f.
327.5  91

*** Table of effects for site ***

site  1           2           3
      3.179       -0.569      -3.120

Standard errors
site  1           2           3
      0.8614      0.8265      0.8926

Standard errors: Average      0.8602
                 Maximum      0.8926
                 Minimum      0.8265
```

***** Table of effects for t1*****

1
1.79

This indicates there are no significant differences between slopes as there is only one value.

```
22 vkeep[fullvcov=m;fittedvalues=fit1;residuals=res1]\
23 terms=site+t1;effects=m1,m2;\
24 sedeffects=sed1,sed2
25 print m1,m2

           m1

site
1          3.179
2          -0.569
3          -3.120

           m2

1          1.179
```

26 print sed1,sed2 (standard errors of the differences used for calculations in y intercept and expected mean calculations)

```
           sed1

site 1     *
site 2     1.195       *
site 3     1.248       1.216       *

           site1       site2       site3

           sed2

t1         0.1297
           t1
```

```
27 graph[nrows=15;ncol=50]res1;fit1

Tabulated statistics site + t1

Rows: site Columns: time
      1           2           3           4           all
1     1.1843      2.3632      3.5420      4.7209      2.9526
2     -2.5642     -1.3855     -0.2067     0.9723      -0.7961
3     -5.1154     -3.9364     -2.7576     -1.5786     -3.3470
all   -2.1651     -0.9826     0.1925      1.3715      -0.3968


Slopes are not significant therefore the difference between expected means
and y intercepts are calculated.

Expected means

Degrees of freedom are 20.63, Bonferoni (bf) adjustment p = 0.05 / 3 =
0.016

site 1 from 2       3.179 + 0.569 / 1.195 = 3.1364 , p = 0.0052
site 1 from 3       3.179 + 3.120 / 1.248 = 5.0473 , p = 0.0001
site 2 from 3       -0.569 + 3.120 / 1.216 = 2.0979, p = 0.0488

Y intercepts

The estimates for y are calculated first

site 1 = 1.1843 - 1.179
       = 0.6640

site 2 = -2.5642 - 1.179
       = -3.7432

site 3 = -5.1154 - 1.179
       = -6.2944

The differences are then obtained

site 1 from 2      0.664 + 3.7432 / 1.195 = 3.6880 , p = 0.0014
site 1 from 3      0.664 + 6.2944 / 1.248 = 5.5757 , p = 0.0001
site 2 from 3      -3.7432 + 6.2944 / 1.216 = 2.098, p = 0.0488

The time it takes for 50% of the prey to be eaten is calculated by the
equation o = a + bt where a = y intercept, b = slope.

site 1. 0.664/1.179 = 0.5632
site 2. 3.7432/1.179 = 3.1649
site 3. 5.1154/1.179 = 4.3388
```

Appendix 4a.

The original immunodot assay protocol developed by Greenstone and Trowell (1994).

1. Specimen preparation.

a) Natural enemies are starved 24-48 hours prior to use.

b) Each individual is allowed to feed on one target prey and then frozen at -80 °C.

2. Homogenate preparation.

a) The insects are thawed and homogenized in phospate saline buffer + bovine serum albumin (PBST+BSA) (3 ml for adults). Each aliquot is divided into 0.1 ml and frozen at -80 °C until required.

b) When required for an assay the aliquots are thawed and centrifuged for three minutes at 14000 x G. One microlitre of the supernatant is extracted with a micropippette and dotted on to a membrane.

c) The membrane is Hybond - C super nitrocellulose membrane cut and placed on a template containing even rows of dots.

d) Each dot represents 1 µl of supernatant. Once all the dots are completed the membrane is dried for 15 minutes.

3. Assay method.

Each step is incubated for 30 minutes at 37 °C, and then the reagant washed out by rinsing six times in water.

a) The membrane is blocked in 5% BLOTTO (Skim milk powder in PBS pH 7.4) containing 0.1% phenylhydrazine and 0.04% hydrogen peroxide.

b) The appropriate amount of MAb (25-150 µl/ml) in Blotto containing 10% New Born Calf serum (NBCS) is added.

c) The second antibody is added at a rate of 1:1000 (Anti – Rabbit Ig biotinylated whole antibody in 2% NBCS in BLOTTO).

d) The streptavidin peroxidase is added at a rate of 1:1500 (in 2% NBCS in PBS pH 7.4).

e) Add the substrate 3-amino-9-ethyl-carbazole (3AEC) and rotate for three minutes at room temperature. Stop the reaction by rinsing in distilled water twice and then twice more in distilled water rotating for two minutes each time.

Appendix 4b.

Specific items required for the immunodot assay techniques in Chapters 6, 7 and 8.

Product Name	Reference #	Supplier
Chapter 6		
Diploma Skim Milk Powder	N/A	Coles Supermarket, Armidale
Hybond – C nitrocellulose membrane	RPN203G	Amersham
NBCS	16010027	GIBCO
BSA	A6798	Sigma
Anti-rabbit Ig biotinylated whole antibody	RPN004	Amersham
Streptavidin HRP	RPN1231	Amersham
Anti- rabbit HRP linked whole antibody	NA934	Amersham
3 - AEC	A5754	Sigma
Chapters 7 and 8		
4 Chloro 1 Napthanol	C6788	Sigma
Anti- mouse HRP linked whole antibody	NA931	Amersham
BM Chemiluminescence POD kit	1500708	Boehringer Mannheim
Fuji New RX xray film	497690	Hanimex

Supplier Addresses

Sigma
PO Box 970
Castle Hill NSW 2154
Fax 02 899 9742

Amersham Australia
PO Box 6001
Baulkam Hills NSW 2351
Fax 02 899 6833

GIBCO
PO Box 4296
Mulgrave VIC 3170
Fax 03 526 773

Hanimex
Old Pittwater Rd.
Brookvale NSW 2100
Fax 02 94662600

Boeringer Mannheim
Unit 2, 10 Anella Ave.
Castle Hill NSW 2154
Fax 1800 066 598

Appendix 5.

The final protocol used in this thesis for testing the use of a polyclonal antibody in immunodot assay.

1. Specimen preparation.

a) Predators are starved 24 - 48 hours prior to use.

b) Each individual is allowed to feed on one target prey and then frozen in liquid nitrogen.

2. Homogenate preparation.

a) The insects are thawed and homogenized in phospate saline buffer + bovine serum albimum (PBST+BSA). Eggs were macerated at a ratio of 1 individual in 200 µl and predators at a ratio of one individual in 200 or 300 µl depending on the body size.

b) Homogenates were centrifuged for three minutes at 14000 x G. One microlitre of the supernatant extracted with a micropippette and dotted on to the membrane(s).

c) The membrane was Hybond - C super nitrocellulose membrane cut and placed on a template containing even rows of dots.

d) Each dot represents 1 µl of supernatant. Once all the dots are completed the membrane is dried overnight.

3. Assay method.

Each step is incubated for one hour at 37 °C with rotation, and then the reagent washed out by rinsing six times in distilled water.

a) The membrane is blocked in 5% BLOTTO (Skim milk powder in PBS pH 7.4).

b) The antibody was used at a ratio of 1:1000 in BLOTTO containing 10% New Born Calf serum (NBCS).

c) The second (whole linked) antibody (Anti-rabbit Ig, horseradish peroxidase linked whole antibody) is added at a rate of 1:300 (in 2% NBCS in PBS pH 7.4).

d) Add the substrate 3-amino-9-ethyl-carbazole (3AEC) and rotate for three minutes at room temperature. Stop the reaction by rinsing in distilled water twice and then twice more in distilled water rotating for two minutes each time.

Appendix 6.

The final protocol established from testing the monoclonal antibody.

1. Assay method.

Each step is incubated for one hour at 37 °C with rotation, and then the reagent washed out by rinsing six times in distilled water.

a) The membrane is blocked in 5% BLOTTO (Skim milk powder in phosphate buffer solution (PBS) pH 7.4).
b) The primary antibody (Hybridoma cell culture supernatant) was used neat (10 ml per petri-dish) or ascites fluid was used at a ratio of 1:1000 in BLOTTO containing 10% New Born Calf Serum (NBCS).
c) The second antibody (Anti-mouse Ig, horseradish peroxidase linked whole antibody) is added at a rate of 1:300 (in 2% NBCS in PBS pH 7.4).
d) Chemiluminescence steps using the POD kit. Prior to the last step, warm solution A (substrate) to 25 °C and add Solution B (starting solution) at 1:100 ratio (125 $\mu l/cm^2$ of membrane). Incubate for a further 30 minutes at room temperature.
e) The substrate is added in the following way.

When ready to use the substrate, transfer to a dark room.
- Wash the membrane in running water for five-ten minutes and blot off excess water,
- add the substrate to cover the membrane for 60 seconds,
- turn all lights off,
- blot off the extra substrate,
- place the membrane between two overheads and remove air bubbles,
- place the membrane (in the overheads) onto x-ray film in an x-ray cassette or between two glass panes inside thick black plastic making sure no light is available.
- expose for 20 seconds.
- remove film and develop for 60, 30, 60 seconds to check exposure time was correct,
- if too sensitive leave the membrane for up to 10 minutes prior to re-exposure. If not re expose for up to four minutes until false negatives appear.
- develop and allow to dry.