A DNA-based approach to study predator-prey trophic interactions within *Brassica* crops: a search for predators of diamondback moth (*Plutella xylostella*)

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To my wife "Sharareh" and daughter "Romina"

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Abstract

Brassica vegetables and oilseeds are economically important crops worldwide. These crops are associated with several destructive and widespread insect pests. In Australia these pests include six species, *Plutella xylostella* (Linnaeus), *Pieris rapae* (Linnaeus), *Hellula hydralis* Guenée, *Helicoverpa punctigera* (Wallengren), *Brevicoryne brassicae* (Linnaeus) and *Myzus persicae* (Sulzer), which are the focus of this research. Among them *P. xylostella* (diamondback moth or DBM) is the most serious and destructive insect pest.

Recently integrated pest management (IPM) strategies and the use of biological control methods have become the preferred approaches to controlling these pests over synthetic insecticides, due in part to the prevalence of insecticide resistance in *P. xylostella*. Pathogens and especially parasitoids play important roles in the control of diamondback moth. As a result, wide-ranging investigations of these natural enemies have been published. However little is known about the potential of predators, which may be able to contribute to control diamondback moth, although some field studies have shown the overall importance of predators in controlling this key pest. The aim of this study was to develop a method that allows study of predator-prey trophic interactions in the field.

While studies of trophic relationships are essential to understand the dynamics of predator-prey interactions, the actual experiments are difficult to perform because arthropod prey and predators are both small and often cryptic. Thus determination of the diets of predatory arthropods in the field can be complicated. Much of the published research on predators has involved manipulation of predators' habitats or use of

artificial arenas. As a result data gathered by these studies may not be realistic and therefore may not show how predators truly behave in the field.

Among techniques that do not interfere with the behaviour of predators, post mortem gut analyses, especially DNA-based methods, have recently received a great amount of attention. A variety of DNA-based approaches have been used to study trophic interactions including restriction fragment length polymorphisms, amplified fragment length polymorphisms, microsatellite variation and amplification of species-specific DNA sequences.

In this research the main objectives were to develop and evaluate a PCR-based approach that allows investigation of trophic relationships among important predators of diamondback moth and five other major pests under field conditions. The research was carried out in five main steps:

1) The abundance and activity of predators in the field were monitored with pitfall traps, sticky traps and a vacuum sampler. Identified predators included species of Coleoptera, Hymenoptera, Hemiptera, Diptera, Neuroptera, Dermaptera, Chilopoda, and Araneae.

2) A partial gene region of the Cytochrome Oxidase subunit I was sequenced from the six selected pest species. Species-specific primers were designed and developed for each of the six pests. Specificity tests confirmed each primer pair specifically amplifies prey DNA without cross-reactivity to predators or other non-target species that are commonly found in the same habitats. These molecular markers also allow amplification of a very small amount of target DNA in the presence of substantially

greater amounts of predator DNA. Although multiplexing of primers could potentially be used to detect the presence of multiple prey species in a single assay, the sensitivity of it compared with singleplex PCR was lower.

3) Some factors that could affect the detectability were evaluated. The detectability was negatively correlated with time. Prey detectability was different in three different predators with the longest retention time observed for *Venator spenceri,* an intermediate time for *Nabis kinbergii,* and the shortest for *Hippodamia variegata*. Subsequent food intake, sex and weight of predator did not influence detection of prey DNA. In *H. variegata* the rate of detection was decreased with increasing temperature. In addition, study uncovered potential sources of error caused by detection of prey DNA following secondary predation.

4) A multiplex PCR-primer system was developed for accurate identification of juveniles of seven species of wolf spiders that are difficult to identify based on morphology. These wolf spiders occur commonly in *Brassica* crops in South Australia. They are *Hogna crispipes, Hogna kuyani, Lycosa godeffroyi*, *Trochosa expolita*, *Venator spenceri, Venatrix pseudospeciosa*, and a new species in a new genus ('Species A'). Diagnostic DNA fragments for each of the target species allowed species identification.

5) Selected predators from the field were evaluated to determine trophic interactions among them and selected species of prey. Study of trophic relationships among all selected predators and their prey in *Brassica* fields by the DNA-based technique showed most selected prey are found in the diets of selected predators therefore

potentially they can be considered as a predators of them. These results will enable future researchers to confidently identify predators that attack six pests of *Brassica* crops.

Overall, this study demonstrated that a PCR-based technique is a valuable and promising tool to investigate trophic interactions among predators and their prey within *Brassica* crops. Many factors affect detection efficiency or rate of target prey DNA detection in the guts of predators, such as time since feeding and temperature. Therefore, future studies of trophic interaction among arthropods should focus on a particular prey and elucidate possible factors affecting detection before undertaking field investigations. If densities of prey and predator can be determined in the field, then determination of key predators of a particular pest will be possible. Moreover, future research may rely on multiplexing of primers for a quicker method to study large numbers of field-collected samples.

Statement

I declare that this thesis is a record of original work and contains no material, which has been accepted for the award of any other degree or diploma in any university or other tertiary institutions. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in text.

I give consent to this copy of my thesis, when deposited in the university library, being available for loan and photocopying.

Reza Hosseini

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Publications

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Abbreviations

°C	Degree centigrade
BSA	Bovine serum albumin
bp	Base pair (s)
cm	Centimetre
CTAB	Cetylmethylammonium bromide
DNA	Deoxyribonucleic
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylene diamine tetraacetic acid
GuSCN	Guanidine thiocyanate
ha	Hectare
Kb	Kilo base pair (s)
l	Litre
M	Molar
mg	Milligram
ml	Millilitre
mg/ml	Milligram per millilitre
µg	Microgram
µl	Microliter
µM	Micromolar
µg/µl	Microgram per millilitre
mM	Milli Molar
min	Minutes
NaCl	Sodium Chloride

NCBI	National Centre for Biotechnology Information
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pg	Picogram
pH	Potential of Hydrogen
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulphate
sec	Seconds
TAE	Tris-acetate / EDTA buffer
TBE	Tris-borate / EDTA buffer
Tris	Tris-hydroxymethyl aminomethane
TE	Tris-EDTA buffer
TB	Tris buffered saline
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
r.p.m.	Rounds per minute
ng	Nanogram
U	Unit
UV	Ultraviolet light
V	Volt
T_m	Melting temperature
×	times
g	Units of centrifugal force
G	Gravity

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Chapter 1: Literature Review and Aims of Study

1. Introduction

In many agroecosystems, natural enemies including pathogens, parasitoids and predators have shown their potential in regulating agricultural pests (DeBach et al. 1976, Luck et al. 1988). Predators have been reported to be effective bio-control agents, but their role has not been elucidated in many agroecosystems (Symondson et al. 2002). In order to understand and evaluate the role of natural enemies, especially predators, in suppressing populations of pests in the natural environment, it is necessary to study trophic interactions among species communities. This is a significant subject in ecological studies and developing a deeper understanding of it is important for development of biological control strategies.

In this review, the background to my research is outlined. It describes the importance of six major *Brassica* pests. The main focus is on the diamondback moth *Plutella xyllostella*, its biology, ecology, management and natural enemies, and impact in vegetable production. Emphasis is placed on predator-prey trophic interactions and assessment of predator feeding activities with traditional methods and a method based on amplification of specific DNA sequences with PCR.

2. *Brassica* crops and their pests

Brassica vegetables and oilseeds are economically important crops worldwide. In South Australia they are cultivated in many regions at varying times of year. Vegetable crops

include cabbage, cauliflower, Brussels sprouts, broccoli and various Chinese cabbages. Many pests from different groups of arthropods are reported in *Brassica* crops. Some of them are very serious and destructive so pest suppression with regular insecticide treatments are required. More than 30 arthropod pests attack *Brassica* crops in Australia (Table 1.1). Among them six species are considered as major pests of *Brassica* in South Australia: *Plutella xylostella*, *Pieris rapae*, *Helicoverpa punctigera*, *Hellula hydralis*, *Brevicoryne brassicae* and *Myzus persicae.*

Table 1.1. Arthropod pests of *Brassica* crops in Australia (Hely et al. 1982)

Order/ Family	Scientific name	Common name
Lepidoptera/ Plutellidae	*Plutella xylostella* (Linnaeus)	Cabbage moth
Lepidoptera/ Pieridae	*Pieris rapae* (Linnaeus)	Cabbage white butterfly
Lepidoptera/ Noctuidae	*Spodoptera litura* (Fabricius)	Cluster caterpillar
Lepidoptera/ Pyralidae	*Crocidolomia pavonana* (Fabricius)	Cabbage cluster caterpillar
Lepidoptera/ Pyralidae	*Hellula hydralis* Guenee	Cabbage centre grub
Lepidoptera/ Noctuidae	*Chrysodeixis* spp.	Loopers
Lepidoptera/ Noctuidae	*Helicoverpa punctigera* (Wallengren)	Native budworm
Lepidoptera/ Noctuidae	*Helicoverpa armigera* (Hubner)	Cotton bollworm
Lepidoptera/ Noctuidae	*Agrotis* spp.	Cutworms
Diptera/ Anthomyiidae	*Delia platura* (Meigen)	Onion maggot
Diptera/ Agromizidae	*Liriomyza brassicae* (Riley)	Cabbage leaf miner
Coleoptera/ Curculionidae	*Listroderes difficilis* Germain	Vegetable weevil
Coleoptera/ Curculionidae	*Graphognathus leucoloma* (Boheman)	White fringed weevil
Coleoptera/ Carabidae	*Clivina biplagiata* Putzeys	A ground beetle

Table 1.1. (Continued)

Order/ Family	Scientific name	Common name
Coleoptera/ Curculionidae	*Desiantha diversipes* (Pascoe)	Spotted vegetable weevil
Coleoptera/ Scarabaeidae	*Heteronychus arator* (Fabricius)	African black beetle
Coleoptera/ Chrysomelidae	*Phyllotreta nemorum* (Linnaeus)	Striped flea beetle
Coleoptera	Elateridae	Wireworms
Coleoptera	Tenebrionidae	False wireworms
Thysanoptera/ Thripidae	*Thrips tabaci* Lindeman	Onion thrips
Hemiptera/ Aphididae	*Brevicoryne brassicae* (Linnaeus)	Cabbage aphid
Hemiptera/ Aphididae	*Myzus persicae* (Sulzer)	Green peach aphid
Hemiptera/ Aphididae	*Lipaphis erysimi* Kaltenbach	Turnip aphid
Hemiptera/ Lygaeidae	*Nysius vinitor* Bergroth	Rutherglen bug
Hemiptera/ Pentatomidae	*Nezara viridula* (Linnaeus)	Green vegetable bug
Hemiptera/ Pyrrhocoridae	*Dindymus versicolor* (Herrich-Schaffer)	Harlequin bug
Orthoptera/ Gryllidae	*Teleogryllus commodus* (Walker)	Black field cricket
Orthoptera/ Acrididae	*Phaulacridium vittatum* (Sjostedt)	Wingless grasshopper
Orthoptera/ Acrididae	*Gastrimargus musicus* (Fabricius)	Yellow-winged locust
Acarina/ Penthaleidae	*Halotydeus destructor* (Tucker)	Red-legged earth mite
Acarina/ Penthaleidae	*Penthaleus major* (Duges)	Blue oat mite

2.1 *Pieris rapae* (Linnaeus)

This European pest became established in Victoria in 1937 and spread rapidly to all States (Waterhouse and Sands 2001). Larvae eat ragged holes in the leaves of host plants, which are generally members of family Brassicaceae. On heavily attacked

plants, only the veins of leaves are left, but less heavily infested plants become stunted and fouled with dark green faecal pellets (Richards 1940). Their pale yellow eggs are laid singly and usually on the undersides of food plant leaves. Mature larvae are velvety green and about 5 cm long. Pupation may occur on host plants or neighbouring objects. There are several generations in a year and over-wintering occurs in the pupal stage. Adults fly erratically and are highly mobile. Together with native natural enemies and granulosis virus, three introduced parasitoids (Wilson 1960) and predators have greatly reduced cabbage white butterfly populations for much of the time in many areas. Nevertheless, damaging populations do occur periodically (Waterhouse and Sands 2001).

2.2 *Helicoverpa punctigera* (Wallengren)

H. punctigera is a native Australian moth and occurs throughout all states (Common 1953). This species and other species of the same genus (e.g., *H. armigera*) are major polyphagous pests in Australia and cause serious damage to a wide range of agricultural crops by feeding on their leaves, flowers and fruiting bodies (Zalucki et al. 1986). Warm moist weather favours these insects. This species has a wide host range and can migrate long distances from breeding sites. The moths are active at night and begin to fly at about dusk. Creamy or white colour eggs are laid on young growing parts of plants, flowers and fruiting structures. Larvae are voracious feeders and, depending on the nature of the host plant, they have different colour patterns. Fully-grown larvae leave the plant and burrow into the soil to a depth on about 10 cm and pupate. They have several generations during a year. A large number of natural enemies have been reported for these pests (Waterhouse and Sands 2001). Insecticides are widely used to

control them, and release of *Trichogramma* spp. and application of *Bacillus thurengiensis* have been made with mixed success in some crops.

2.3 *Hellula hydralis* Guenée

This pest has usually only a minor importance on *Brassica* crops but may occasionally do severe damage, especially to radish, turnip and cabbage. Hot dry weather is favourable to the development of this pest. However, it is most likely to be troublesome in autumn and may also occur in spring. The moths are about 12 mm long. Eggs are laid on the younger parts of the plants and the younger larvae burrow into the centre of the growing point. Older larvae bore into the compact head of cabbage and cauliflowers. The tunnels are sealed with webbing and frass. Fully-grown larvae are about 12 mm long, yellowish with brown strips. They pupate in the tunnels. Young plants attacked by this pest usually wither or die (Hely et al. 1982, Waterhouse and Sands 2001). Usually regular treatment for cabbage white butterfly and other pests will prevent injury from *H. hydralis* (Hely et al. 1982).

2.4 *Brevicoryne brassicae* (Linnaeus)

This aphid of European origin now occurs throughout the world and can be a serious pest of *Brassica* vegetables wherever they are grown. Warm dry conditions suit the cabbage aphid. Adult cabbage aphids are slate grey, globular and covered with a waxy bloom. Infestations are usually found on the upper surfaces of leaves. In warmer conditions, a generation takes about 2 weeks. Many *Brassica* species serve as hosts of this aphid in Australia. As colonies of the aphid build up, infested leaves curl in and protect the colonies. Infested plants stop growing and leaves become mottled and distorted. At high population density, younger plants may wilt and die, and even at low

population densities honeydew production may still render plants unsuitable to market. *B. brassicae* has a large number of natural enemies, including braconid wasps, syrphids and coccinellids. These biocontrol agents have clearly had an important effect in suppressing cabbage aphid populations (Waterhouse and Sands 2001).

2.5 *Myzus persicae* (Sulzer)

The polyphagous, cosmopolitan aphid *Myzus persicae* is widespread in Australia. It is capable of damaging plants both directly and through virus transmission on wide range of host plant species. In Europe, where this species is thought to originate, *M. persicae* over-winters as eggs on its primary host, the peach. After several generations on peach, dark green to black winged females move to other hosts like cabbage, peas, potatoes and so on (Hely et al. 1982). In Australia, *M. persicae* does not over-winter on peaches. Instead it breeds throughout the year on a range of host plants (Maelzer 1981). Young nymphs develop in about 6 days to adult. Infections on leaves produce distortion follow by dropping off. Large amounts of honeydew are produced which lead to heavy growth of sooty moulds and inhibition of photosynthesis. *M. persicae* is reported to transmit more than 100 virus diseases worldwide and a number of these occur in Australia, such as cucumber mosaic virus (Stubbs 1955, Kennedy et al. 1962, Thackray et al. 1998). This pest has a large number of natural enemies including parasitoids, predators and fungi.

All above-mentioned pests are less important than the diamondback moth, *Plutella xylostella*. However, in some cases and under some conditions, they may become even more damaging pests. But overall many characteristics make *P. xylostella* more serious

and destructive than other species. For this reason, *P. xylostella* pest is considered as a key pest of *Brassica* crops.

3. Biology, ecology and management of diamondback moth

3.1 Diamondback moth as a pest

The diamondback moth (DBM), *Plutella xyllostella* (L.) (Syn. *P. maculipennis* (Curtis); Lepidoptera, Plutellidae), is the most serious insect pest of *Brassica* vegetables worldwide (Butt and McEwen 1981, Talekar and Lee 1985, Talekar et al. 1985, Talekar et al. 1986, Waterhouse and Norris 1987, Shelton et al. 1988, Talekar and Yang 1991, Honda 1992, Muckenfuss et al. 1992, Poelking 1992, Talekar 1992, Talekar and Shelton 1993). The annual cost for managing this destructive pest is estimated to be in excess US$ 1 billion (Shelton et al. 1997). Although the diamondback moth is believed to have originated from the Mediterranean area (Harcourt 1954), it now occurs wherever *Brassica* crops are grown and is believed to be the most widely distributed of all Lepidoptera (Meyrick 1928, Talekar and Shelton 1993). This pest is also widespread in Australia (Waterhouse and Sands 2001).

The diamondback moth is capable of becoming resistant to a wide range of insecticides. There are reports that it has become resistant to most synthetic insecticides used in the field (Johnson 1953, Talekar et al. 1985, Talekar et al. 1990). For example, resistance to pyrethroid insecticides has been identified in populations of diamondback moth from vegetable growing areas in South Australia (Endersby and Ridland 1997, Baker and Kovaliski 1999, Endersby et al. 2003).

It has been reported that the absence of effective natural enemies is a major cause of the diamondback moth's pest status in most parts of the world (Lim 1986). One possible reason for the lack of effective biological control is the ability of diamondback moth to migrate long distances (Talekar and Shelton 1993). As a result, it can establish populations in newly planted *Brassica* crops more rapidly than its natural enemies. Also natural enemies may be eliminated by the use of broad-spectrum synthetic insecticides (Gazzoni et al. 1999)

3.2 Life cycle

The ovoid eggs of the diamondback moth are laid singly or in groups (Fig. 1.1B) on the undersides (Bhalla and Dubey 1986) or upper sides of leaves (Waterhouse and Norris 1987). Newly hatched larvae are whitish yellow to pale green with a brown heads, but older larvae are pale green (Fig. 1.1C) (Bhalla and Dubey 1986, Chelliah and Srinivasan 1986). First instars are leaf miners. Mature larvae feed from the lower surface of young leaves and usually consume all the tissue except the wax layer on the upper surface, thus creating a window in the leaf (Harcourt 1957, Ooi 1986, Talekar and Shelton 1993). The diamondback moth has four instars and the duration of the larval period depends on temperature (Waterhouse and Norris 1987). At the end of the fourth instar, a loose cocoon (Fig. 1.1D) is constructed on the leaf surface and approximately two days of quiescence mark the prepupal stage (Talekar and Shelton 1993). The duration of the pupal period depends on temperature (Harcourt 1957, Lu and Lee 1984, Chelliah and Srinivasan 1986, Hoy 1988). An extensive recent investigation has studied the development and survival of diamondback moth at constant and alternating temperatures (Liu et al. 2002). This study revealed that diamondback moth reared at

constant temperature could not develop from egg to adult outside the temperature range of 8-32°C. However, data indicated that diamondback moth could develop to a significant degree at temperatures outside 8-32 °C under fluctuating temperature regimes, especially in the lower temperature range.

The adult is a slender, grayish brown moth. When resting, a creamy yellow dorsal band with three distinct diamond shapes gives the moth its common name (Fig. 1.1A) (Waterhouse and Norris 1987). Females mate only once after emergence and ovipositing may begin immediately after copulation (Moller 1988) on the day of emergence (Harcourt 1957). Each female lays about 160 eggs over 10 days (Waterhouse and Norris 1987).

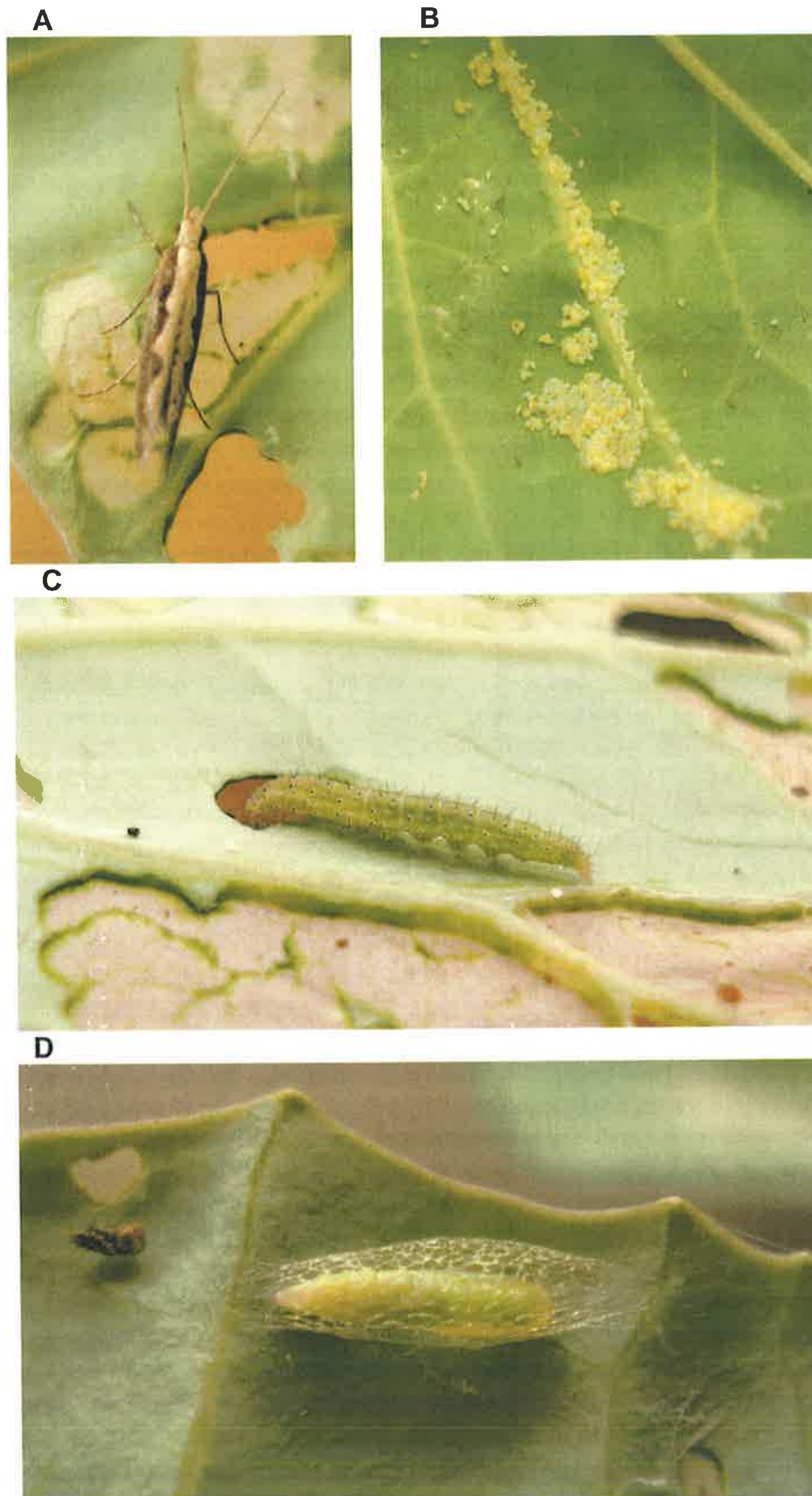

Fig. 1.1. Developmental stages in *Plutella xylostella*. A. adult, B. Eggs, C. Larva, D. Pupa .

3.3 Host plants

The diamondback moth usually feeds on species from the Brassicaceae (Gupta and Thorsteinson 1960a,b, Harcourt 1986, Horn 1987, Poelking 1992, Talekar and Shelton 1993, McLaughlin et al. 1994). The host range of the diamondback moth is primarily limited to *Brassica* species because they contain mustard oils and their glucosides (Gupta and Thorsteinson 1960a,b, 1960b, Nayar and Thorsteinson 1963, Hillyer and Thorsteinson 1971). Host plants include cultivated crops and a large number of weeds (Harcourt 1957, Rai and Tripathi 1985, Harcourt 1986, Louda 1986, Crafford and Chown 1987, Talekar and Shelton 1993, Muhamad et al. 1994). Alternative weed hosts are especially important in maintaining diamondback moth populations in temperate countries in spring before *Brassica* crops are planted (Talekar and Shelton 1993). Recently, Lohr and Rossbach (2004) found a field diamondback moth population on pea crops (*Pisum sativum*) in Kenya and demonstrated that this population could survive well on both pea and kale. This study suggests that even the so-called specialist phytophagous insects may show strong experience-induced responses to non-host plants.

3.4 Seasonal dynamics and migration

There are many overlapping generations in a year and breeding continues as long as the weather is warm (Waterhouse and Norris 1987). In temperate climates diamondback moth has 4-6 generations in a year (Harcourt 1963, Tabashnik et al. 1987) while in tropical climates like Hawaii about 15-20 generations have been reported (Caprio and Tabashnik 1992).

There is no evidence that diamondback moth diapauses or hibernates at any of its life stages (Atwal 1955, Harcourt and Cass 1966, Yamada and Umeya 1972). Studies show that diamondback moth could not be collected during the coldest seasons in Japan (e.g. Honda 1992); however, all life stages can be found at all times in tropical areas.

In some years populations of diamondback moth may erupt into outbreaks. These outbreaks are highly weather-dependant. Warm and dry conditions promote outbreaks because higher temperatures increase the rate of diamondback moth development (Endersby 2004).

The migration of diamondback moth is an important factor in its geographical distribution, and can influence its population dynamics (Chu 1986, Talekar and Shelton 1993). Available reports on the ability of diamondback moth to migrate long distances show that long distance flights cause considerable variation in diamondback moth population densities at various geographical locations (Chu 1986, Honda et al. 1992).

3.5 Mortality factors affecting diamondback moth

Many factors (density dependent and density independent) can influence population densities of diamondback moth. Weather is a density independent factor (Harcourt 1986) responsible for most of the variation in survival of diamondback moth populations as rainfall reduces the survival of young larvae (Harcourt 1954, 1963, Chin 1974, Talekar and Lee 1985, Talekar and Shelton 1993). Physiological death (i.e. unhatched eggs; Wakisaka et al. 1992) is another mortality factor. However, natural

enemies, including pathogens, parasitoids and predators, cause density dependant mortality of diamondback moth (Furlong et al. 2004, Wang et al. 2004).

3.6 IPM of diamondback moth

Farmers have depended on insecticides for the control of diamondback moth since the 1960's (Talekar and Shelton 1993). The development of resistance to different insecticides led to research on alternative control measures and the development of integrated pest management (IPM) (Talekar and Shelton 1993, Talekar and Yang 1993). An IPM program for diamondback moth can involve a combination of methods such as intercropping (Srinivasan 1984, Chelliah and Srinivasan 1986, Magallona 1986, Talekar et al. 1986), sprinkler irrigation (Nakahara et al. 1986, Tabashnik and Mau 1986, Talekar et al. 1986), trap cropping (Talekar et al. 1986, Srinivasan and Krishna Moorthy 1992, Waterhouse 1992, Luther et al. 1996), crop rotation and destruction of plant residues after harvest (Poelking 1992), use of "soft" insecticides (Poelking 1992, Waterhouse 1992), use of plant resistance (Lin et al. 1984, Dickson et al. 1990, Perfect 1992, Waterhouse 1992), use of pheromone traps (Nemoto et al. 1992, Ohno et al. 1992) and sticky traps (Rushtapakornchai et al. 1992) and use of natural enemies, especially parasitioids (Wilson 1960, Goodwin 1979, Hamilton 1979, Lim 1986).

4. Role of natural enemies in IPM of diamondback moth

The genetic resistance, environmental, occupational and consumer health problems created by insecticides have forced field entomologists to place greater reliance on biological control by natural enemies. Natural enemies can cause density dependent

mortality, which affects the seasonal fluctuations of populations of the diamondback moth (Iga 1985). There are some examples of suppression of diamondback moth populations using pathogens, and especially parasitoids (Wagge and Cherry 1992, Waterhouse 1992), but, surprisingly, information on natural predation is limited for diamondback moth.

4.1 Pathogens

Diseases caused by microbial control agents are direct mortality factors that affect the survival of young larvae (Poelking 1992, Wakisaka et al. 1992, Riethmacher and Kranz 1994). They include bacteria, viruses and enthomopathogenic fungi. *Bacillus thuringiensis* has been the most commercially successful insecticidal bacterium (Lacey and Goettel 1995), however, its use on diamondback moth has been somewhat limited due to the development of resistance against it (Kirsch and Schmutterer 1988a,b). Diamondback moth can be infested by two types of virus, nuclear polyhedrosis virus (NPV) and granulosis virus (GV). They can be applied for successful control of diamondback moth (Grzywacz et al. 2004). Diamondback moth populations are commonly regulated by two entomophthoralean species, *Zoophthora radicans* and *Erynia blunckii* (Stavely et al. 2004). They contribute to the natural regulation of diamondback moth populations worldwide (Pell et al. 2001). These pathogens can cause substantial mortality of larval diamondback moth under moist conditions (Furlong et al. 1995, Yeo et al. 2001, Vickers et al. 2004).

4.2 Parasitoids

More than 90 species of hymenopterous parasitoids are associated with the eggs, larvae and pupae of diamondback moth (Oatman and Platner 1969, Yarrow 1970, Goodwin 1979, Lim 1986, Cordero and Cave 1992). However, apparently only about 60 of these 90 species are important (Talekar and Shelton 1993). A list of 20 parasitoids of the diamondback moth is presented in a review by Waterhouse and Sands (2001), most of which are native to Australia. Investigations of parasitoids of diamondback moth have revealed that genera with high control capacity include *Diadegma*, *Cotesia, Apanteles* (Waterhouse 1992) and *Microplitis* (Lim 1986). In Australia the most important parasitoids are *Diadegma semiclausum*, *Diadegma rapi*, *Diadromus collaris*, *Apanteles ippeus* and *Cotesia plutellae.* Among them, *D. semiclausum* and *C. plutellae* have been used successfully in controlling diamondback moth populations and provide a model for the basis of a successful IPM program (Wilson 1960, Goodwin 1979, Waterhouse and Sands 2001, Wang et al. 2004).

4.3 Predators

Studies on other agricultural systems show predators can be important factors in the control of harmful insects (Symondson et al. 2002), but surprisingly little attention has been given to documenting and assessing the rolc of predators in diamondback moth management. Some scientists suggest predation by polyphagous predators such as birds, spiders and some other arthropods is implicated as a major mortality agent for the diamondback moth (Chelliah and Srinivasan 1986, Wakisaka et al. 1992). Syrphid larvae, coccinellids, heteropterans and chrysopids are possible predators of

diamondback moths, but quantitative assessments of their impact have not been made (Oatman and Platner 1969, Alam 1992). Likewise, a number of predatory arthropods were reported as important sources of diamondback moth mortality in South Africa (Ullyett 1947). These predators included staphylinids, wasps of the genus *Polistes*, syrphids, chrysopids, hemerobiids and anthocorids. Among them, syrphids and anthocorids were thought to cause the greatest mortality. Many of these predators were attracted initially to aphids and switched to the diamondback moth as the aphid population declined. Ullyett (1947) also followed diamondback moth populations through several periods and recorded total mortality between 83 and 92%. Of this amount, 23% was attributed to predation. In an investigation in a South Carolina, USA collard field, a range of predators associated with diamondback moth was collected (Muckenfuss et al. 1992). Predators were from three families of spiders and 13 families of insects (Table 1.2). This suggests that there may be a similarly high level of diversity among predators that attack the diamondback moth in other agricultural ecosystems. In this study, predation of eggs and larvae of the diamondback moth involved the use of exclusion cages in the field, which indicated that predators accounted for up to 72% of larval mortality. Predation by *Pardosa milvina* (Hentz)(Arachnida, Lycosidae) was studied in laboratory cages. These studies showed that *P. milvina* consumed about one larva per day. It was postulated that this spider could be an effective member of the predator complex to control diamondback moth (Muckenfuss et al. 1992).

In a study at Hawaii's Poamoho Research Station, USA, Hooks et al. (2003), found that broccoli plants protected by birds and spiders as predators sustained less damage from caterpillars and the plants had greater productivity compared to control plants.

In a quantitative evaluation of biotic mortality factors affecting diamondback moth in southeast Queensland, (Australia) by exclusion predators from caged cabbage plants, estimated losses due to predation ranged 2-85% (Furlong et al. 2004). This study also indicated that Araneae (Lycosidae, Oxyopidae) were the most abundant predators but Coleoptera (Carabidae, Staphylinidae and Coccinellidae) and Hemiptera were also relatively abundant on commercial *Brassica* farms.

Table 1.2. List of arthropod predators associated with diamondback moth in a South Carolina collard field (USA)(Muckenfuss et al. 1992)

Order / Family	Scientific name
Arachnida/ Lycosidae	*Pardosa milvina* (Hentz)
Arachnida/ Lycosidae	*Pardosa pauxilla* Montgomery
Arachnida/ Lycosidae	*Pardosa delicatula* Gertsch & Wallace
Arachnida/ Linyphiidae	*Eperigone fradeorum* (Berland)
Arachnida/ Linyphiidae	*Florinda coccinea (*Hentz)
Coleoptera/ Coccinellidae	*Hippodamia convergens* Guerin-Meneville
Coleoptera/ Coccinellidae	*Coleomegilla maculata* (DeGeer)
Coleoptera/ Coccinellidae	*Scymnus* spp.
Coleoptera/ Coccinellidae	*Coccinella septempunctata* L.
Coleoptera/ Carabidae	*Calosoma sayi* Dejean
Hemiptera/ Nabidae	*Nabis amerricoferus* Carayon
Hemiptera/ Pentatomidae	*Podisus maculiventris* (Say)
Hemiptera/ Reduviidae	Reduviidae
Hemiptera/ Anthocoridae	Anthocoridae
Hemiptera/ Lygaeidae	*Geocoris punctipes* (Say)
Hemiptera/ Lygaeidae	*Geocoris uliginosus* (Say)
Neuroptera/ Hemerobiidae	Hemerobiidae
Neuroptera /Chrysopidae	Chrysopidae
Diptera/ Syrphidae	Syrphidae
Dermaptera/ Labiduridae	Labiduridae
Hymenoptera/ Vespidae	*Polistes* spp.
Hymenoptera/ Formicidae	*Solenopsis invicta* Buren

5. Assessment of a predator's potential to control a pest

A big challenge in IPM is to assess the action and impact of biological control agents. Unlike parasitoids that develop inside or on their hosts, predators often leave no clues after feeding. Therefore predation is one of the most difficult ecological interactions to study. In order to overcome this problem ecologists have used a number of approaches to obtain data on predation.

5.1 Functional response

The functional response describes the relationship between prey density and the rate of prey consumption by individual predators. Evaluations of functional responses have been made to evaluate the potential of a particular predator to regulate the density of its prey (Murdoch and Oaten 1975, Schenk and Bacher 2002). Ecologists have used estimates of functional response in order to select natural enemies for biological control (Gitonga et al. 2002, Lester and Harmsen 2002, Schenk and Bacher 2002, Omkar et al. 2003). However, the functional response of a generalist predator to a single prey species determined in the laboratory may be of little value for predicting a predator's responses to a variety of prey types and densities as found under natural conditions (O' Neil 1997, Lester and Harmsen 2002, Schenk and Bacher 2002).

5.2 Prey preference

Studies on the voracity and feeding preference of predators are important steps in assessing the potential of a biological control agent (Weseloh 1988, Hazzard and Ferro

1991, Heong et al. 1991, Lucas et al. 1997). However, such studies have typically been done on predators under laboratory conditions, so results may not apply to the behaviour of predators in the field, where environmental conditions and limitations on predator movement are substantially different.

5.3 Predator exclusion

Field caging and predator exclusion techniques are two other methods to estimate predation rate. In the first instance, predator and prey are confined in a cage under field conditions and the prey's rate of increase is compared with that found in a control cage (Barry et al. 1984). In the second instance, insect predators are excluded from some plants and prey survival is compared to plants that are exposed to predators (Kring et al. 1985, Sunderland 1988a, Furlong et al. 2004, Wang et al. 2004). Exclusion is the most appropriate experimental method for testing whether natural enemies have the potential to control the pest population (DeBach and Huffaker 1971) and can provide information on the impact of a specific natural enemy or a community of natural enemies (Luck et al. 1988). However, exclusion cage experiments involve disturbance of the system under study and can change characteristics of the micro environment, such as light intensity, temperature, humidity and wind speed (Hand and Keaster 1967), which subsequently may have an effect on predator and prey behaviour (Faeth and Simberloff 1981).

Other general experimental approaches for evaluating natural enemies include:

I) introduction and augmentation, II) removal of natural enemies, III) direct observation, IV) chemical evidence of natural enemy feeding and V) faecal analysis.

5.4.1 Introduction and augmentation

In this technique quantification of pest densities is compared before and after release of natural enemies in an experimental area (DeBach et al. 1976). In this method the actual rate and potential impact of specific natural enemies can be studied. However it cannot provide such information for communities of interacting natural enemies (Luck et al. 1988).

5.4.2 Removal techniques

Removal techniques include the insecticidal exclusion check method (DeBach et al. 1976, Kenmore et al. 1984) and hand removal as a means of assessing the effectiveness of natural enemies (Fleschner et al. 1955, Fleschner 1958, Matsumoto and Nishida 1966, Way and Banks 1968). However, the hand removal technique is extremely time consuming and can be used only on relatively inactive invertebrates. In the insecticidal exclusion method, insecticides change the fauna of the field and may directly affect the reproduction and survival of the pest (Bartlett 1968).

5.4.3 Direct observation

Field observations of predation events either directly or using video recording techniques are often the most useful means of both determining predation rates and identifying the prey and the predator species (Carter and Dixon 1982, Carter et al. 1984, Legaspi et al. 1996, Heimpel et al. 1997, Munyaneza and Obrycki 1998, Meyhöfer 2001). Valuable behavioural data can also be collected during observations (Kareiva and Odell 1987, Völkl 1992). However, direct observation of predation in the field is difficult because of the time of predator's activity (nocturnal or diurnal) and also time

consuming because, in most cases prey and predators are both cryptic and easily disturbed.

5.4.4 Chemical evidence of natural enemy feeding

Prey marking is another technique to identify predator species or to estimate predation rates. Markers used in this technique include radioactive isotopes (McDaniel and Sterling 1979, McCarty et al. 1980, Elvin et al. 1983), rare elements (Stimmann 1974, Shepard and Waddill 1976), or dyes (Hawks 1972, Elvin et al. 1983). This method has been used to determine the predators of eggs of pests (McDaniel and Sterling 1979, McCarty et al. 1980). However, it can be expensive, and users of radioactive isotopes must be properly trained and have the necessary equipment to perform the assay. Furthermore, safety regulations should be considered.

5.4.5 Faecal analysis to estimate consumption rates

This method is involving with collecting and weighing faeces produced by predators. If predators are collected from the field at particular times and maintained under standard conditions in the laboratory, by weighing of faeces produced in different time intervals the rate of food consumption can be estimated (Phillipson 1960). However, this method is time consuming, and also the predator's gut evacuation rate and weight of faeces produced are affected by degrcc of hunger, temperature and abundances of prey, which makes faecal analysis unreliable (Sunderland 1988a).

5.5. Post mortem gut analysis

In order to overcome problems of above-mentioned techniques, post-mortem gut analysis of predators that are collected from the field can provide valuable information about the diet of the predator without influencing their natural behaviour.

Studies of trophic relationships in order to understand the dynamics of predator-prey interactions are essential but can be complicated issue for ecologists (Hoogendoorn and Heimpel 2001). Even when the predator complex is well documented, it is extremely difficult to obtain data on a food chain and predation rate in the field. Because prey and arthropod predators are both small and often cryptic, determining the diet of predatory insects in the field can be complicated. To date several methods have been applied to overcome these difficulties. Each technique for identifying predator gut contents has its strengths and weaknesses.

5.5.1 Gut dissection analysis

Microscopic analysis of a predator's gut contents by dissecting its contents is one technique for determining the diets of predators and predation rate (Sunderland 1975, Walker et al. 1988, Aussel and Linley 1994, Powell et al. 1996, Sleaford et al. 1996, Triltsch 1997). The majority of predators are fluid feeders and solid prey remains are never found in their guts, therefore this method is only applicable for chewing predators that ingest relatively large prey fragments (Aussel and Linley 1994, Powell et al. 1996, Triltsch 1997). Expertise is needed to recognize and identify prey remains, however gut dissection is simple and does not require sophisticated equipment.

5.5.2 Chromatography

In this method paper or gas chromatography is used to identify the prey species pigment in the guts of predators (Putman 1965, 1967, Knutsen and Vogt 1985). However, some prey species cannot be completely separated by this method. Quantification of predation is not possible because factors such as life stage of prey and alternative prey have quite significant effects on interpretation of results.

5.5.3 Electrophoresis

Polyacrylamide gradient gel electrophoresis is used to detect diagnostic prey proteins (esterases) in the guts of predators by analysis of isozymes (Murray and Solomon 1978, Wool et al. 1978, Giller 1982, Castanera et al. 1983, Giller 1984, Wool et al. 1984, Solomon et al. 1996, Camara at al. 2003, Traugott 2003). However, this technique in some cases has low specificity when a single predator gut contains the remains of several different preys (Walrant and Loreau 1995).

5.5.4 Serological methods

Serological methods have also been used to assess predator-prey interactions (Rothchild 1971, Vickermann and Sunderland 1975, Borcham and Ohiagu 1978, Gardner et al. 1981, Crook and Sunderland 1984). They are based on immunological assays using polyclonal or monoclonal antibodies for prey-specific proteins. The most widely used technique has been enzyme-linked immunosorbent assays (ELISA). These assays detect prey material (antigens) in the guts of predators by their reaction with antibodies

obtained from a vertebrate such as a rabbit that has been sensitised against the prey (Greenstone and Hunt 1993, Hagler et al. 1995, Powell et al. 1996, Hagler and Naranjo 1997, Symondson et al. 1997, Hagler 1998, Agustí et al. 1999a, Symondson et al. 1999a). This method can be used to determine the absence or presence of prey in the gut with an accuracy that depends on factors such as temperature, meal size, time since feeding, resistance of the target epitope to digestion, prey size and predator species (Hagler et al. 1997, Symondson et al. 1997, Hagler 1998, Symondson et al. 1999b). However, although it is sensitive and specific, it can be time consuming and expensive to develop (Greenstone 1996).

5.5.5 DNA-based approaches

In consideration of the disadvantages of the above-mentioned techniques, molecular techniques have been developed based on specific DNA sequences for the identification of closely related species. Some of the molecular DNA-based techniques include restriction fragment length polymorphism (RFLP) analysis (Cuthbertson et al. 2003), sequencing of mitochondrial DNA (mtDNA) or ribosomal cistrons (rDNA) and introns, microsatellites (simple sequence repeats; SSRs) (Symondson 2002), randomly amplified polymorphic DNA (RAPD) markers (Agustí et al. 1999b), and amplified fragment length polymorphisms (AFLP) (see Sheppard and Harwood 2005 for a review). However, PCR-based techniques developed to identify a unique target sequence of the prey DNA in the gut contents of predators has proved to be highly sensitive and effective (Agustí et al. 1999a, Chen et al. 2000) and could largely replace other techniques (Symondson 2002). A noteworthy point about this technique is that it is

possible to differentiate a target prey from similar species using prey-specific DNA sequences.

In PCR-based techniques, the length of the DNA fragment and number of copies of target sequences (Zaidi et al. 1999) are factors determining the relative success of detection of prey DNA in the guts of predators. Studies to date have shown that larger fragments of DNA break down more rapidly during digestion than shorter fragments. Therefore shorter DNA sequences should be targeted (Zaidi et al. 1999, Agustí et al. 2000, Hoogendoorn and Heimpel 2001, Agustí et al. 2003a). It has also been shown that multiple-copy genes considerably increase the probability and duration of detection in the gut of predators (Zaidi et al. 1999). Thus, in order to detect prey DNA in the gut contents of predators, it is necessary to consider these factors in the selection of gene sequences that have the appropriate characteristics for diagnostic studies. Nuclear ribosomal DNA and mitochondrial DNA are good candidate gene sequences for this type of work.

The attributes of mitochondrial genes seem to make them particularly suitable for this work, as well as evolutionary studies. These attributes include 1) mtDNA can easily be extracted from alcohol-stored materials, in some instances from dried museum specimens and from ancient insects held in amber, 2) there are hundreds or thousands of copies per cell, 3) there is no sexual recombination, 4) there is considerable conservation of sequences and structures across the metazoa, 5) the range of mutational rates varies in different regions (Hoy 1994).

Mitochondrial DNA in eukaryotic organisms is a small circular molecule ranging in size from 15 to 18kb (Wilson et al. 1985). The molecule is made up of 37 genes coding for 22tRNAs, 2rRNAs and 13 protein-coding genes (Clary and Wolstenholme 1985, Crozier and Crozier 1993, Mitchell et al. 1993). Most of the mtDNA is involved in coding genes with a general lack of introns, large families of repetitive DNA, pseudogenes and large spacer sequences. The mtDNA consists of the following major coding regions in sequence: ND2, CO1, CO2, ATP, C3, N3, ND5, ND4, ND6, Cyt b, ND1, 16S, 12S and the A+T regions (positions correspond to sequences of *Drosophila yakuba* Burla (Diptera: Drosophilidae; Clary and Wolstenholme 1985). Among mtDNA genes cytochrome oxidase subunit I is as one of the largest protein-coding genes. It has a mix of highly conserved and variable regions closely adjacent to each other that make the COI gene particularly useful for the development of universal primers to be used in evolutionary and population studies (Simon et al. 1994, Caterino et al. 2000), and designing specific primers, allowing closely related species to be separated (Zhang and Hewitt 1996).

With the advent of PCR, universal primers have become available for the amplification of mitochondrial genes such as COI to a broad range of taxa (Folmer et al. 1994, Simon et al. 1994). For amplification of different fragment sizes of COI genes in insects, a few pairs of universal primers have been reported by Simon et al. (1994) and Folmer et al. (1994) (Fig. 1.2).

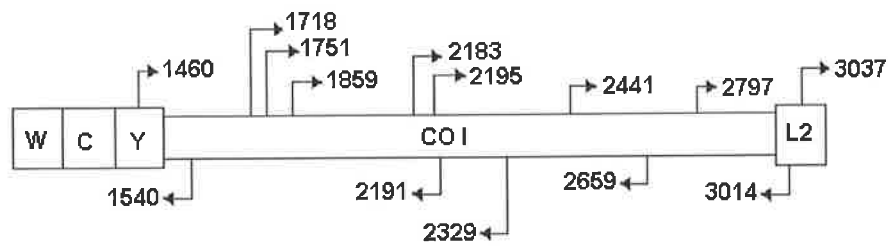

Fig. 1.2. Schematic figure of mitochondrial DNA, cytochrome oxidase subunit I and its universal primers, which can amplify a partial of this gene (Simon et al. 1994).

A number of target genes have been used by researchers in molecular detection of prey DNA in the gut contents of predators; unidentified genes have been used in RAPD analyses (Agustí et al. 1999b, Agustí et al. 2000). In many cases specific gene sequences have been targeted including esterase nuclear genes (Zaidi et al. 1999), cytochrome oxidase subunit I gene (COI) (Agustí et al. 2003b), cytochrome oxidase subunit II gene (COII) (Chen et al. 2000), 12S rDNA (Dodd et al. 2003) and the internal transcribed spacer I region in nuclear ribosomal RNA (Hoogendoorn and Heimpel 2001, Ma et al. 2005). In this project, with consideration of the many suitable characteristics of COI, we have selected the COI locus as the targeted gene for diagnosis of prey DNA.

To date, DNA-based techniques have been developed to detect predation on pests including mosquitoes (Diptera: Culicidae) (Zaidi et al. 1999), moths (Lepidoptera: Noctuidae, Crambidae, Plutellidae) (Agustí et al. 1999b, Hoogendoorn and Heimpel 2001, Ma et al. 2005), whiteflies (Homoptera: Aleyrodidae) (Agustí et al. 2000), aphids (Homoptera: Aphididae) (Chen et al. 2000), psyllids (Homoptera: Psyllidae) (Agustí et

al. 2003a), molluscs (Dodd et al. 2003), and non-pest prey such as Collembola (Agustí et al. 2003b).

An important issue in DNA-based approaches is the possible limitations that should be considered before any application to the field for interpretation of predation. As it is known in predators, digestion rate has a positive correlation with time and temperature (Hoogendoorn and Heimpel 2001). Therefore, time and temperature are critical factors for field studies, which should be investigated in laboratory feeding trials. Unfortunately DNA-based approaches cannot distinguish among predation, secondary predation and scavenging (Foltan et al. 2005, Juen and Traugott 2005, Sheppard et al. 2005). As a result, if someone is going to use predation data to model the predation rates in the field, then one should take into account the potential errors of secondary predation or scavenging. The possibility of secondary predation should be investigated in controlled experiments, and correction factors applied to estimated predation rates based on the outcomes of this research.

6. Molecular identification of arthropod species: a taxonomic approach

The ability to accurately identify species is fundamental to ecological research (Hogg and Hebert 2004). Traditionally, identification of species is based on morphological characteristics. Unfortunately for an increasing number of groups of arthropods, there is a shortage of experts required to carry out identification via traditional taxonomy. Moreover, morphological taxonomic keys are often useful only for a particular life stage or sex, and many species cannot be identified reliably as juveniles. The usefulness of

DNA-based techniques has filled the gap between available taxonomic expertise and the need for an identification capability (Tautz et al. 2003). Molecular identification of species also solves the problem of the identification of cryptic species or immature stages of some taxa. The techniques used for identification of species in different studies include: RFLP primers (Clark et al. 2001, Brunner et al. 2002, Fanello et al. 2002, Goswami et al. 2005), COI gene sequencing (Malgorna and Coquozb 1999, Morlais and Severson 2002, Hogg and Hebert 2004, Saigusa et al. 2005), ITS1 and ITS2 gene sequencing (Silva et al. 1999, Mukha et al. 2000, Gallego and Galián 2001), RAPD (Roehrdanz et al. 1993, Chang et al. 2001). In some cases this involves identification of single species using specific PCR primers (Zhu and Greenstone 1999, Chang et al. 2001), whereas in others it involves multiplexing of PCR primers. Multiplex PCR simultaneously amplifies several fragments in a single reaction. Under certain conditions, several species can be identified using a single PCR followed by an electrophoretic separation of amplified DNA fragments (e.g. Greenstone et al. 2005).

7. Summary

Alternative control methods for diamondback moth are needed as a result of its resistance to available chemical and natural insecticides. Pathogens and especially parasitoids play important roles in the control of diamondback moth, and a wide-range of investigations has confirmed the efficacy of these natural enemies (Wagge and Cherry 1992, Vickers et al. 2004). However, little information is available on potential predators, which may contribute to IPM strategies for this pest.

Effective biological control agents can serve as the basis for pest management strategies. Studies of various agricultural systems show that predators can suppress some harmful insects (Symondson et al. 2002, Grundy 2004). The review of available literature reveals a variety of predators from different taxa can attack diamondback moth. Predators have been collected from fields infested with diamondback moth, but their impact or even trophic interactions on this pest have not been thoroughly assessed. Therefore our knowledge about predators in *Brassica* crops is insufficient and there is no information on their trophic relationships with particular prey species and also their potential to suppress pests, especially diamondback moth.

Molecular identification of difficult taxa of predators and also prey DNA in the gut contents of predators based on PCR has proved to be highly effective and sensitive, and is rapidly replacing other techniques to assess the diets of predators.

8. Aims and significance of study

In the current research project, the main aim was to study the trophic interactions of predators and prey in *Brassica* crops, with a focus on the six most damaging pests. To evaluate the diets of predators collected from the field and assess their potential as biological control agents, the following objectives were developed:

- To identify the most abundant and active predators in the field. Collection and identification of predators associated with *Brassica* pests was performed to achieve this aim. In this regard, a multiplex PCR-primer system was developed for identification of wolf spiders, which are morphologically difficult to identify.

- To develop PCR markers for the major pests of *Brassica* crops. Markers were developed for six pests to enable detection of their remains in the gut contents of predators. Prior to application of this technique in field studies, possible limitations of DNA-based approach were investigated. Detection limits depend on a complex interaction of factors, which affect interpretation of field data. Therefore, in this study some of the factors affecting the detectability of prey DNA in the gut contents of predators were determined.
- To determine which common predators feed on the six selected pests of *Brassica* crops. Predators were collected from the field and tested with specific markers to detect remains of these pests in their gut contents.

Overall, in this study, for the first time, trophic interactions of the common predators and their prey in Australian *Brassica* crops were elucidated. The results enabled us to identify predators that attack six pests of *Brassica* crops and clarified their role in biological control strategies.

Chapter 2: Materials and methods

1. Introduction

General materials and methods, which were often used in various experiments, are described in this chapter. Also a general list of solutions and buffers utilised in the described protocols and experiments are listed at the end. Specific material and methods are described in the appropriate chapters.

2. Insect cultures

2.1 *Plutella xylostella* (diamondback moth) culture

Larval samples used in this study were obtained from a culture of *P. xylostella* kept at the Waite Campus, The University of Adelaide. The conditions for rearing were 25±1°C and a photoperiod of 14L: 10D. Caterpillars were fed on cabbage plants until pupation. A few days before pupation, plants were transferred to a cage and to confine adults upon emergence. Adults were collected with a small vacuum cleaner modified into an aspirator and put into another cage for collecting eggs. Two or three plants were introduced into this cage to collect eggs (Fig. 2.1A).

2.2 *Acyrthosiphon kondoi* (blue green aphid) culture

In order to rear blue green aphid, *Acyrthosiphon kondoi*, firstly lucerne plants (var. Hunter River) were grown in 21 cm diameter pots in the greenhouse. Young plants were moved to a growth chamber (25 ±1°C, 60 ±5% RH, 12L: 12D) and were infested by blue green aphids from a colony obtained from South Australian Research and

Development Institute (SARDI). Each week newly emerged offspring were collected for subsequent feeding trials (Fig. 2.1B).

2.3 *Ephestia kuehniella* (Mediterranean Flour Moth) culture

Adults of *E. kuehniella* initially were obtained from a culture that is maintained at the Waite Campus, The University of Adelaide. Firstly, the adults were place in a plastic box (22×14×7.5 cm) with food for larvae (oat bran, wheat germ, brewer's yeast at proportions of 10:2:1 by volume, respectively). Female moths laid eggs on this food and larvae fed on it until pupation. Rearing was done in an incubator at 25 ±1°C and a photoperiod of 14L: 10D. In order to collect the eggs of *E. kuehniella,* the bottom of a plastic bottle was cut off and then covered by cheesecloth mesh. This mesh was large enough for females to pass their abdomens through to oviposit on paper below. Adults were collected from rearing boxes and transferred to the plastic bottles to lay eggs. Eggs were collected every day from papers and stored at 4°C until used in future feeding trials (Fig. 2.1C).

2.4 *Hippodamia variegata* (spotted amber ladybird) culture

To establish a culture of *H. variegata*, a batch of eggs was obtained from IPM Technologies PTY Ltd. (Hurstbridge, VIC, 3099). The culture of ladybirds was maintained in an incubator at 25 ±1°C at Waitc Campus, The University of Adelaide. A photoperiod of 14L: 10D was applied. A colony of ladybird was reared separately in transparent plastic cups (7.5 cm diameter × 4.5 cm hight) provided with a piece of wet filter paper and blue green aphids, rose aphids (*Macrosiphum rosae*) and/or eggs of Mediterranean flour moth as food (Fig. 2.1D).

2.5 *Drosophila melanogaster* (vinegar fly) culture

Vinegar flies were initially obtained from the School of Molecular and Biomedical Science, The University of Adelaide. Flies were reared in small plastic bottles containing a prepared diet mix (Table 2.1). The rearing was under a 14L: 10D photoperiod regime at 25 ±1°C.

Table 2.1. Recipe for *Drosophila* diet[1]

Vinegar fly diet ingredient	Per one litre
Fresh yeast	186 g
Polenta (maize meal)	100 g
Treacle	143 g
Agar (J grade)	10 g
Water	870 ml
Tegosept[2]	25 ml
Acid mix[3]	15 ml

[1]Food mixture was obtained from School of Molecular and Biomedical Science, The University of Adelaide.

[2] Tegosept (per 1 L)

Methylparahydroxybenzoate (100g)

100% ethanol (1 L)

[3] Acid Mix (per 1 L)

Orthophosphoric acid (47 ml)

Propionic acid (473 ml)

Water (distilled) (480 ml)

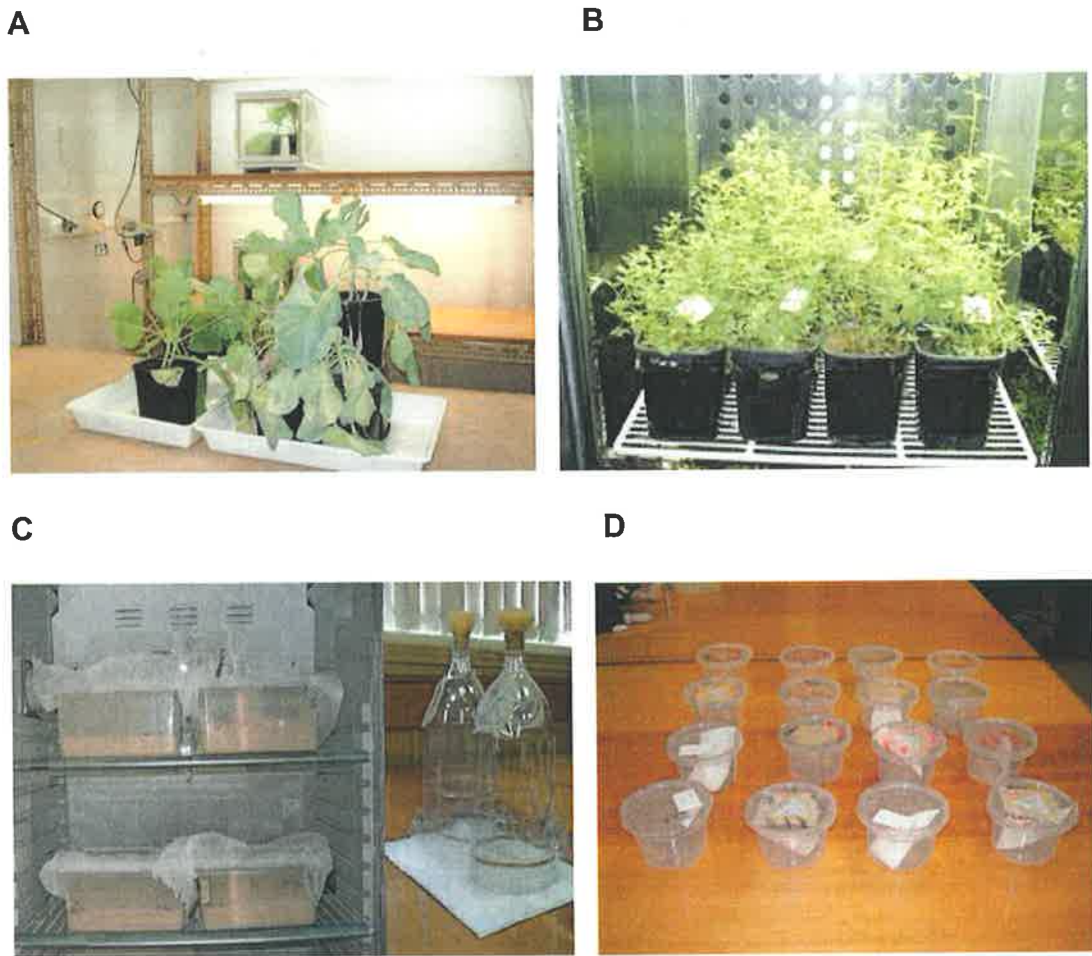

Fig. 2.1. Insect culturing methods. (A) Culture of *Plutella xylostella* on cabbage plants; (B) culture of *Acyrthosiphon kondoi* on lucerne in a growth chamber; (C) culture of *Ephestia kuehniella* on cereal food in an incubator, (left) rearing box; funnel shape bottles for egg collection, (right); (D) culture of *Hippodamia variegata* on food provided in plastic cups.

3. Extraction of DNA

3.1 Extraction of DNA by Qiagen DNeasy ® kit

DNA extraction from arthropod specimens was done according to the manufacturer's protocol with some modifications as follows:

1) Depending on the size of the sample, 180-250 µl PBS was added to cover the body of specimens in a 1.5 ml microcentrifuge tube.

2) The sample was homogenized using a disposable microtube plastic pestle.

3) 5 µl of RNase A (10 mg/ml) was added to the homogenate and incubated for 5 min at room temperature.

4) Then 20 µl of proteinase K and 200 µl buffer AL (provided by manufacturer) was added to the tube and mixed thoroughly by vortexing.

5) The mixture was incubated at 70°C for 10 min.

6) Following incubation, 200 µl ethanol (96-100 %) was added to the sample and mixed thoroughly by pipetting.

7) The homogeneous mixture was put into a DNeasy® Mini Spin (QIAGEN) column placed in a 2 ml collection tube.

8) The tube was centrifuged at 8000 rpm for 1 min.

9) Collection tube was discarded and the column was placed in a new 2 ml collection tube.

10) 500 µl of buffer AW1 (provided by manufacturer) was added to the column.

11) The tube was centrifuged for 1 min at 8000 rpm.

12) 500 µl of Buffer AW2 (provided by manufacturer) was added to the column.

13) Tube was centrifuged for 5 min at 14000 rpm.

14) The column was placed in a clean 1.5 ml microcentrifuge tube and 100 µl buffer AE (provided by manufacturer) was added directly onto DNeasy® membrane (QIAGEN).

15) Tube was incubated at room temperature for 5 min and then centrifuged for 1 min at 8000 rpm.

16) Collected DNA was stored at -20°C.

3.2 Extraction of DNA by the Phenol/Chloroform method

1) 400 µl homogenisation buffer was added to a clean 1.5 ml microcentrifuge tube containing a specimen and then ground and homogenized using a sterile plastic pestle free of any nucleic acids.

2) After adding 5 µl proteinase K (20 µg/µl), samples were incubated at 56°C for 15 min.

3) For removal of RNA, 0.5 µl RNase-A (10mg/ml) was added and the tube was incubated at 37°C for 15 min.

4) After adding 200 µl of phenol, the sample was gently mixed by inversion and left at room temperature for 5 min. The tube was centrifuged as 12500 *g* at room temperature for 5 min. The upper liquid phase was collected carefully by avoiding the interphase, and transferred to a new clean tube.

5) To remove phenol residue, 200 µl of chloroform was added, mixed gently and then left at room temperature for 5 min.

6) The mixture was centrifuged again at 12500 *g* for 5 min. The upper liquid phase was removed and transferred to a new tube.

7) To 200 µl of collected supernatant, 400 µl of cold (kept at –20°C overnight) 100% ethanol and 10 µl of 5 M NaCl were added. The tube was inverted gently several times and placed on ice for 20 min.

8) To precipitate DNA, the sample was centrifuged at 12500 *g* for 20 min at room temperature, the pellet was washed with 200 µl 70% ethanol and centrifuged at full speed (15800 *g*) for an additional 5 min.

9) The pellet was dried in a heating block at 60°C for 5 min.

10) Finally, the DNA pellet was redissolved in 100 µl of sterile double-distilled water or TE and stored at –20°C.

3.3 Extraction of DNA by silica method

This technique was adapted from Boom et al. (1990) and Höss and Pääbo (1993).

1) Tissue from each specimen was placed in a 1.5 ml microcentrifuge tube and 200 µl of lysis buffer was added. Tissue was ground thoroughly with a disposable pestle and the pestle was rinsed with 300 µl of lysis buffer into the tube.

2) After mixing the contents, the tube was kept at 60°C for 15 min.

3) The microcentrifuge tube was centrifuged at 12000 *g* for 1 min to remove debris.

4) 400 µl of supernatant was transferred to a new tube, and then 400 µl of lysis buffer and 32 µl of silica suspension were added to the tube. The contents of each tube were left to settle for at least 10 min at room temperature, and then the tube was agitated by vortexing to suspend the silica.

5) The suspension was separated in a centrifuge at 12000 *g* for 15 sec.

6) The supernatant was discarded by aspiration.

7) 800 µl of washing buffer was added to clean the silica and, following agitation, it was centrifuged and the supernatant discarded.

8) Then 800 µl of washing ethanol was added, agitated and centrifuged for 15 sec.

9) The pellet was dried in a heating block at 60°C for 5 min.

10) In order to elute DNA from the silica, the pellet was re-suspended in 75 µl TE and incubated at 55°C for 15 min.

11) The solution was centrifuged for 1-2 min and the supernatant containing DNA was transferred to a new tube. To collect more DNA, the last step was repeated until a total volume of 150 µl was processed. The extracted DNA was stored at -20°C.

4. Quantification of DNA

In this study two methods were used to measure the concentration of purified DNA.

4.1 Spot blot test

1 µl spots of an appropriate serial dilution of DNA concentration standard (DNA marker) (e.g. 100, 50, 25, 12.5, ... µg/µl) were pipetted onto the surface of a 1% agarose gel containing ethidium bromide (0.5 µg/µl). Next to these standards 1 µl of DNA of samples were pipetted. The spots were allowed to dry and then photographed under UV illumination. The amount of DNA was estimated by comparing the intensities of the photographed samples spots with the ones of the standards.

4.2 Spectrophotometry

The amount of DNA was measured and analysed by a NanoDrop® ND-1000 spectrophotometer by using 1 µl of sample following the instruction manual of the manufacturer (NanoDrop Technologies Inc, Wilmington, DE, USA .).

5. Isolation and purification of DNA from agarose gels

After running PCR products on a 1.5-1.7 % low-melting agarose gel in 1× TAE or TBE containing ethidium bromide (0.5 µg/µl), the desired DNA fragment was cut out by

visualizing the DNA band in the gel on a UV illumination box. The gel pieces were transferred into a new, clean microcentrifuge tube and weighed. The Qiaquik gel extraction kit (Qiagen Inc., Germany), Eppendorf Perfectperp® gel cleanup kit (Eppendorf, Germany), or the Wizard ™ PCR Prep DNA purification kit (Promega, USA) were used to isolate and purify DNA restriction fragments out of agarose according to the manufacturer's instructions.

6. DNA sequencing

For DNA sequencing two methods were used.

1) By using the ABI PRISM® BigDye terminator Cycle Sequencing Ready Reaction kit (version 3), the sequencing reaction was set up as follows:

BigDye	2 µl
Reaction mix	2 µl
Primer (3.2 pmol)(Forward or reverse)	1 µl
Template DNA (30-90ng)(PCR product)	X µl
dH_2O	X µl

The volume of water was determined by template DNA's volume in the reaction. The sequencing reaction was put in a thermocycler that was programmed with the following cycle sequencing programme.

i) 95 °C for 5 min

ii) 96 °C for 10 sec

iii) 50 °C for 5 sec

iv) 60 °C for 4 min

v) Goto step 2, for 24 cycles

vi) END

To purify reaction products for sequencing, 30 µl of deionized water and 10 µl of the sequencing reaction were mixed and put in a 1.5 ml microcentrifuge tube. Then 60 µl of 100% isopropanol was added. The tube was vortexed smoothly and left at room temperature for 30 min. Then the tube was centrifuged for 20 min at high centrifuge speed (15800 *g*). Supernatant was discarded immediately and afterward 250 µl isopropanol 75% was added to the pellet. After a brief vortexing, the tube was centrifuged for 5 min at high centrifuge speed (15800 *g*). All supernatants were carefully aspirated and the pellet was dried in a heating block at 65°C for 5 min. DNA sequencing was performed by the Institute of Medical and Veterinary Sciences (IMVS), Adelaide.

2) In the second method, PCR-labelling was performed. After purification of the PCR product from the gel and determination of DNA concentrations with the appropriate method (described above), 10-40 ng of PCR product and 6.4 pmol of the primer (forward or reverse) were mixed with dH_2O in a 1.5 ml tube to prepare a total volume of 12 µl. Afterwards samples were submitted for sequencing at the Australian Genome Research Facility Ltd. (AGRF), Brisbane.

6.1 Sequence analysis

Raw sequence data were reviewed and refined by CHROMAS (version 2.31; http://www.technelysium.com.au), FinchTv (version 1.4.0; http://www.geospiza.com/finchtv/) and SeqEd (version 1.0.3; for Macintosh, Applied Biosystems). The sequence similarities were compared with those that existed in the GeneBank database by BLAST search (the Basic Local Alignment Search Tool)

accessed via the NCBI website (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov/blast).

All edited sequences were aligned using GENEDOC (http://www.psc.edu/biomed/genedoc) or Clustal W (version 1.82; http:// www.ebi.ac.uk/clustalw/) to find the variation among sequences and to design specific primer pairs for each species.

8. Buffers and solutions used in this study

PBS

NaCl	8 g
KH_2PO_4	0.24 g
Na_2HPO_4	1.44 g
KCl	0.2 g

Homogenisation buffer

Tris-HCl 1M (pH 8.0)	4 µ
EDTA 0.5 M (pH 8.0)	8 µl
10% SDS	40 µl
H_2O	348 µl

10× TBE

Tris-base	108 g
Boric acid	55 g
EDTA 0.5 M (pH 8.0)	20 ml

50× TAE

Tris-base	242 g
Glacial acetic acid	57.1 ml
EDTA 0.5 M (pH 8.0)	100 ml

TE

Tris-HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

EDTA 0.5 M (pH 8.0)

93 g disodium ethylenediaminetetraacetate was added to 400 ml H_2O. It was stirred on a magnetic stirrer. pH was adjusted to 8.0 with NaOH (about 10 g NaOH). The solution dispensed into 500 ml and sterilized by autoclaving.

NaCl 5 M

292.2 g NaCl in 800 ml H_2O was dissolved then the volume was adjusted to 1 l. For sterilization the bottle was autoclaved.

Tris 1 M (pH 8.0)

121.1 g Tris-base was dissolved in 800 ml H_2O, and then the volume was adjusted to 1-l. Also pH was adjusted to 8.0 by adding HCl.

10% SDS

10 g of sodium dodecylsulfatc (SDS) was dissolved in 90 ml H_2O by heating at 68°C to assist dissolution. Before adjusting the volume to 100 ml, pH was adjusted to 7.2 by adding HCl.

Washing buffer used for specimens cleaning

969 µl H_2O; 20 µl 5 M NaCl; 10 µl 1 M Tris-HCl pH 8.0; 1 µl 1 M $MgCl_2$

Buffers for DNA extraction by Silica method

Lysis buffer

1) 10 ml 0.1 M Tris-HCl (pH 6.4) was added to 12 gr of GuSCN then heated in water bath (60°C) to dissolve GuSCN.

2) 2.2 ml of 0.2 M EDTA (pH 8.0) was added and mixed.

3) 250 µl Triton X-100 was added and mixture was wrapped in aluminium foil for storage.

Washing buffer

1) 10 ml 0.1 M Tris-HCl (pH 6.4) was added to 12 gr of GuSCN then heated in water bath (60°C) to dissolve GuSCN.

2) 2.2 ml of 0.2 M EDTA (pH 8.0) was added and mixed. Mixture was wrapped in aluminium foil for storage.

Silica suspension

1) 50 ml of dH_2O was added to 6 g Silica (Sigma#S5631). After vortexing, it was allowed to settle over night at room temperature.

2) 43 ml of liquid was aspirated. The tube was refilled with water and shaken and allowed to settle for 6-10 h at room temperature.

3) 44 ml of upper layer of liquid was aspirated and 60 µl of 10 M HCl was added to it. After vortexing, a series of aliquots was made.

4) each tube was wrapped with aluminium foil for storage.

Washing Ethanol

For preparation of 100 ml of washing ethanol, 70 ml ethanol 100% was mixed to 250 µl 4 M NaCl and the mixture adjusted to 100 ml with water.

Chapter 3: Predators associated with *Brassica* spp.

1. Introduction

Predators are important agents in the biological control of insect pests and many studies suggest that they may have substantial effects on pest populations (Chelliah and Srinivasan 1986, Wakisaka et al. 1992, Furlong et al. 2004, Wang et al. 2004). However, little is known about the diversity and the impact of predatory arthropods on pests of *Brassica* crops. Shelton et al. (1983) reported on extensive ground-dwelling predators in cabbage fields in central New York State, and Schmaedick and Shelton (2000) have documented a list of predators associated with *P. rapae* in cabbage fields of New York State, U.S.A. In an investigation in a South Carolina, U.S.A. collard crop, a range of predators associated with diamondback moth was collected (Muckenfuss et al. 1992).

In the current study, pitfall traps, sticky traps and a vacuum sampler were used to monitor arthropod predators and their relative abundance in broccoli crops in South Australia. This work was done to provide an initial characterisation of the predators that occur in this system.

2. Materials and methods

2.1 Field study

Studies were conducted in a broccoli field on a commercial vegetable farm at Currency Creek, South Australia (35°41′S, 138°75′E) in February and March in 2005. The

experimental site was approximately 3.85 ha in size (Fig. 3.1, 3.2A). Broccoli was planted each week in a sequence of bays running from the western (youngest) to the eastern (oldest) side of the field, in total comprising 11 bays. During experiments no fungicides or insecticides were applied in the field.

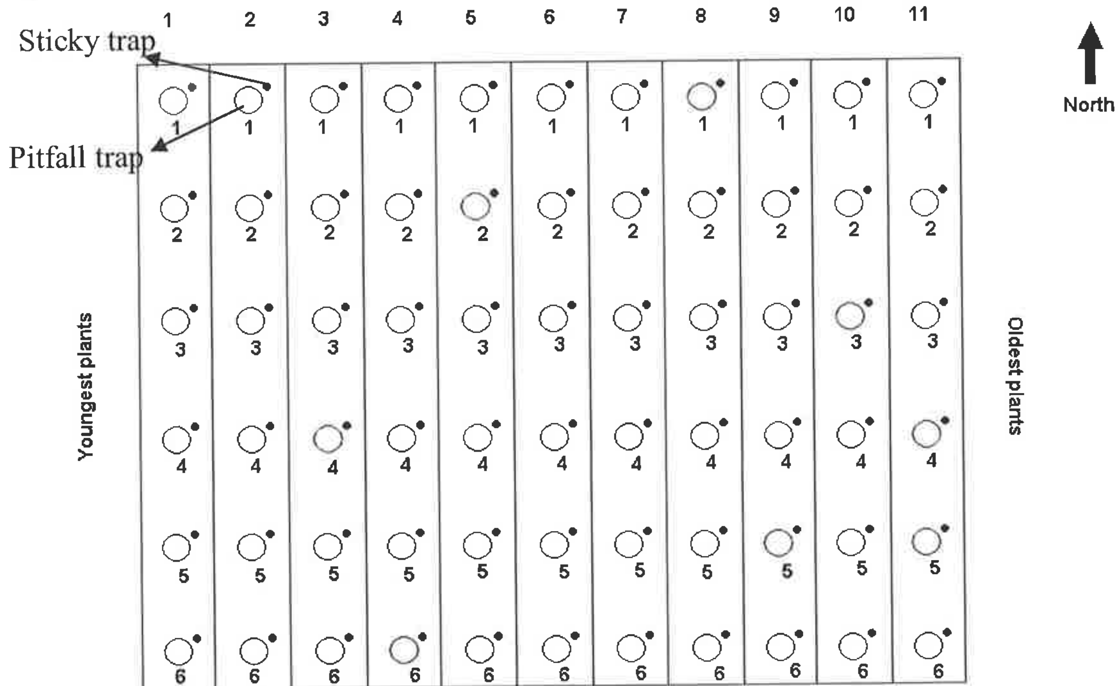

Fig. 3.1. Layout of field where predators were sampled. The field was approximately 3.85 ha, measuring 208 m North-South and 185 m East-West. All bays were planted to 24 rows of broccolini, except bay 2 which had 18 rows.

2.2 Sampling

Predators were sampled in a grid across the experimental crop. Pitfall traps consisting of a 300 mL plastic cup (7.5 cm diameter) were placed inside sections of plastic pipe, which were buried in the soil (Fig. 3.2C). Half of each pitfall trap was filled with saturated salt water and detergent to reduce the water tension. In each bay, 6 pitfall traps

were placed along a transect at equal distances, with a total of 66 locations around the field (Fig. 3.1). The traps were replaced on 21/2/2005, 24/2/2005, 28/2/2005, 3/3/2005 and 7/3/2005. Captured arthropods were held at 4°C until they could be examined. Sticky yellow traps (10 cm×15 cm; Seabright Laboratories, CA, USA), each with approximately 75 cm square adhesive area, were placed adjacent to each pitfall trap (Fig. 3.2B). During the study period, sticky traps were replaced twice on 21/2/2005 and 28/2/2005. In order to check if the pitfall and sticky traps were catching a representative sample of predators, a vacuum sampler (Makita model RBL 250, Makita Corporation, Japan) was used to collect foliar-dwelling predators across the study site on 21/2/2005 and 28/2/2005 (Fig. 3.2D). The vacuum sampler used in this study had a 12cm diameter aperture. A gauze collection bag (25 cm deep) was inserted into the suction tube and attached by an elastic band to collect the arthropods that were sucked from the plants. Sampling was performed by slowly moving the vacuum through the foliage from the tops to bottoms of plants. The collection was done on 21/2/2005, and 28/2/2005 across the field but mainly from older plants in last two bays (bays 10 and 11). Collection bags were removed and sealed from the vacuum while the device was still running. The bags were kept chilled on ice until they were returned to the laboratory and placed at –80°C overnight to kill the arthropods until the contents were counted and identified. The most conspicuous species of predators found in the vacuum samples are reported here. Apart from conventional sampling methods described above, a separate sampling method was used for collection of wolf spiders by headlamp at night (Wallace 1937). Collection was done inside and outside (harvested crops and a border strip of native vegetation) of the experimental site. Predatory arthropods were identified to the lowest taxonomic level possible and the abundance of the most common species was

determined by calculation of the mean of the total collected specimens over the course of study.

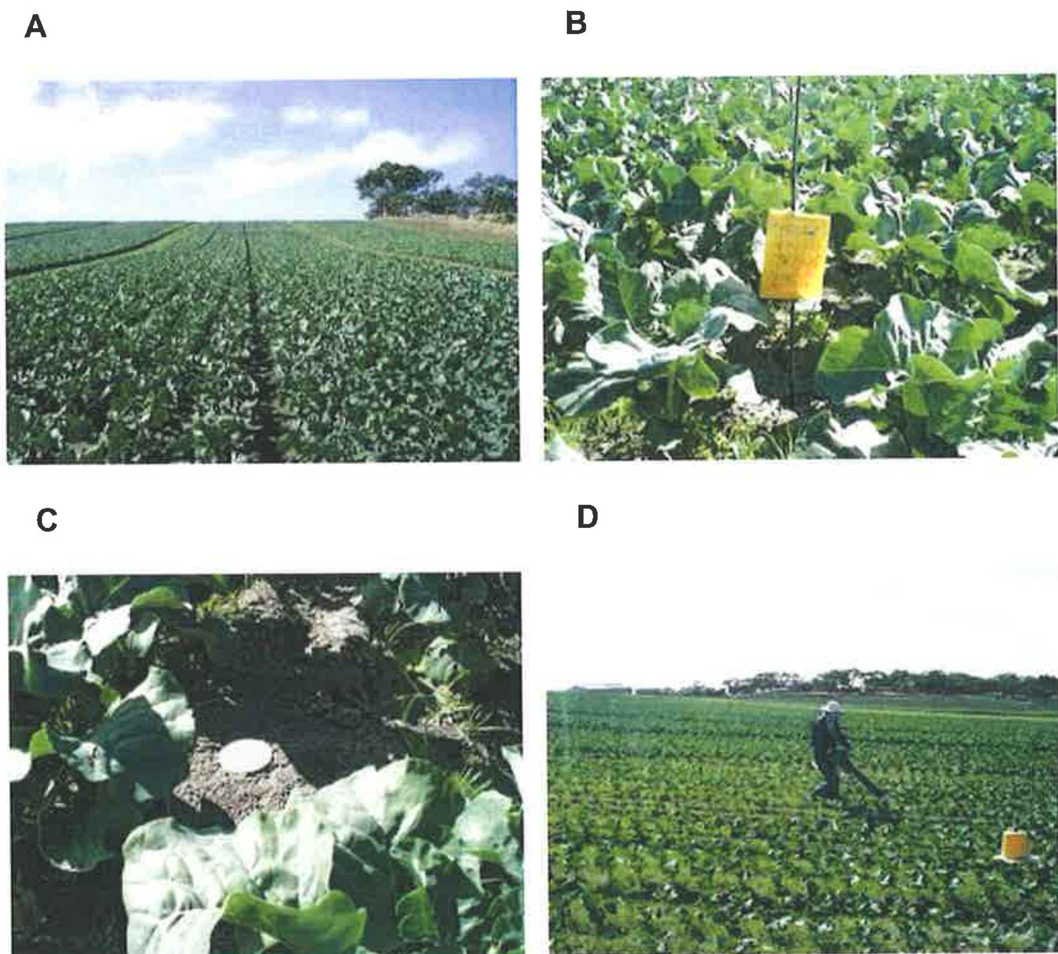

Fig. 3.2. Experimental location and methods used to assess the fauna of predators in a broccoli crop. (A) Experimental site in broccoli field at Currency Creek; (B) sticky trap; (C) pitfall trap; (D) vacuum sampler.

3. Results

Eight orders of predatory arthropods were collected in pitfall traps over the course of the study (Table 3.1). Three species of wolf spiders were collected in pitfall traps inside the crop. A further four species were collected from adjacent areas by headlamp at night. These were *Lycosa godeffroyi* (L. Koch), *Hogna crispipes* (L. Koch), *Hogna kuyani* Framenau, Gotch and Austin and *Venatrix pseudospeciosa* Framenau and Vink. Lycosidae (Arachnida), Formicidae and Coccinellidae (Insecta) comprised the largest number of specimens caught in pitfall traps (Fig. 3.3).

Table 3.1. Predators collected in pitfall traps in a crop of broccoli at Currency Creek, South Australia, February-March 2005.

Order	Family	Species
Coleopetra	Staphylinidae	*Philonthus* sp.
	Cocclinellidae	*Diomus notescens* (Blackburn)
		Hippodamia variegata (Goeze)
		Coccinella transversalis Fabricius
	Carabidae	*Simodontus fortnumi* (Castelnau)
		Mecyclothorax ambiguus (Erichson)
		Anomotarus crudelis (Newman)
		Gnathoxys humeralis Macleay
		Dicrochile goryi (Boisduval)
		Rhytisternus cyathoderus (Chaudoir)
		Pseudoceneus sp.
		Notagonum submetallicum (White)
Hymenoptera	Formicidae	*Machomyrma* sp. (Myricinae)
		Notoncus sp. (Formicinae)
		Rhytidoponera sp. (Ponerinae)
		Iridomyrmex sp. (Dolichoderinae)
		Pheidole sp. (Myrmicinae)
		Myrmecia sp. (Myrmiciinae)
		Camponotus sp. (Formicinae)
	Sphecidae	Unknown spp.

Table 3.1. (Continued)

Order	Family	Species
Hemiptera	Miridae	*Creontiades dilutus* (Stål)
	Nabidae	*Nabis kinbergii* Reuter
	Lygaeidae	*Euander lacertosus* (Erichson)
	Pentatomidae	*Oechalia schellenbergii* Guérin-Méneville
	Reduviidae	*Coranus granosus* Stål
Neuroptera	Hemerobiidae	*Micromus tasmaniae*(Walker)
Diptera	Syrphidae	Unknown spp.
Dermaptera	Forficulidae	Unknown spp.
Chilopoda		Unknown spp.
Araneae	Linyphiidae	*Erigone* sp.
	Salticidae	Unknown spp.
	Tetragnatidae	Unknown spp.
	Gnaphosidae	Unknown spp.
	Miturgidae	Unknown spp.
	Lycosidae	*Trochosa expolita* (L. Koch)
		Hogna sp.
		Venator spenceri Hogg

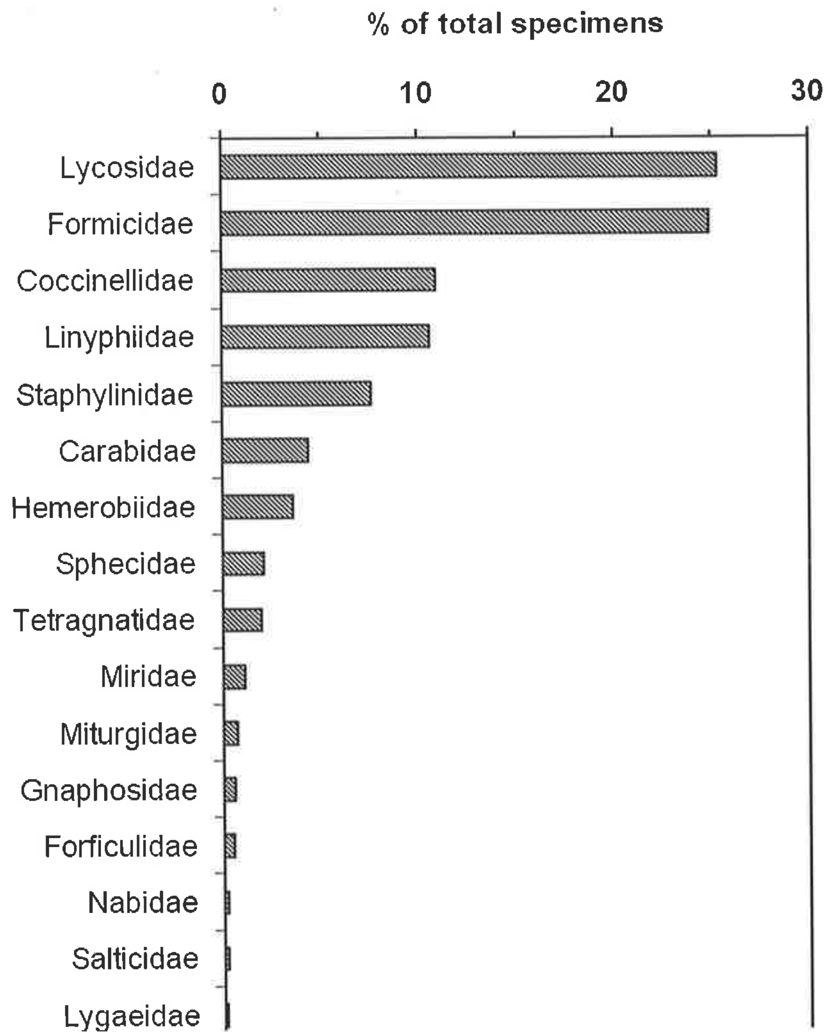

Fig. 3.3. Relative abundance of predators collected in pitfall traps at Currency Creek, South Australia, February-March 2005 as indicated by the % of total predator specimens collected.

The spatial distributions of species varied. *Iridomyrmex* sp. (Formicidae) was the limited to the first 4 bays, where the youngest plants were planted, while for the rest of the field another species, *Pheidole* sp. (Formicidae), was dominant (Fig 3.4A). The distribution of the many species appeared to be random (e.g. Linyphiidae, Fig 3.4B). *H. variegata* and *O. schellenbergii* was most frequently collected in association with older plants that had flowers in bays 10 and 11, while most of *Philonthus* sp.

specimens collected from younger plants (Fig. 3.4C). *T. expolita* (Araneae: Lycosidae) was collected over the entire field (Fig. 3.4D).

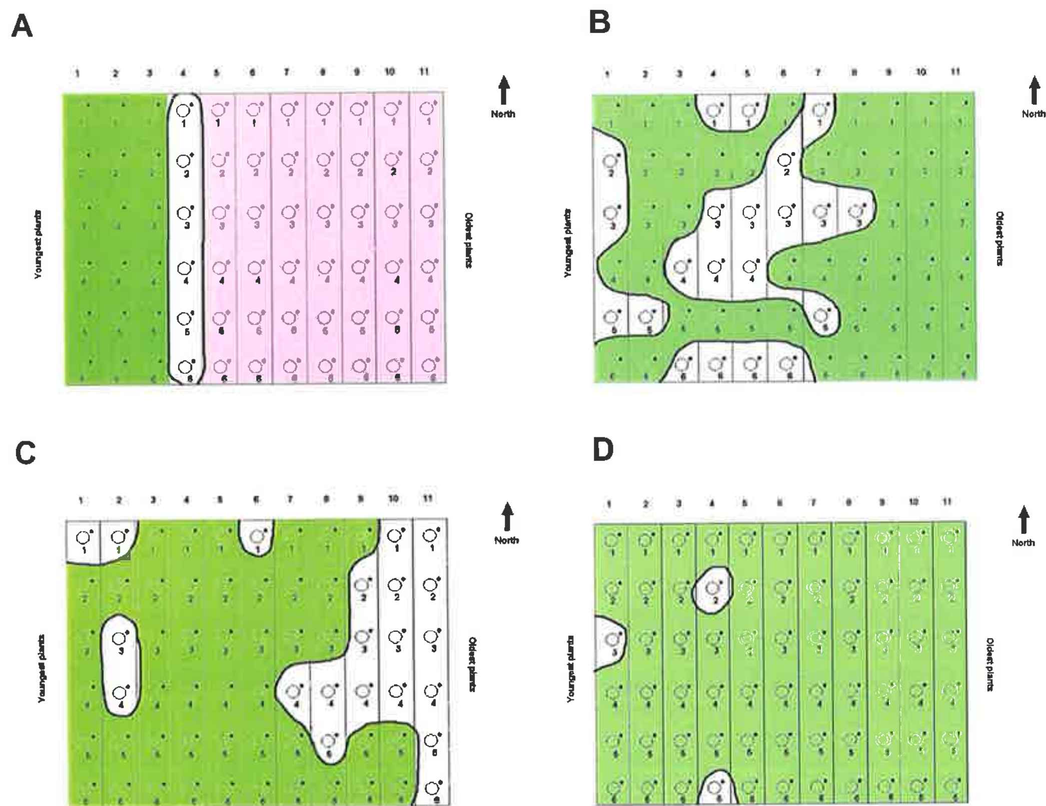

Fig. 3.4. Distribution maps showing where some of the more abundant predators were present in the field. (A) Ants (Green, *Iridomyrmex* sp.; Purple, *Pheidole* sp.; white, both species); (B) Linyphiidae (Green); (C) *Philonthus* sp. (Green); (D) *Trochosa expolita* (Green). No indication of abundance is implied by these figures.

Compared to pitfall traps, fewer species from only three orders were captured on sticky traps (Table 3.2). The absence of spiders on sticky traps is noteworthy. The green mirid, *Creontiades dilutus*, was the most abundant predator collected on sticky traps (Fig. 3.5).

Table 3.2. Common predators found on sticky traps in a crop of broccoli at Currency Creek, South Australia, February-March 2005.

Order	Family	Species
Coleopetra	Staphylinidae	*Philonthus* sp.
	Coccinellidae	*Coccinella transversalis* Fabricius
		Hippodamia variegata (Goeze)
		Diomus notescens (Blackburn)
Neuroptera	Hemerobiidae	*Micromus tasmaniae* (Walker)
Hemiptera	Miridae	*Creontiades dilutus* (Stål)

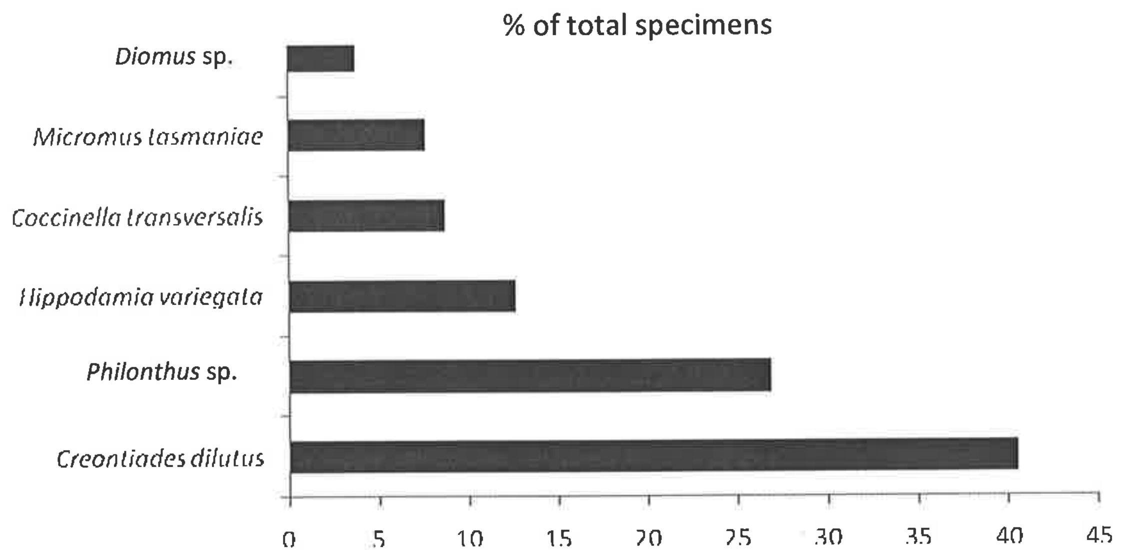

Fig. 3.5. Relative abundance of predators collected on sticky traps in a crop of broccoli at Currency Creek, South Australia, February-March 2005 as indicated by the % of total predator specimens collected.

M. tasmaniae, N. kinbergii and *O. schellenbergii* were the most conspicuous predatory species collected by the vacuum sampler (Table 3.3, Fig 3.6), that is the numbers of these species were greatest. However, the vacuum sampler is not collected large numbers of non-target species and sorting of specimens was difficult. Therefore quantification of these samples was not considered to be a true indicator of abundance and was not done. Each of the conspicuous species collected with the vacuum sampler was also collected in either the pitfall of sticky trap samples. Fig. 3.7, 3.8, 3.9 show some of arthropod predators collected in this study.

Table 3.3. Common predators collected with a vacuum sampler in a crop of broccoli at Currency Creek, South Australia, February 2005.

Order	**Family**	**Species**
Coleopetra	Coccinellidae	*Coccinella undecimpunctata* Linnaeus
		Coccinella transversalis Fabricius
		Hippodamia variegata (Goeze)
		Diomus notescens (Blackburn)
Neuroptera	Hemerobiidae	*Micromus tasmaniae* (Walker)
Hemiptera	Nabidae	*Nabis kinbergii* Reuter
	Pentatomidae	*Oechalia schellenbergii* Guérin-Méneville
	Miridae	*Creontiades dilutus* (Stål)

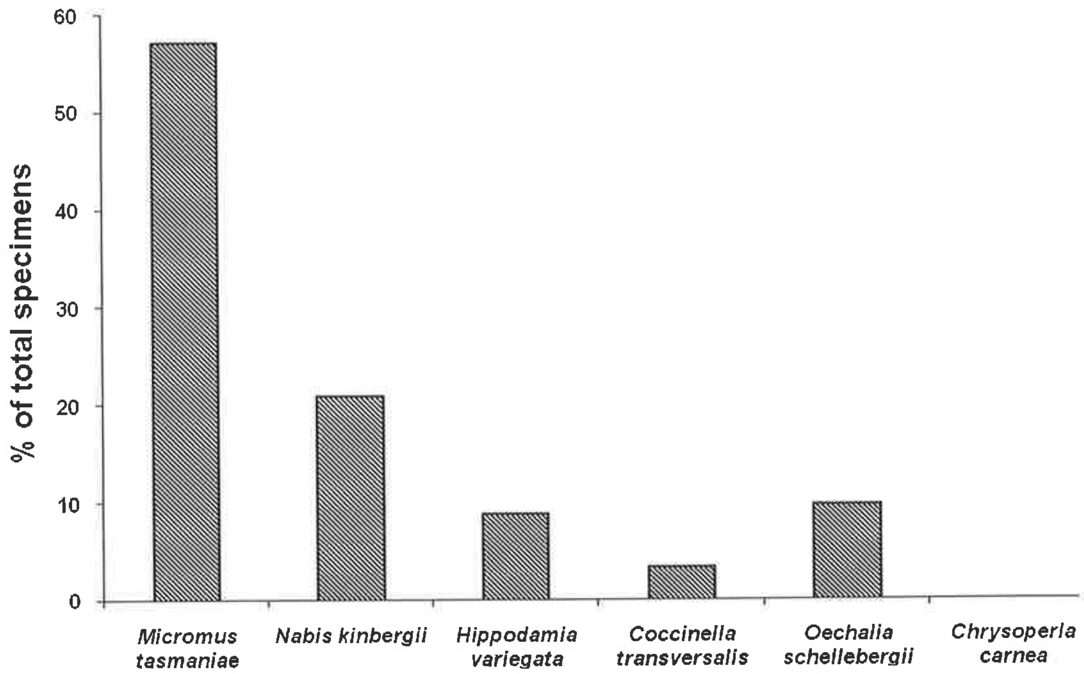

Fig.3.6. Relative abundance of predators collected by vacuum sampler at Currency Creek, South Australia, February-March 2005 as indicated by the % of total predator specimens collected.

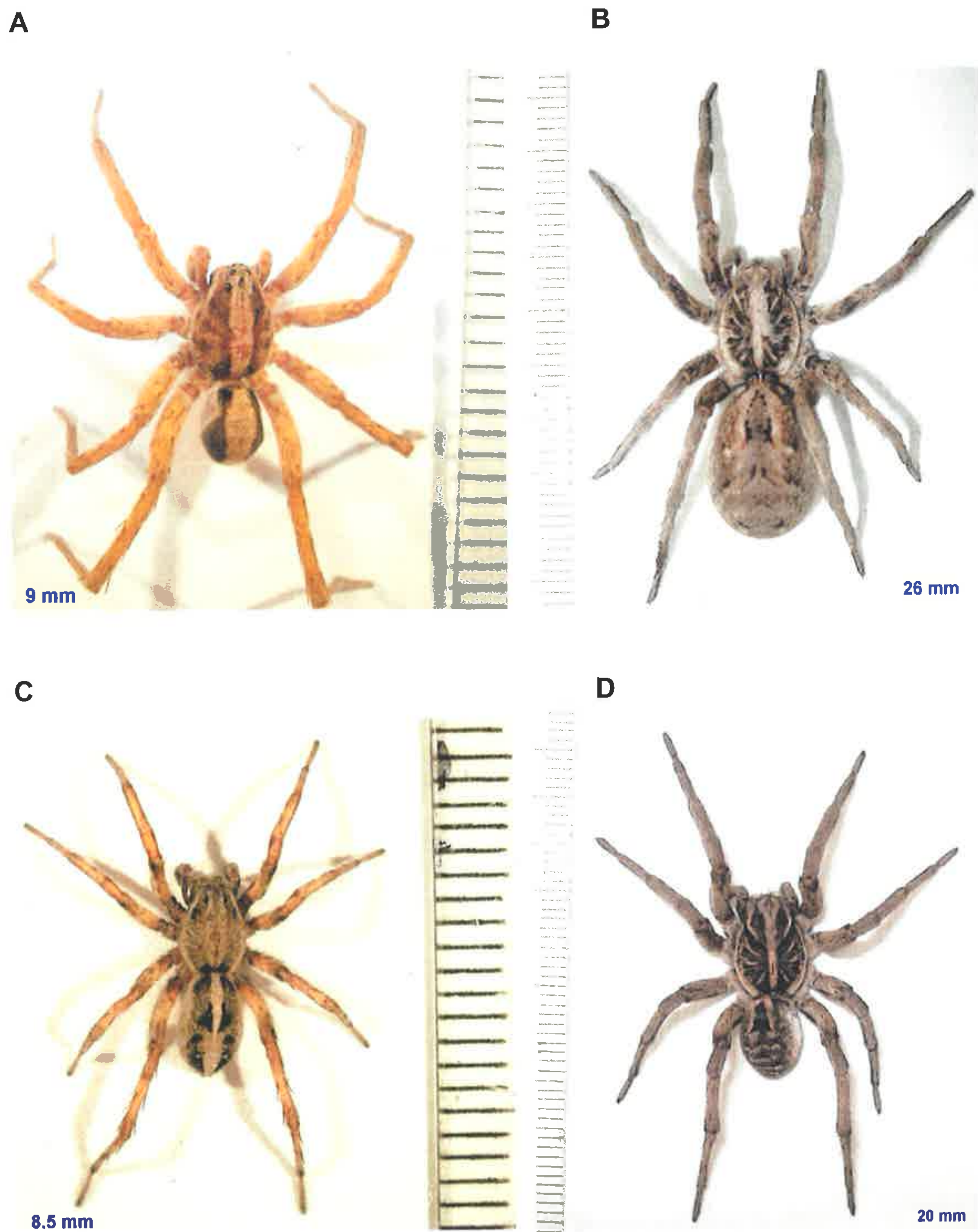

Fig. 3.7. Common wolf spiders collected in this study. (A) *Venatrix pseudospeciosa*; (B) *Venator spenceri*; (C) *Trochosa expolita*; (D) *Lycosa godeffroyi*.

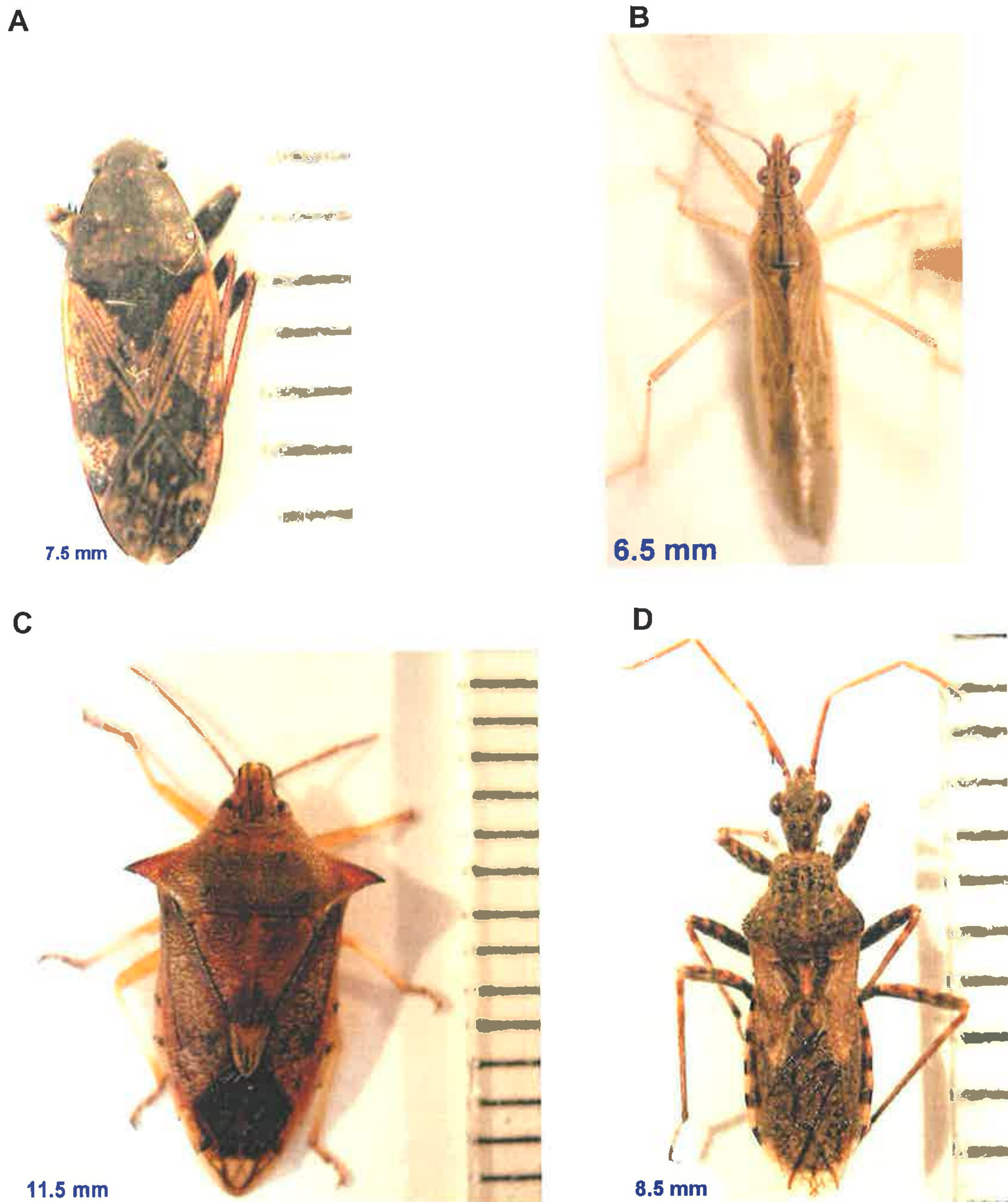

Fig. 3.8. Common hemipteran predators collected in this study. (A) *Euander* sp.; (B) *Nabis kinbergii*; (C) *Oechalia schellenbergii*; (D) *Coranus* sp.

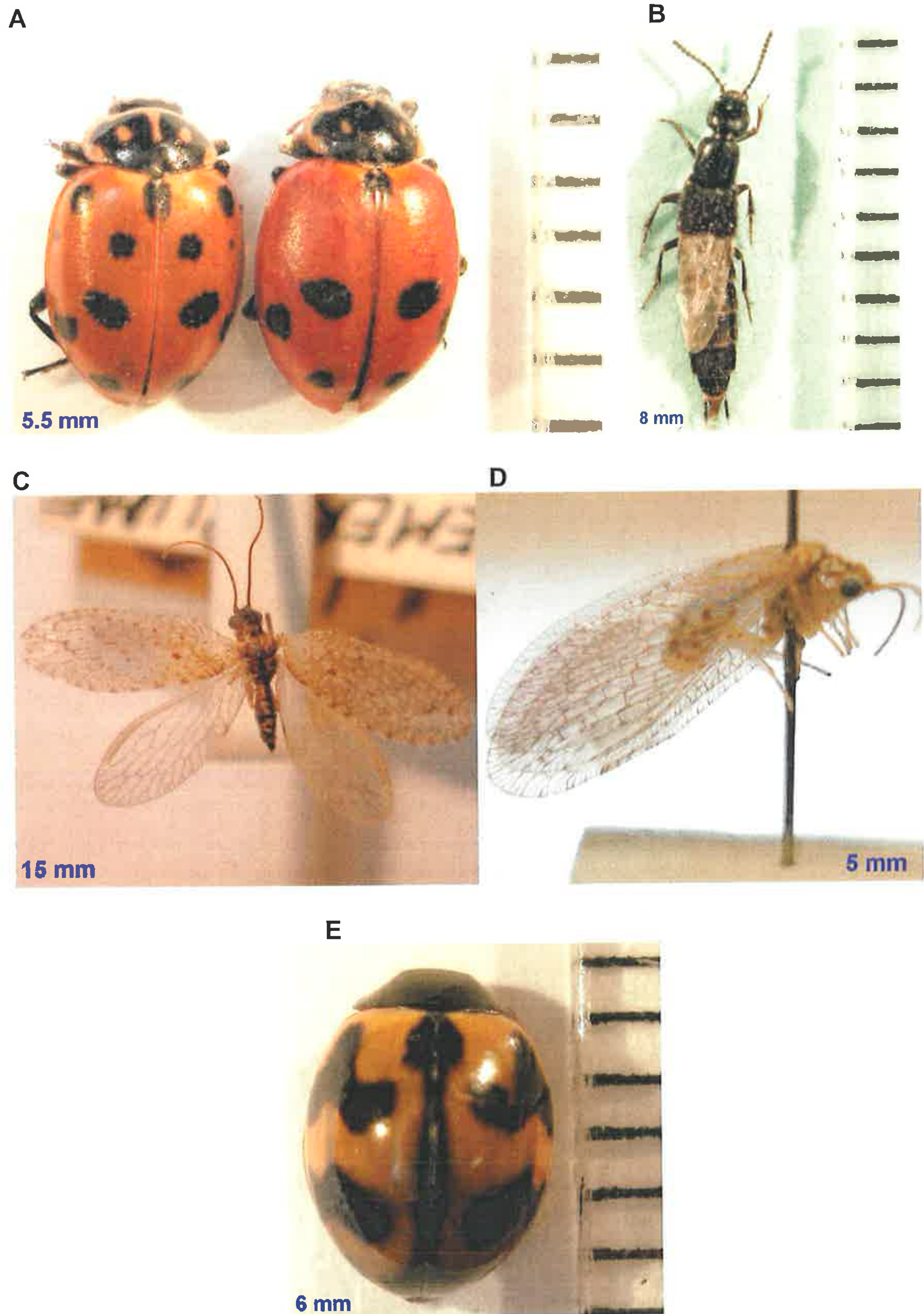

Fig. 3.9. Other predators collected in this study. (A) *Hippodamia variegata*; (B) *Philonthus* sp.; (C, D) *Micromus tasmaniae*; (E) *Coccinella transversalis*.

4. Discussion

This study has provided an inventory of the predatory arthropods associated with *Brassica* crops in South Australia. The results clearly indicated advantage of multiple sampling methods for determination of abundance of arthropod predators in the broccoli field as an example of *Brassica* crops. Each sampling method collected a different number of arthropod predators. Pitfall traps collected the largest numbers of species and specimens. The ground dwelling predators are clearly numerically significant in this cropping system. Sticky traps collected fewer species. It is noteworthy that only one of the most conspicuous species collected with the vacuum sampler, *M. tasmaniae*, was also captured on sticky traps. This suggests that some of the more abundant predators, such as *N. kinbergii* and *O. schellenbergii*, either do not fly often or avoid capture on the sticky traps. In order to have a better understanding of the abundance of predators found in *Brassica* crops, it is necessary to use several collection methods during growing season in subsequent years for target ground and plant dwelling species.

An important issue regarding to collection of predators is their daily period of activity. Leathwick and Winterbourne (1984) reported that predator densities on lucerne were over four times higher at night than in the day. Fewkes (1961) found more Nabidae in sweep net samples taken from grassland at night. Green (1999) showed that four of 21 spider families in citrus orchard were collected nocturnally. In our study, the pitfall traps and sticky traps captured nocturnal and diurnal arthropod predators. However, no attempts were made to collect foliage dwelling nocturnal predators by vacuum sampler. Therefore, if the aim of study was to determine the abundance of predators, taking samples during the day only would be unlikely to be yield a fully representative range

of species. As a result comparison of data obtained from sampling at different times of day may be of limited value.

Wolf spiders were the most commonly collected ground predators in pitfall traps, although Formicidae, Coccinellidae, Linyphiidae, Staphylinidae and Carabidae were also abundant. Namoto (1989) showed that lycosid spiders were an important agent of mortality for third and forth instar diamondback moth. Muchenfuss et al. (1992) reported the wolf spider *P. milvina* is an important member of the predator complex in South Carolina, USA *Brassica* crops, where it was the most frequently collected predator in pitfall trap sampling. Furlong et al. (2004) indicated that spiders (Lycosidae and Oxyopidae) were the most abundant insectivorous predators present on commercial *Brassica* farms in the Lockyer Valley, Queensland, however, Coleoptera (Carabidae, Staphylinidae and Coccinellidae) and Hemiptera were also relatively abundant.

Sampling indicated that the spatial distribution of predators may be affected by plant development and possibly interspecific competition. For example, *H. variegata*, which was the most abundant species of Coccinellidae, was most common in traps placed among flowering plants. It is likely that this species was feeding on aphids that were associated with these older plants. Hence the selective occurrence of the coccinellids on older plants could have been due to the response of prey to the plants, rather than a direct response of the predators to plant development. Spatial separation of the two most abundant species of ants, *Iridomyrmex* sp. and *Pheidole* sp., suggested competitive displacement between them, but this requires further study.

Chapter 4: Development of PCR markers for major pests of *Brassica* crops

1. Introduction

One of the most difficult aspects of studying invertebrate predators is assessing their feeding behaviour under natural conditions. Knowledge of prey items and rates of predation are important in determining the effects of predators on prey populations and are especially critical in evaluating the effectiveness of a predator as a biological control agent (Hayes and Lockley 1990). Unfortunately, detection of predation is technically difficult because prey and predator are relatively small and cryptic in most cases (Greenstone 1996, Naranjo and Hagler 1998). In order to overcome these difficulties, biochemical techniques have been developed and used to identify prey species in predators' diets. Monoclonal and polyclonal antibodies (Symondson 2002) and enzyme-electrophoresis (Traugott 2003) can be used to determine which prey has been consumed by a predator. However, amplification of specific prey DNA using the polymerase chain reaction has proven to be more practical and cost-effective in detecting prey remains. It can be used to detect specifically small amounts of prey DNA in the gut contents of invertebrate predators (Symondson 2002, Agusti et al. 2003a, Harper et al. 2005, Juen and Traugott 2005).

PCR-based techniques are rapidly replacing other biochemical techniques because molecular biology facilities are widely available and prey-specific primers can be used in different contexts once they have been designed. This technique has been successfully used for the detection of a variety of prey remains in predators' gut

contents (Zaidi et al. 1999, Chen et al. 2000, Agusti et al. 2003a, Agusti et al. 2003b, Harper et al. 2005, Juen and Traugott 2005, Read et al. 2006).

Brassica vegetables and oilseeds are economically important crops; approximately 3.1 and 26.1 million ha respectively were grown worldwide in 2004 (Food and Agriculture Organization of the United Nations 2007). These crops are associated with several destructive and widespread insect pests. Total damage caused by these pests is substantial. In Australia, the pests of *Brassica* crops include the lepidopterans *Plutella xylostella*, *Pieris rapae*, *Hellula hydralis*, *Helicoverpa punctigera* and two aphids, *Brevicoryne brassicae* and *Myzus persicae*. Integrated pest management (IPM) systems and the use of biological control methods are preferred approaches to controlling these pests over insecticides due to the prevalence of insecticide resistance in diamondback moth (Shelton et al. 1997).

Generalist predators can play a major role in the control of agricultural pests (Symondson et al. 2002). Studies showed *Brassica* crops have a rich fauna of predators (Chapter 3). The impact of predators on pests of *Brassica* crops has not been thoroughly studied and therefore their potential in suppression of major pests of *Brassica* has not been elucidated. It is essential to develop a reliable technique to evaluate the diets of key predatory species in order to understand their role in pest suppression.

Ma et al. (2005) developed a species-specific marker for *P. xylostella* based on the internal transcribed spacer (ITS-1) of the ribosomal gene. This specific primer pair was used to detect prey in the gut contents of two polyphagous predators, *Nabis kinbergii* and *Trochosa expolita* (reported as *Lycosa* sp.). In a pilot study of predation of

diamondback moth on cauliflower and broccoli farms near Virginia, South Australia, the remains of *P. xylostella* were detected in the gut contents of both species of field-collected predators.

In the current study, the mitochondrial gene coding for cytochrome oxidase subunit I (COI) was selected as a source of diagnostic DNA fragments..

The aims of the present study were: 1) to develop species-specific primers for each of the six major pests of *Brassica* crops, 2) to test their specificity and sensitivity against non-target species and other common predators (Note that any false positive results can affect the interpretation of predation, especially when generalist predators can feed on a wide range of prey species (Sunderland 1975). Thus specificity and sensitivity of each primer set are critical and have to be tested before any application in field studies.), and 3) to test the potential of multiplexing of two primer pairs to assess the potential for simultaneous detection of DNA from two different target prey species.

2. Materials and methods

2.1 Sample collection

Six species of crop pests of *Brassica* spp. and predators and non-predatory arthropods were collected from different farms in South Australia, but mainly from Pitchford's broccoli farm (Currency Creek, South Australia) during 2004 and 2005 (Table 4.1). In addition, *P. xylostella* and *P. rapae* were obtained from a laboratory culture. Collection was done by hand, vacuum sampler or insect net for day active invertebrates and with a headlamp at night for wolf spiders (Wallace 1937). Identification of species (pests and

predators) was done morphologically following the most current relevant taxonomic keys and confirmed by comparison to identified-specimens in the Waite Insect and Nematode Collection (Waite Campus, The University of Adelaide, South Australia). Wolf spider samples were identified by Volker W. Framenau (Department of Terrestrial Invertebrates, Western Australian Museum, Perth, WA). Generally, collected samples were preserved either in 70% ethanol or stored at -20°C for future molecular work. Some live predators were kept individually in a plastic cup (7.5 cm diam.× 4.5 cm) provided with a piece of wet cotton wool in the laboratory at room temperature for subsequent feeding experiments.

2.2 DNA extraction

DNA of individual specimens was extracted from legs in order to avoid contamination with gut contents but DNA of predators used for feeding trials were extracted from the whole body (methods described in Chapter 2). DNA of six species of *Brassica* plants was extracted by the Phenol/Chloroform method (Chapter 2).

Preliminary studies showed DNA extracted by the Phenol/Chloroform method from the whole bodies of spiders could not be reliably amplified by PCR. Therefore DNA from spiders was extracted using a method that incorporated silica (Chapter 2). DNA concentration was measured for all DNA extracts (Chapter 2).

2.2 PCR and sequencing

Two universal primers, C1-J-1718 as forward and C1-N-2191 as reverse primer (Simon et al. 1994), were used to amplify a portion of the mitochondrial cytochrome oxidase subunit I (COI) gene of the six species of *Brassica* pests. Amplification was performed

in 50 μl total volume of reaction buffer containing 150 μM dNTPs (Fisher Scientific Inc., USA), 2 mM $MgCl_2$, 0.4 μM each primer, 1 U of *Taq* DNA polymerase (Biotech international Ltd., Australia) and 8 μl of DNA template (20-40 ng). The reaction mix was put into a 0.2 ml PCR tube and amplification was performed in a PTC-200 thermocycler (MJ Research, MA, USA) with the following temperature profile: 95°C for 2 min followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min and a final elongation at 72°C for 5 min. COI specific fragments of each species were extracted, purified and then sequenced (Chapter 2).

2.4 Sequence alignment and primer design

COI fragments were sequenced from two individuals per species in both forward and reverse directions. Sequencing results were reviewed and then edited (Chapter 2). Pairs of primers were designed for each of the six species according to their sequence variations, especially in regions that were unique to each species. Primer design guidelines proposed for the design of efficient and specific primers by Innis and Gelfand (1990) and Saiki (1990) were followed. The primer-primer interactions were analysed using the program "Oligonucleotide Properties Calculator" (http://www.basic.northwestern.edu/biotools/oligocalc.html). Primers were synthesized by Geneworks, Adelaide, South Australia. For optimisation of each primer pair, a gradient PCR program was performed by using gradient thermocycler (PTC-200) with the following temperature profile: 35 cycles at 94°C for 30 sec, 53°C as the lower temperature and 65°C as the higher temperature for 30 sec, 72°C for 60 sec. A first cycle of denaturation was carried out at 95°C for 2 min and a last cycle of extension was performed at 72°C for 5 min.

2.5 Singleplex and Multiplexing of PCR primers

Singleplex PCR was performed in 25 µl total volume of reaction buffer containing 150 µM dNTPs (Fisher Scientific Inc., USA), 2 mM $MgCl_2$, 0.4 µM each of primer pairs for each species (Table 4.2), 1 U of *Taq* DNA polymerase (Biotech international Ltd., Australia) and 4 µl of DNA template. To evaluate the ability of multiplex PCR for simultaneous detection of two target prey DNA fragments, a multiplex PCR procedure was developed and optimised to multiplex the primer pairs DBM-F-2/DBM-R1-1 and HH-F-1/HH-R-1 (Table 4.2) for *P. xylostella* and *H. hydralis,* respectively. These primers have the same annealing temperature but each pair can amplify fragments of different size. Based on a 25 µl reaction volume, the multiplex PCR reaction mix consisted of 150µM dNTPs (Fisher Scientific Inc., USA), 2 mM $MgCl_2$, 0.4 µM each of the *P. xylostella* primers, 0.2 µM each of the *H. hydralis* primers, 1 U of *Taq* DNA polymerase (Biotech international Ltd., Australia), 1× of reaction buffer provided by the manufacturer, 0.8 µg/µl BSA (Promega, Madison, USA) and 4 µl of DNA template. The cycling conditions for singleplex PCR were 95°C for 2 min followed by 35 cycles of 94°C for 30 sec, 30 sec at the specific annealing temperature for each specific primer pairs (Table 4.2), and 72°C for 1 min and final extension at 72°C for 5 min. For multiplex PCR, annealing temperature used in cycling program was 58°C for 30 sec and the rest of conditions were the same as singleplex.

2.6 Primer specificity and sensitivity

The specificity of the primer pairs was tested separately for each of the designed primers by attempting to amplify target DNA (at least 10 individuals) from six *Brassica* pests and other invertebrates, including a representative sample of most abundant

Diptera, Hymenoptera, and selected arthropod predators as well as an aphid and a lepidopteran, and six species of *Brassica* crops collected from the field (Table 4.1). In order to test if the designed primers would amplify specific DNA in the presence of predator DNA even in high amounts, extracted DNA of each species was diluted and mixed with DNA of a selected predator (*T. expolita*). The concentration of predator's DNA was 20 ng and constant in all mixtures, while the ratio of predator DNA to target species DNA mixtures varied (Table 4.3).

Table 4.1. List of arthropods and *Brassica* plants used to test specificity.

Scientific name	Order: Family	Common name
Plutella xylostella (L.)	Lepidoptera: Plutellidae	Diamondback moth
Pieris rapae (L.)	Lepidoptera: Pieridae	Cabbage white butterfly
Hellula hydralis Guenee	Lepidoptera: Pyralidae	Cabbage centre grub
Helicoverpa punctigera Wallengren	Lepidoptera: Noctuidae	Native budworm
Agrotis sp.	Lepidoptera: Noctuidae	Cutworm
Brevicoryne brassicae (L.)	Homoptera: Aphididae	Cabbage aphid
Myzus persicae (Sulzer)	Homoptera: Aphididae	Green peach aphid
Acyrthosiphon kondoi Shinji	Homoptera: Aphididae	Bluegreen aphid
Unknown fly	Diptera	Fly
Diadegma semiclausum (Hellén)	Hymenoptera: Ichneumonidae	none
Cotesia plutellae (Kurdjumov)	Hymenoptera: Braconidae	none
Nabis kinbergii (Reuter)	Heteroptera: Nabidae	Pacific damsel bug
Oechalia schellenbergii (Guerin-Meneville)	Heteroptera: Pentatomidae	Predatory shield bug
Creontiades dilutus (Stål)	Heteroptera: Miridae	Green mirid
Philonthus sp.	Coleoptera: Staphylinidae	Rove beetle

Scientific name	Order: Family	Common name
Diomus notescens (Blackburn)	Colepotera: Coccinellidae	Minute two-spotted ladybird
Coccinella transversalis Fabricius	Colepotera: Coccinellidae	Transverse ladybird
Micromus tasmaniae (Walker)	Neuroptera: Hemerobiidae	Tasman's lacewing
Hogna crispipes (L. Koch)	Araneae: Lycosidae	Wolf spider
Hogna kuyani Framenau	Araneae: Lycosidae	Wolf spider
Venatrix pseudospeciosa Framenau & Vink	Araneae: Lycosidae	Wolf spider
Lycosa godeffroyi (L. Koch)	Araneae: Lycosidae	Wolf spider
Venator spenceri Hogg	Araneae: Lycosidae	Wolf spider
Trochosa expolita (L. Koch)	Araneae: Lycosidae	Wolf spider
Brassica oleracea var. capitata	Brassicaceae	Cabbage
Brassica oleracea var. botrytis	Brassicaceae	Cauliflower
Brassica oleracea var. gongylodes	Brassicaceae	Kohlrabi
Brassica oleracea var. italica	Brassicaceae	Broccoli
Brassica campestris. var pekinensis	Brassicaceae	Chinese cabbage
Brassica rapa	Brassicaceae	Canola

2.7 Specificity and Sensitivity of Multiplex PCR

Apart from testing specificity and sensitivity of primers in the singleplex PCR, separate assays were performed to test specificity and sensitivity of multiplex PCR. Primer pairs DBM-F-2/DBM-R1-1 and HH-F-1/HH-R-1 were used for multiplexing. Four different DNA template mixtures were tested in multiplex PCR: 1) a serial dilution of *P.*

xylostella DNA mixture with DNA of predator, 2) a serial dilution of *P. xylostella* DNA mixture with a 0.8 ng constant DNA of *H. hydralis* and DNA of predator 3) a serial dilution of *H. hydralis* DNA mixture with a DNA of predator, and 4) a serial dilution of *H. hydralis* DNA mixture with a constant 1 ng DNA of *P. xylostella* and DNA of predator. The concentration of serial dilution for *P. xylostella* and *H. hydralis* DNA ranged from 16.8 ng to 2 pg and 11.6 ng to 0.02 pg, respectively and the concentration of the predator's DNA (*T. expolita*) was 20 ng and constant in all mixtures.

2.8 Detection of prey DNA in gut contents of predators

Two experiments were performed to test the ability to detect DNA from two different prey species in the gut contents of predators. In the first experiment, three species of field-collected predators (5 specimens each of *T. expolita*, *N. kinbergii* and *H. variegata*) were starved at room temperature for at least seven days. After this period each predator was fed a 2nd or 4th instar of *P. xylostella*. In the second assay 5 starved *T. expolita* were fed on 2nd instar of *P. rapae*. Predators were frozen at –20 °C immediately after consuming their prey for subsequent molecular assay.

3. Results

3.1 Primer design and specificity and sensitivity

The readable fragments sequence from the COI gene of each of the six species varied in length (*P. xylostella* 481 bp, *P. rapae* 478 bp, *B. brassicae* 502 bp, *H. punctigera* 514 bp, *M. persicae* 478 bp and *H. hydralia* 492 bp). Sequences were submitted to the National Centre for Biotechnology Information Genebank (http://www.ncbi.nlm.nih.gov; Table 4.2). The sequences of these species were aligned

and, on the basis of diagnostic differences among sequences, one species-specific pair of primers was designed for each species (Table 4.5). Optimised annealing temperature ranged from 58°C to 64°C for each primer pair (Table 4.2). The target sequences amplified by the six primers pairs range from 200 to 307 bp (Table 4.2, Fig 4.1). All primer pairs proved to be highly specific against non-target DNA and could amplify the expected fragment size only in the presence of respective target species DNA (Fig. 4.1). Results revealed the highest and lowest detection sensitivity among the designed primers belong to *H. hydralis* with 0.02 pg and *H. punctigera* with 27.3 pg (Table 4.3). As little as 2 pg of the DNA of the key pest species *P. xylostella* could be detected in singleplex PCR (Fig. 4.2).

Table 4.2. Species-specific primer sequences designed from the COI mtDNA of six common pests of *Brassica* crops, optimal PCR annealing temperatures, amplification fragment sizes and GeneBank accession numbers for the COI gene fragments.

Species	Primer name	Sequence	Accession no.	Annealing temp. (°C)	Fragment size
P. xylostella	DBM-F-2	5′-TGTTTATCCTCCTTTATCTTCA-3′	AY898745	58	293
	DBM-R1-1	5′-CTCCTGCAGGATCAAAGAAG-3′			
P. rapae	PR-F-1	5′-AGTGTACCCCCCACTTTCTT-3′	AY898746	60	222
	PR-R-1	5′-ACTGGTAATGATAATAGTAAAAGT-3′			
H. punctigera	HP-F	5′-CTCATGGAGGAAGATCTGTA-3′	AY898747	64	270
	HP-R	5′-CTCCTCCTCCAGCAGGAT-3′			
B. brassicae	BB-F-1	5′-TTGATTACTCCCTCCATCAC-3′	AY898748	60	307
	BB-R	5′-TCCAGCTAATACTGGGAGA-3′			
M. persicae	MP-F	5′-TGATTATTACCACCCTCAT-3′	AY898749	58	247
	MP-R	5′-TGGAAATAAAGGGATTTGG-3′			
H. hydralis	HH-F-1	5′-TGGTGGAAGATCAGTTGATC-3′	DQ387063	58	200
	HH-R-1	5′-CTCCAGCTAATACTGGTAGT-3′			

Table 4.3. Sensitivity of all six *Brassica* pests primer pairs. The DNA of target prey was mixed with 20 ng of DNA from *Trochosa expolita* in a range of dilutions. Prey DNA could be detected at the indicated sensitivity threshold across the range of dilutions shown.

Species	Minimum and maximum proportion of predator: target species DNA in mixtures	Sensitivity in singleplex PCR
H. hydralis	1.7: 1 / 10^6: 1	0.02 pg
P. xylostella	1.2: 1 / 11111: 1	2 pg
P. rapae	2: 1 / 2083: 1	9.6 pg
H. punctigera	1.4: 1 / 732.6: 1	27.3 pg
B. brassicae	2.1:1 / 153846:1	0.13 pg
M. persicae	2.4: 1 / 1282: 1	15.6 pg

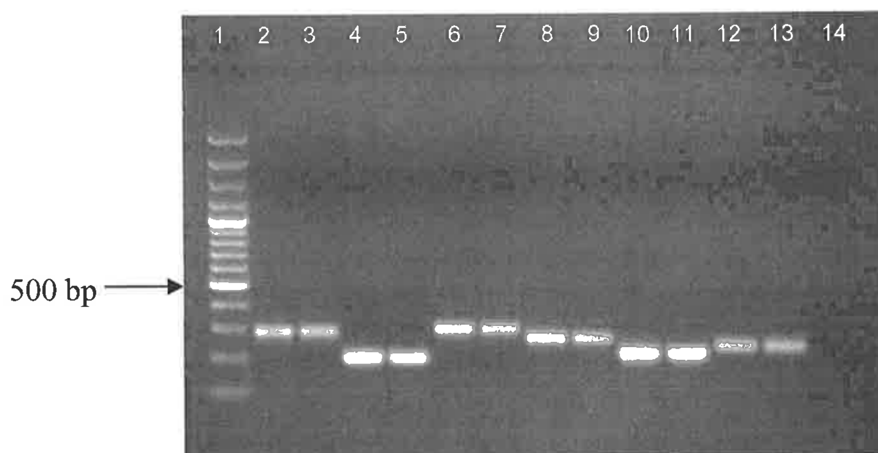

Fig. 4.1. Agarose gel electrophoresis of polymerase chain reaction (PCR) amplified DNA using all six pest specific primer pairs for the target species, lane 1, 100 bp molecular marker, lanes 2-3, *P. xylostella* (293 bp), lanes 4-5, *H. hydralis* (200 bp), lanes 6-7, *B. brassicae* (307 bp), lanes 8-9, *H. punctigera* (270 bp), lanes 10-11, *P. rapae* (222 bp), lanes 12-13, *M. persicae* (247 bp), lane14, negative control.

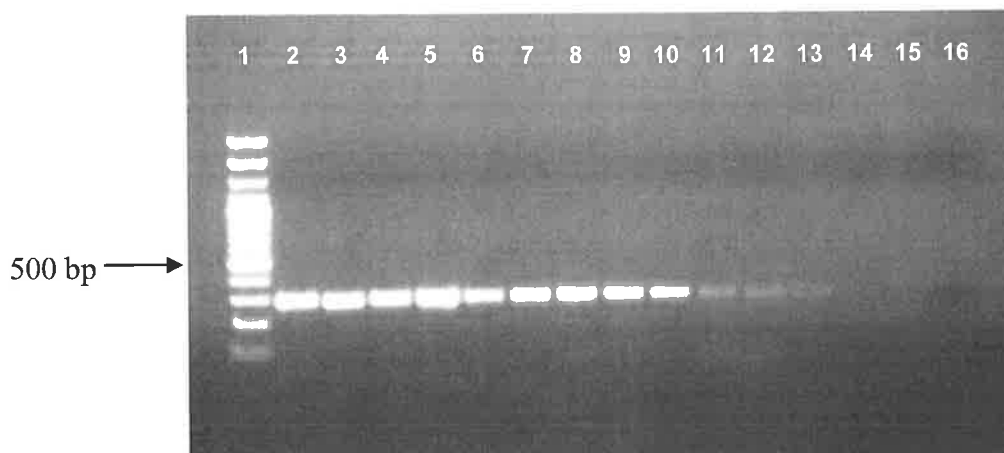

Fig. 4.2. Concentration-response trial to determine the sensitivity of *P. xylostella* primer pairs (DBM-F-2/DBM-R1-1). Prey DNA was serially diluted in a constant concentration of 20 ng of DNA from *T. expolita*, lane1, 100 bp molecular marker, lanes 2-15 are, 16.8 ng, 8.4 ng, 4.2 ng, 2.1 ng, 1.05 ng, 0.526 ng, 0.262 ng, 0.131 ng, 0.065 ng, 0.032 ng, 0.0164 ng, 0.0082 ng, 0.0041 ng, 0.002 ng (total DNA in PCR) respectively, lane 16, negative control.

3.2 Specificity and sensitivity of Multiplex PCR

Multiplex PCR with DBM-F-2/DBM-R1-1 and HH-F-1/HH-R-1 demonstrated the possibility of simultaneous detection of *P. xylostella* and *H. hydralis* and selectivity against other non-target species (data not shown).

Multiplex PCR showed less sensitivity compare with singleplex PCR. In the multiplex PCR assay, detection sensitivity of *H. hydralis* primers was 8-fold (mixture with predator DNA) and 140-fold lower (mixture with *P. xylostella* DNA) compared to singleplex PCR. Likewise, *P. xylostella* primer pairs in the mixture with predator DNA had the same sensitivity as singleplex PCR, however their sensitivity in the mixture with *H. hydralis* DNA was 8.2-fold lower than with singleplex PCR (Table 4.4).

Table 4.4. Comparison of sensitivity of *H. hydralis* and *P. xylostella* primer pairs in singleplex and multiplex PCR in two different DNA mixtures (predator and opposite prey + predator).

Species	Sensitivity in Singleplex PCR		Sensitivity in Multiplex PCR	
	Mix with Predator's DNA	Mix with opposite target DNA + Predator's DNA	Mix with Predator's DNA	Mix with opposite target DNA + Predator's DNA
H. hydralis	0.02 pg	0.02 pg	0.16 pg	2.8 pg
P. xylostella	2 pg	2 pg	2 pg	16.4 pg

3.3 Detection of prey DNA in the gut contents of predators

DNA of target pests *P. xylostella* and *P. rapae* could be reliably detected in the gut contents of *T. expolita*, *N. kinbergii* and *H. variegata* (Fig. 4.3)

Fig. 4.3. Detection of target prey DNA in the gut contents of predator, using primer pairs DBM-F-2/DBM-R1-1 and PR-F-1/PR-R-1. lanes 1,8,15,22, 100 bp molecular markers, lanes 2-6, *T. expolita* fed on *P. xylostella* larvae, lanes 9-13, *N. kinbergii* fed on *P. xylostella* larvae, lanes 16-20, *H. variegata* fed on *P. xylostella* larvae, lanes 23-27, *T. expolita* fed on *P. rapae*, lanes 7,14,21,28, negative controls.

4. Discussion

Species-specific molecular markers were developed for six *Brassica* pests. The designed primer pairs were tested against many possible non-target invertebrate species found in broccoli fields as well as *Brassica* plants with no amplification detected for non-target species, which indicates that these primer pairs were highly specific for the target species. In the only published study of molecular detection of *Brassica* crop pests in predators' gut contents, Ma et al. (2005) showed that using a primer set based on the ITS-1 region not only amplified target DNA but also larger non-specific fragments from *Brassica* plants. The current study aimed to identify specific sequences that are 300 bp or smaller to ensure reasonable detection times, because it has been known that prey DNA is fragmented in the predators' gut by digestion enzymes. As a result the detection time of prey DNA depends on the length of the amplification product (Hoogendoorn and Heimpel 2001, Agusti et al. 2003a). Larger fragments become undetectable in the gut more rapidly than smaller ones (Zaidi et al. 1999, Agusti et al. 2000). However, some studies showed there is no difference in detection rate within a range of fragment lengths. For example Chen et al. (2000) found no difference in the detection rates of *S. avenae* fragments shorter than 246bp. In similar cases, studies showed there was no difference in detection rates of DNA fragments between 175 and 387 bp in feeding experiments with cockchafer prey (Juen and Traugott 2005), and detection for a 127 bp fragment was not significantly higher than for a 463 bp fragment of the *A. solstitiale* (Juen and Traugott 2006).

The sensitivity thresholds of species-specific primers in prey detection achieved by PCR is an important issue because it indicates whether a single prey consumed by a predator

is sufficient for detection (e.g. Zaidi et al. 1999, Chen et al. 2000, Admassu et al. 2006). In this study a concentration-response trial of prey DNA in the presence of a constant concentration of predator DNA was performed for all six primers pairs. Detection limits ranged from 0.02 pg to 27.3 pg (Table 4.3). This result is comparable to the highest detection sensitivity levels achieved in a study on PCR used to detect parasitism; Traugott et al. (2006) reported detection limits ranging between 0.6 pg and 46.8 pg of DNA from a parasitoid of *P. xylostella*. Results in this study showed that the presence of a second non-target DNA in the singleplex PCR does not influence the detectability of target DNA in all primer pairs examined. For example *H. hydralis* DNA was detectable in the presence of 10^6 times the concentration of *T. expolita* DNA. Thus DNA can be extracted from the whole body of a predator for DNA extraction without any dissection of the gut or its contents. This is a big advantage in field studies and makes the analysis of samples with small predators easier and quicker. In a study of earthworm residues in predators' gut contents (Admassu et al. 2006), sensitivity of designed primers was determined to be 0.15 ng/μl of earthworm DNA in the presence of 243 times the amount of predators' DNA. Likewise, Sheppard et al. (2004) showed that, despite the presence of large amounts of predator tissue, there was no evidence that non-target material could mask the detection of very small amounts of prey DNA.

Some workers used another approach to determine the sensitivity threshold of primers. For example Agusti ct al. (2003b) defined the sensitivity of designed primers at 10^{-5} dilution of a target sample and Chen et al. (2000) determined sensitivity to be 10^{-7} aphid equivalents of DNA. However, the concentration of the extracted DNA depends on the size of sample and extraction method used, therefore results will vary with smaller or larger specimens.

Results revealed the possibility of applying multiplex PCR for the simultaneous detection of at least two prey species in one reaction. This is a big advantage in increasing the efficiency of PCR amplification. However, the results indicated that the ratio of concentrations of DNA from target prey is an important factor in detection of each species in a multiplexing system. On the other hand, if the concentration of *H. hydralis* is higher than *P. xylostella*, the latter prey may either not be detected or only observed as a very faint band in the electrophoresis gel. A possible explanation may be the competition between DNA of target species. For this reason sensitivity of primers in multiplex PCR was less than singleplex PCR. This was obvious when DNA of *H. hydralis* was mixed with a constant concentration of *P. xylostella* and vice versa. The sensitivity of primers decreased in the multiplex PCR reaction from 0.02 pg (in singleplex PCR) to 2.8 pg for *H. hydralis* and from 2 pg to 16.4 pg for *P. xylostella* (Table 4.4). In the multiplex PCR assay with primers of *P. xylostella* and one of its parasitoids, detection sensitivity of the parasitoid primers was determined to be 4-fold lower compared to singleplex PCR, but the sensitivity of *P. xylostella* primers was not determined in the multiplex PCR reaction (Traugott et al. 2006). Harper et al. (2005) developed a multiplex PCR incorporating fluorescent markers to detect mitochondrial DNA fragments from more than 10 prey species simultaneously in the gut contents of generalist predators. However, the higher cost of techniques and equipment used is a limiting factor in thc application of this method in ecological investigations. Moreover the sensitivity of the multiplex systems was not tested with varying ratios of prey DNA to examine the effect on detection of each species.

Competition between DNA from different target species is a known phenomenon in multiplex PCR (Markoulatos et al. 2002). For example, in a study on simultaneous detection of four bacterial pathogens (Stralin et al. 2005), while there was a clear dominant band in the gel electrophoresis from one pathogen, a relatively weaker PCR band was identified for the pathogen with the lower concentration. Consequently a strong band for one pathogen in the gel electrophoresis may be associated with a decreased sensitivity for detection of other species by multiplex PCR. Therefore in future further investigation should be done to examine the real sensitivity of each primer in multiplex PCR before any application in the ecological studies.

This study has demonstrated that species-specific primers targeting six pests of *Brassica* crops can be used for ecological studies of *Brassica* pests and to screen field-caught predators for prey species consumption. Multiplexing of primers has potential for application in ecological investigations but further study is needed to establish the sensitivity of the method in each case. This study reinforces the diagnostic utility of the mitochondrial COI gene in studies of predator diets. Partial sequences of COI in the order of 200-300 bp can readily be detected in mixed DNA samples.

Table 4.5. Alignment of COI sequences from six pest species of *Brassica* crops. Species-specific primers of each species are highlighted.

```
                       *        20         *        40         *        60         *        80
Plutella    : ---------------------------------.......................T..T..................... :  48
Helicoverpa : ...........G..............A.................A..............C.................T... :  81
Pieris      : ---------------..........................C...........C........................... :  66
Hellula     : ------------..........................T..C..............T..T..................... :  69
Brevicoryne : ---------..................A.....A.....TG.........GT.A..T......T..........T...... :  72
Myzus       : ---------------------------------A.....TG..........T...........T.......C..T.....C :  48
              GGAGGATTTGGAAATTGATTAGTTCCTTTAATATTAGGAGCTCCTGATATAGCTTTCCCACGAATAAATAATATAAGATTT

                       *       100         *       120         *       140         *       160
Plutella    : ...C....T..............C...T.......C...........T..............T..T........T.....T : 129
Helicoverpa : ........T..T...........T...C.T..................................................T : 162
Pieris      : ..............T........T...T.................................................G... : 147
Hellula     : ...C.TT.......A........C...C..................................T.................. : 150
Brevicoryne : ........C..T..A...C...T.A.........GT..TTTTT.AA.TA.T......A................TA....T : 153
Myzus       : ......T....A..........T.A.........GT..TTTTT.AA.TA.T......A................TA..... : 129
              TGATTACTACCCCCCTCATTAACATTAATAATTTCAAGAAGAATTGTAGAAAATGGAGCAGGAACAGGATGAACAGTTTAC

                       *       180         *       200         *       220         *       240
Plutella    : ..T..TT....T..A.....C.................T..C..................C.T........C..T...... : 210
Helicoverpa : ........T..................G..........T..A..............C..CC.T........T......... : 243
Pieris      : ........T..T....................TTCT.....A........C........A...........T......... : 228
Hellula     : ........T.....A........C...G.T...............C....A.............................. : 231
Brevicoryne : ..A..T......AA..............ATAAT.TT............A...............................A : 234
Myzus       : ..A..CT.....AA.........A....ATAAT.TT............A..........A....................A : 210
              CCCCCACTATCATCTAATATTGCTCATAGAGGAAGATCAGTTGATTTAGCTATTTTTTCTTTACATTTAGCAGGAATTTCT
```

Table 4.5. (Continued)

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                   *       260         *       280         *       300         *       320
Plutella   : ...........G.................A...................G....GGA..G..........GT......... : 291
Helicoverpa: ..T.............................................T........T....T..............A... : 324
Pieris     : .............................A...............CGT..T.GA........................... : 309
Hellula    : ..C..........................C....................T...GG.C.T..T.................. : 312
Brevicoryne: ..........................CTG...A...C.A.......T.CCT.........AA...AA.......T...C.T : 315
Myzus      : ...........................TG...A..CT.A.......T.CC...C......AA...AA.C.....C...... : 291
             TCAATTTTAGGAGCAATTAATTTTATTACTACTATTATTAATATAAAAATAAATAATATATCATTTGATCAAATACCTTTA

                  *       340         *       360         *       380         *       400
Plutella   : .....T.....T..A........................C...............................T......... : 372
Helicoverpa: ...A.T........A........T..AT...........C.....T................T........T...C.T... : 405
Pieris     : .....T.....T...............T.AC.T...C.......AT........C..........C.........C.T... : 390
Hellula    : ...........T..G...........CT.A..............A........A.....T.....A.....T......... : 393
Brevicoryne: ...CC....T..A..TT...................A.T........C.....A.....T.....A............... : 396
Myzus      : ...CC....T..A..TT...................A.T......T....T...C.......T.................. : 372
             TTTGTATGAGCAGTTGGAATTACAGCTATTTTATTATTATTATCTCTACCAGTTTTAGCAGGAGCTATTACAATATTATTA

                 *       420         *       440         *       460         *       480
Plutella   : .....T.....C...........C..C.....T..T........G.................C.................. : 453
Helicoverpa: ............C.T.....A..T........T..T..T...........C.................C............ : 486
Pieris     : ....................C..............G...........T.....A...C.T.........C........... : 471
Hellula    : ..T..........................C...........T..G.................C.................C : 474
Brevicoryne: ..T..T..........................T................................................ : 477
Myzus      : ..T..T..T................................G.....T..C..A..C..G..................... : 453
             ACAGACCGAAATTTAAATACTTCATTTTTTGACCCAGCAGGAGGAGGAGATCCTATTTTATATCAACATTTATTTTGATTT
```

Table 4.5. (Continued)

```
                    *        500         *        520
Plutella    : ........C..................------------ : 481
Helicoverpa: .....A..C..A................------------ : 514
Pieris      : .....G.---------------------------------- : 478
Hellula     : ...........A......----------------------- : 492
Brevicoryne: .........................---------------- : 502
Myzus       : .........................---------------- : 478
              TTTGGTCATCCTGAAGTTTATATTTTAT
```

Chapter 5: Factors affecting detectability of prey DNA in the gut contents of predators: A PCR-based method

1. Introduction

The polymerase chain reaction (PCR) has been used effectively to amplify prey residues in the gut contents of a range of arthropod predators (e.g. Spider: Greenstone and Shufran 2003, Coleoptera: Chen et al. 2000, Sheppard et al. 2004, Hemiptera: Agusti et al. 2003b). The assay uses species- or group-specific primers to amplify prey DNA in the gut contents of predators (Symondson 2002). Several species can be identified based on different diagnostic fragment sizes, which are observed as bands in the stained agarose gel following electrophoresis. The detection of prey DNA in the gut contents of predators is potentially affected by a number of environmental and physiological factors. Therefore, the use of field data obtained by PCR-based methods to model predation rates (Mills 1997) requires careful evaluation of all possible factors that may affect interpretation of field-derived material.

Various factors may affect the detectability of prey residues in the gut contents of predators. Biological factors include the degree of digestion as indicated by time since feeding (Chen et al. 2000, Hoogendoorn and Heimpel 2001, Agusti et al. 2003a,b, Shepperd et al. 2005, Read et al. 2006), gender (Hoogendoorn and Heimpel 2001, Shepperd et al. 2005), weight (Hoogendoorn and Heimpel 2001), size, developmental stages and species of predator (Hoogendoorn and Heimpel 2001), prey species (Harper et al. 2005, Juen and Traugott 2006), and meal size and subsequent food intake (Agusti et al. 1999a, Hoogendoorn and Heimpel 2001, Juen and Traugott 2005). Temperature (Hoogendoorn and Heimpel 2001) is a physical factor that can affect the rate of

digestion and hence the detectablility of prey remains. The sensitivity and stability of primers (Leon et al. 2006, Admassu et al. 2006), multiple copy gene sequences (Zaidi et al. 1999), the size of amplification products (Agusti et al. 1999b, 2000, Chen et al. 2000, Hoogendoorn and Heimpel 2001), DNA extraction methods and amount of template DNA in PCR (Juen and Traugott 2005, 2006) are methodological factors that have been reported to affect detectability of prey DNA.

Few studies have attempted to quantify the potential influence of secondary predation or scavenging on detectability of prey DNA (Harwood et al. 2001, Calder et al. 2005, Sheppard et al. 2005), which could lead to overestimation of primary predation in field studies. Dennison and Hodkinson (1983) were unable to detect any first prey after secondary predation using polyclonal antisera. Harwood et al. (2001) reported a fully replicated and quantitative study of secondary predation within a three species interaction (aphid-spider-beetle) using an aphid-specific monoclonal antibody to detect a prey antigen. They found that secondary predation was only detected when the beetles were killed immediately after consuming at least two spiders, which were in turn eaten immediately after consuming aphids. Sheppard et al. (2005) also used an aphid-specific PCR-primer to detect secondary predation in an aphid-spider-beetle model. Their results showed detection of secondary predation in beetles up to 4 hours after they consumed spiders that had digested their aphid prey within 4 hours. In PCR-based studies, it is impossible to distinguish between primary predation, secondary predation and scavenging. Therefore distinguishing predation from scavenging requires additional research to interpret the mode of ingestion (Foltan et al. 2005, Juen and Traugott 2005). In this chapter, the possible factors affecting prey detection using PCR-based methods are discussed. Variables that can affect the outcome of gut content analysis are

examined on three model predator species with three different types of mouthparts and likely differences in digestive physiology: the wolf spider *Venator spenceri* Hogg. (Araneae: Lycosidae), the spotted amber ladybird, *Hippodamia variegata* (Goeze) (Coleoptera: Coccinellidae), and the Pacific damsel bug, *Nabis kinbergii* (Reuter) (Hemiptera: Nabidae). These arthropods are common predators in *Brassica* crops in Australia (Chapter 3). The aims in this chapter were to explore: 1) the effects of time since feeding (determination of half-life for each predator under study), temperature, the predator's gender, weight and species on detection of prey, larval diamondback moth (DBM), *Plutella xylostella* (L.), 2) effects of subsequent feeding on detectability of first prey, and 3) the potential variation in detecting active secondary predation in cannibalistic (prey-spider-spider) and intraguild predatory (prey-beetle-spider) food chains. These data are required before entering into field investigations for the determination of the key predators of *P. xylostella*.

2. Materials and methods

2.1 Arthropods

The three species of predators that were the subjects of this study were prepared in different ways for the experiments. Wolf spiders were collected by headlamp at night (Wallace 1939) from Pitchford's broccoli farm, Currency Creek, South Australia (35° 41′ S, 138° 75′ E). Specimens were maintained individually at room temperature in plastic cups (7.5 cm diam.× 4.5 cm) provided with a piece of wet cotton wool and larvae of Mediterranean flour moth (*Ephestia kuehniella* Zeller) as food. The spotted amber ladybird was obtained from a culture, which was initiated from a batch of eggs that was provided by IPM Technologies Pty Ltd, Hurstbridge, Victoria, Australia. The culture of ladybirds was maintained in an incubator at 25±1°C and reared separately in transparent

plastic cups (7.5cm diameter × 4.5 cm height) (see Chapter 2 for details). Damsel bugs, *N. kinbergii*, were collected from a broccoli field at Currency Creek and on weeds at the Waite Campus, the University of Adelaide, Urrbrae, South Australia. Field-collected damsel bugs were maintained in plastic cups (7.5 cm diam.× 4.5 cm) at room temperature, and were provided with a wet cotton dental roll and a piece of cabbage leaf, but no prey, until used in experiments.

Two species were used as prey in the experiments. Larvae of *P. xylostella* were obtained from a laboratory culture that was reared on cabbage plants at 25±1°C (Chapter 2). A culture of vinegar fly, *Drosophila melanogaster,* was reared in plastic bottles (125 ml) (Chapter 2).

All predators were starved prior to experimentation. In order to prevent cannibalism among wolf spiders and damsel bugs, each specimen was individually confined in a transparent plastic cup (7.5cm diameter × 4.5 cm height) and starved for at least 7 days at room temperature. Moisture was supplied by adding a 2 cm wet cotton dental roll and a piece of cabbage leaf. Newly-emerged adult ladybirds were removed from the rearing culture and individually maintained in a 1.5 ml centrifuge tube provided with a drop of water and starved for 2 days. Following starvation, 5 specimens each of wolf spiders, ladybirds, and damsel bugs were frozen at -80°C to be used as negative controls.

2.2 Factors affecting detection of prey DNA in primary predators

The first experiment investigated the effects of temperature and time since feeding on the detection of prey remains in wolf spiders. Each spider was offered one live 4th instar *P. xylostella* in a transparent plastic cup (7.5 cm diameter × 4.5 cm height) at room

temperature and allowed to consume it for 30-45 min to finish its meal completely. Once the spider had eaten its whole meal, it was either immediately frozen (t=0), or maintained for 2, 4, 8, 16, 24, 48, 72, 96, 120 h at 15, 20, 25, or 30°C in a constant temperature incubator and then frozen at -80°C for subsequent assay. For each time interval and temperature, at least 5 individuals were used. In all experiments spiders of similar body size were used (10-15mm).

Trials involving adult *H. variegata* and *N. kinbergii,* were modified to accommodate their size and biology. As these predators were smaller than the wolf spiders, they were fed one live 2nd –3rd instar *P. xylostella* in a 1.5 ml Eppendorf tube at room temperature. Predators were allowed to consume the whole prey for 45 min. The predators, which had eaten the meal, were immediately frozen (t=0) or maintained for further specified time periods. Following feeding, *H. variegata* was held at 15, 20, 25 or 30°C for 2, 4, 6, 8, 16, 24, 32, 48 h until freezing at -80°C. *N. kinbergii* was held only at 20°C and the time intervals after feeding were 2, 4, 8, 16, 24, 32, 48, 72, 96, 120 h. Each time interval experiment was repeated at least 5 times. The sex and weight of each *H. variegata* was determined prior to assay, but these characteristics were not recorded for *N. kinbergii.* Individuals that failed to feed or had died before the end of the trial were discarded.

2.3 Effect of second prey intake on detectability of first prey DNA

After starving for seven days, 19 *V. spenceri* were fed one live 4th instar *P. xylostella* individually in plastic cups (7.5 cm diameter × 4.5 cm height). After consumption of the whole larva, each wolf spider was placed into a Petri dish (9 cm diameter × 2 cm height) provided with wet filter paper and more than 40 vinegar flies. They were allowed to feed on this second prey species overnight. Afterwards each wolf spider was

held for a further 16 h before it was frozen at –80 °C. The experiment was conducted in an incubator at 20±1°C.

2.4 Secondary predation

Two experiments were conducted to determine the detectability of prey DNA in a secondary predator after prey was first consumed by a primary predator. *P. xylostella* was the prey. In one food chain, wolf spiders cannibalised smaller wolf spiders. In the other food chain, wolf spiders fed on ladybird beetles that had consumed *P. xylostella*. Prior to experiments, wolf spiders were starved for seven days and ladybirds for two days. For primary predation, newly emerged adult ladybirds were fed one 2nd instar *P. xylostella* and small wolf spiders (7-8 mm body length) were fed one 4th instar *P. xylostella*. Primary predators were then presented to a large wolf spider (> 15 mm body length). In all of these cases the first predator was offered for consumption to the second predator immediately after it had consumed the prey, *P. xylostella*. Beetles or spiders that failed to eat their meal were discarded from the experiment. Following secondary predation, spiders were maintained for a further 20 h at room temperature before freezing at –80 °C. Gut analysis included totals of five wolf spiders that were intraguild predators and 14 wolf spiders that were cannibals.

2.5 DNA extraction and PCR amplification using DBM-specific primers

The presence of prey remains in the guts of predators was determined using the following protocol. DNA was extracted with a silica-based method (Chapter 2). After extraction, DNA of each sample was diluted in 100 µl TE and stored at -20°C until it was analysed. DNA concentration was measured using a spectrophotometer (ND-1000: NanoDrop technologies Inc., Wilmington, DE, USA) according to the manufacturer's

manual. In this study, primers were used that specifically amplify a fragment of the cytochrome oxidase subunit I gene of *P. xylostella*. The forward primer was DBM-F-2 (5′-TGTTTATCCTCCTTTATCTTCA-3′) and the reverse primer was DBM-R1-1 (5′-CTCCTGCAGGATCAAAGAAG-3′)(Chapter 4). These primers amplify a 293bp fragment. PCR amplification was performed in 25 μl total volume containing, 2.5 μl of 10× reaction buffer (Fisher Scientific Inc., USA), 150 μM dNTPs (Fisher Scientific Inc., USA), 2 mM $MgCl_2$ (Fisher Scientific Inc., USA), 0.4 μM each primer, 1 U of *Taq* DNA polymerase (Biotech international Ltd., Australia) and 4 μl of DNA template (10 ng/μl). The reaction mix was transferred into a 0.2 ml PCR tube and amplification was performed in a PTC-200 thermocycler (MJ Research, MA, USA) with the following temperature profile: 95°C for 2 min followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min. A final strand elongation at 72°C was performed for an additional 5 minutes. PCR products (8 μl) were separated on 1.8% agarose gel containing ethidium bromide for staining following electrophoresis at 100 V.

2.6 Statistical analysis

Effects of time since feeding, temperature, sex and weight of predator on the frequency of detection of prey DNA were analysed using logistic regression. These factors were included as main effects plus a time × temperature interaction term in the full statistical model. Stepwise elimination was used to remove those factors that had no significant effect on detection of prey. As the experiment with *N. kinbergii* involved only one temperature, data for this species were analysed using a logistic regression of the frequency of detection vs. time since feeding. The half-life of detection for each predator was estimated from the final fitted curve. The half-life characterizes the

detection period for prey DNA. Data were analysed using STATGRAPHICS Centurion XV version 15.2.00 and Microsoft EXCEL 2000.

3. Results

3.1 Factors affecting detection of prey DNA in primary predators

Time since ingestion of prey was the primary factor that influenced the detection of prey DNA. Detection frequency decreased with increasing time since ingestion and increasing temperature in *H. variegata*, but there was no significant influence of gender, weight, or the interaction of time and temperature on detection (Table 5.1). In wolf spiders detection of prey DNA decreased with increasing time since ingestion, while there was no significant effect of temperature or the interaction of time and temperature (Table 5.2). In *N. kinbergii,* detection also decreased with increasing time since feeding (χ^2=61.543, df= 1, P = 0.00001). The half-life of detection varied among the three species of predators from 17.1 h in *H. variegata*, to 36.1 h in *N. kinbergii* and 49.6 h in *V. spenceri* (Fig. 5.1-3).

Table 5.1. Results of logistic regression on detection of *P. xylostella* DNA in *H. variegata*.

Factor		Full model		Final model	
	df	χ^2	*Prob*	χ^2	*Prob*
Time	1	3.72282	0.0537	79.8452	0.00001
Temperature	1	11.4962	0.0007	22.8819	0.00001
Weight	1	0.0425103	0.8367		*ns*
Sex	1	0.758458	0.3838		*ns*
Time×Temperature	1	0.0285949	0.8657		*ns*

Table 5.2. Results of logistic regression on detection of *P. xylostella* DNA in *V. spenceri*.

Factor		Full model		Final model	
	df	χ^2	*Prob*	χ^2	*Prob*
Time	1	1.02043	0.3124	82.7635	0.00001
Temperature	1	0.308709	0.5785		*ns*
Time×Temperature	1	0.746845	0.3875		*ns*

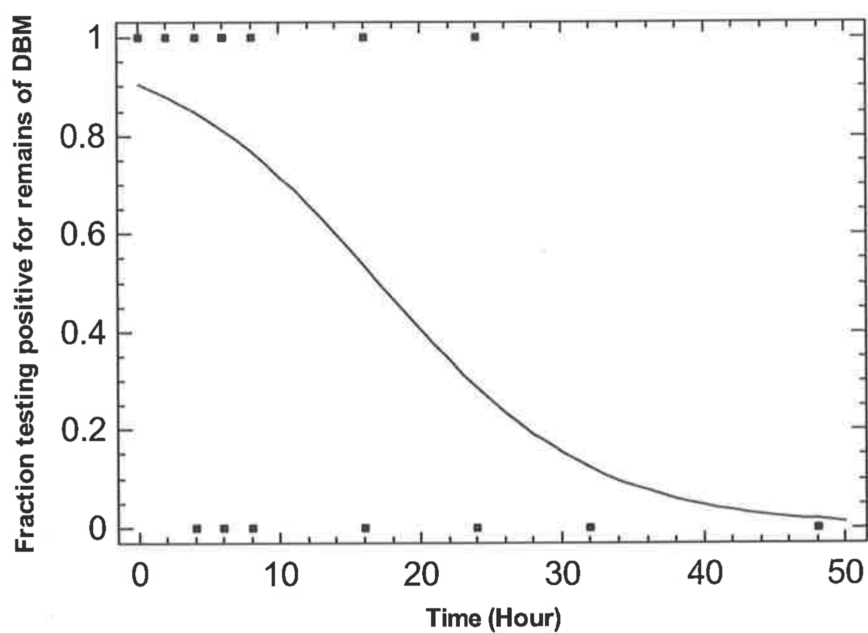

Fig. 5.1. Fitted line from logistic regression of time since feeding on detectability of DNA of *P. xylostella* in *H. variegata* held at 20°C.

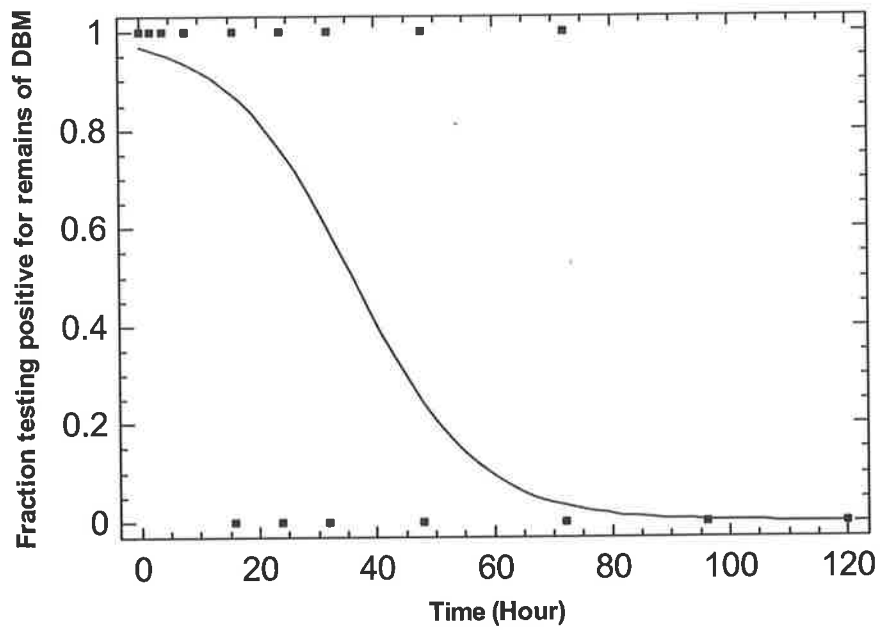

Fig. 5.2. Fitted line from logistic regression of time since feeding on detectability of DNA of *P. xylostella* in *N. kinbergii* held at 20°C.

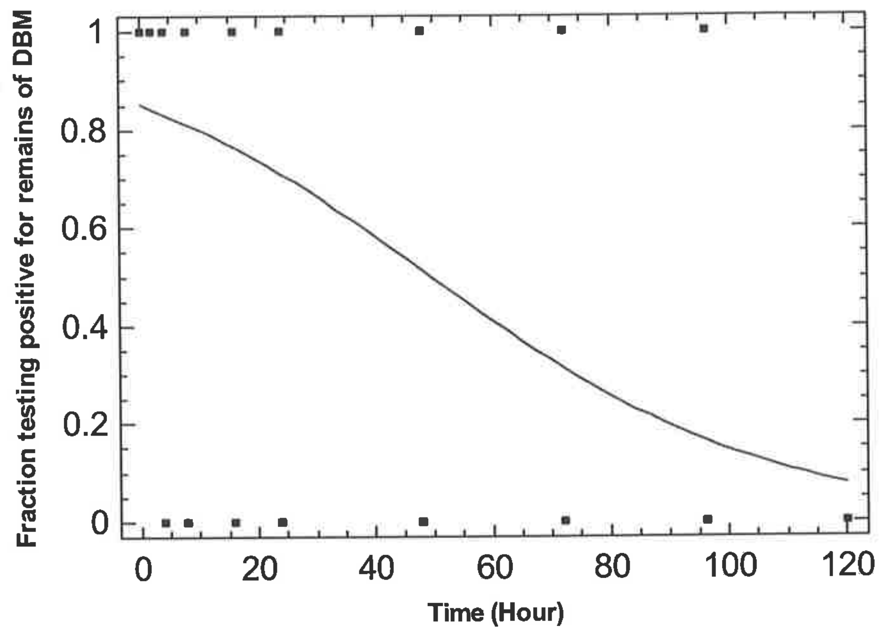

Fig. 5.3. Fitted line from logistic regression of time since feeding on detectability of DNA of *P. xylostella* in *V. spenceri* held at 20°C.

3.2 Effect of second food intake

Consumption of vinegar flies by wolf spiders did not inhibit the amplification of DNA of *P. xylostella* that were consumed previously. Sixteen out of 19 wolf spiders tested positive to target DNA of *P. xylostella* after 16 hours of feeding on *D. melanogaster*. All spiders had consumed all of the flies during this period.

3.3 Detection of *P. xylostella* DNA after secondary predation

Experiments showed that DNA of prey is detectable in two types of secondary predation (Fig. 5.4). Twenty hours after secondary predation, DNA of *P. xylostella* was detected in 13 out of 14 (93%) wolf spiders (second predators) that had consumed a smaller wolf spider (cannibalistic predation) and 4 out of 5 (80%) wolf spiders that had been fed on a ladybird that had consumed *P. xylostella* (intraguild predation).

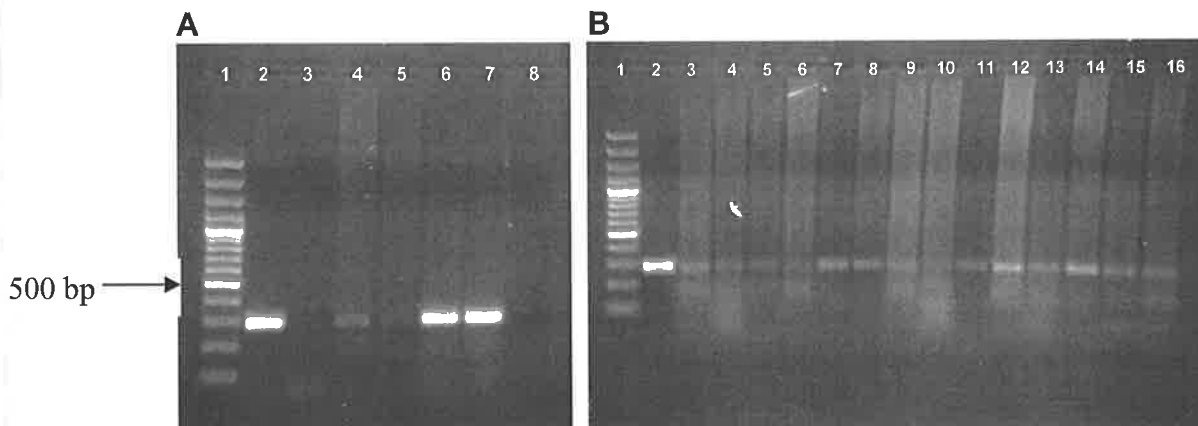

Fig. 5.4. Detection of secondary predation by *V. spenceri* (A) intraguild predation: lane 1, 100bp molecular marker ladder, lane 2, positive control, lanes 3-7 wolf spiders fed on *H. variegata*, lane 8, negative control. (B) cannibalistic predation: lane 1, 100bp molecular marker ladder, lane 2, positive control, lanes 3-16, wolf spiders fed on smaller wolf spiders (negative control for both analysis was the same (lane 8-A) as the same master PCR mixture was used in both assays).

4. Discussion

Results from time-since-feeding experiments revealed that detectability of prey DNA decreased with increasing time intervals. This fact has been shown before in other studies with different taxonomic groups of predators (eg. Hoogendoorn and Heimpel 2001, Agusti 2003b). Determination of detectability half-life (T_{50}) for each predator under study is essential because a reliable indicator for the relative importance of any given predator taxon requires the knowledge of how quickly the signal decays inside the predator (Greenstone and Hunt 1993, Chen et al. 2000). This value was in fact the longest for *V. spenceri* (49.6 h) and the shortest for *H. variegata* (17.1 h). As Chen et al. (2000) suggested, in practice, the predator species with the shortest half-life for DNA of a particular species of prey could be given a detectability weighting of 1.0 and the half-lives of all other predator species would be divided by the benchmark half-life to give a detectability weighting for each predator species. In the current study, *H. variegata* would be assigned a weight of 1.0 for detectability of *P. xylostella* DNA, whereas *N. kinbergii* and *V. spenceri* and would receive a weightings of 2.1 and 2.9, respectively. Relative weighting for each predator species consuming the pest can be determined if the density of each predator species could be determined by absolute methods (e.g. Sunderland and Topping 1995, Michels et al. 1997). It is important to consider that a short prey detection period could limit the application of gut content analysis by PCR-based methods in the field (Hagler and Naranjo 1997). In this study a detection period of at least 17 hours was considered adequate to identify residues of prey in the gut contents of at least 50% of examined predators.

In the experiment with *H. variegata* as a predator, it was showed higher temperatures resulted in a reduction in detectability within given time periods, presumably as a result

of increased rates of digestion. These results concur with earlier findings for the detectability of prey in predator guts. Hoogendoorn and Heimpel (2001) used four ITS-1 primers for *Ostrinia nubilalis*, which amplify four different fragment sizes, to study predation in the ladybird *Coleomegilla maculata*. They showed that temperature has a significant negative effect on the number of bands when *C. maculata* fed on prey eggs. Sopp and Sunderland (1989) reported that the detection period generally declines with increasing temperature when using ELISA to detect prey-specific proteins among selected predator species in the families Linyphiidae, Carabidae or Staphylinidae. In a similar experiment performed at five different temperatures (15, 20, 25, 30 and 35°C), Hagler and Naranjo (1997) showed that the proportion of positive responses to prey residues and duration of median detection intervals of prey (pink bollworm egg) decreased as temperature increased in one ladybird and two hemipteran predators. These studies suggest that temperature is an important factor and should be considered in the evaluation of predators' gut contents obtained from field samples. In the *Brassica* growing areas of South Australia, temperatures of 40°C and above have been reported during January, February and March. However, the mean daily temperature during the growing season rarely exceeds 35°C (Bureau of Meteorology 2007). Therefore it would be expected that target prey DNA could be detected longer in the gut contents of predators in the moderate months (May - June, September - October) compared with warmer summer time.

In wolf spiders only an effect of time on detection was observed and unexpectedly temperature did not significantly affect the detectability of prey DNA. The exact reason for this unexpected result is unknown. However, it might be due to the spiders' specific digestion system. Among arthropod predators, spiders have resting metabolic rates that

are 50-75% lower than those of other invertebrates (Anderson 1970, Greenstone and Bennett 1980). This enables them to survive longer during periods of prey shortage (Nakamura 1972, Anderson 1974, Greenstone and Bennett 1980). In a similar result, Sopp and Sunderland (1989) found that linyphiid spiders exhibited very long detection periods, even at high temperatures. Hoogendoorn and Heimpel (2001) did not detect an effect of temperature on detectability of prey DNA in larvae of *C. maculata*, but gave no explanation for the lack of a temperature effect. Thus, although temperature is expected to affect detectability of prey remains in most predators, there are clearly some exceptions.

Three different predators were tested to examine whether species differences can be a possible factor affecting the detection of prey. Among the predator species tested, *H. variegata* had the shortest, and wolf spiders the longest, detection period. Using an ELISA method, Sopp and Sunderland (1989) showed that different taxonomic groups of predators have different detection periods. Ma et al. (2005) found differences in detection time between two predators when using ITS-1 primers. Findings in this study were comparable to their results, but the maximum period of detectability in our results was 96 h for wolf spiders and 72 h for *N. kinbergii*, compared with 72 h and 16 h using their primer sets.

Possible intrinsic factors affecting the detection period are a predator's gender, size and also developmental stage. A study by Sheppard et al. (2005), using a PCR-based technique to compare male and female *Pterostichus melanarius*, did not show any difference in the detection period between the sexes, despite the fact that females are significantly larger than males (Lindroth 1974). Hoogendoorn and Heimpel (2001) also

did not find any influence of predator's sex, weight or developmental stage on detectability. In this study it was also found no difference in detection period between male and female of *H. variegata*, while females were larger, and consequently weighed more than males. However, Symondson et al. (1999a) reported that the detection period for males of *P. melanarius* was 30% longer than for females feeding on the slug *Arion hortensis* using monoclonal antibodies. This was in contrast with results presented by Harwood et al. (2001) that did not reveal any differences between genders in the carabid beetle *Poecilus cuperus* or the spider *Tenuiphantes tenuis*. It is important to note that most laboratory experiments, including the results presented here, typically do not take differences in mobility and activity into account and confined insects in the laboratory may be less active than their counterparts in the field. Males of most species tend to be more active than females (Harwood et al. 2001, Sheppard et al. 2005), which may affect the digestion rate and, as a result, detectability of prey DNA in their gut contents (Sunderland et al. 1987, Topping and Sunderland, 1992, Harwood et al. 2001). This is a topic that requires further investigation to clarify (1) the impact of activity levels on the duration of prey detectability and (2) the extent of differences between laboratory and field experiments.

The presence of additional food in the gut contents of *V. spenceri* did not reduce the detectability of prey DNA compared with the detection results when the spiders fed on only one species. Lovei et al. (1988, 1990) also found subsequent food intake did not reduce detection period or antigen decay and Hoogendoorn and Heimpel (2001) showed that the second prey did not inhibit the amplification of target prey DNA. However, surprisingly Symondson and Liddell (1995) found subsequent feeding on alternative prey has a profound effect upon detection period in *P. melanarius*, extending it by

nearly 50% when a monoclonal antibody was used to detect prey remains. Harper et al. (2005) found a similar result when using a PCR-based approach for *P. melanarius*. The detection period was 26.3 h when the carabid was not fed alternative prey, but was 35.6 h when fed on additional alternative prey. Both results revealed that feeding on additional food causes longer detection periods in *P. melanarius*, perhaps due to a reduction in digestion rate. This suggests that studies aimed at elucidating the effects of the digestive physiology of predators on detection of prey remains may be worthwhile in future.

Results of secondary predation trials showed the possibility of detection of prey DNA in both cannibalistic and intraguild predation, and therefore highlight a potential significant source of error for interpretation of data on detection of DNA in the guts of predators. In a study by Sheppard et al. (2005), the maximum detection period of 8 h was determined for an aphid-spider-beetle model but it was 20 h in the current study for both cannibalistic and intraguild predation. Therefore these results confirmed that the presence of a spider in the food chain allows a longer detection period of secondary predation as suggested by Sheppard et al. (2005). However, additional experiments are needed with different time intervals, prey and predator species in different food chains to clarify how long prey can be detected following secondary predation. Where secondary predation is possible, predators should be screened for the remains of other common predators as well as common herbivores. Thosc species that test positive for both predators and herbivores should be considered to be likely instances of secondary predation, which can be investigated in feeding trials.

In conclusion, the detection efficiency or rate of target prey DNA detection in the gut contents of predators is affected by a number of factors. Additional factors not considered here include effect of meal size, fragment size, stability and sensitivity of primer, DNA extraction method, and different prey group. Therefore the development of diagnostic markers for a specific prey species is only the first step in the study of predator-prey interactions in the natural environment. Additional factors have to be considered in order to interpret predation data obtained by molecular markers from field-collected predators. This is unlikely ever to be easy, and will depend on a thorough knowledge of the biology of both predators and prey.

Chapter 6: Molecular identification of wolf spiders

1. Introduction

To determine the role and effectiveness of predators in the control of insect pests in the field, a detailed understanding of the biology of the species involved, in particular their behaviour and ecology, is essential. Accurate species identification is a precondition for achieving this aim. Traditionally, identification of species is based on morphological characteristics, but morphological keys are often useful only for a particular life stage or gender, and many species cannot be identified reliably as juveniles. Wolf spiders (Araneae: Lycosidae) are important predators of insect pests in vegetable crops (Hummel et al. 2002). However, species in this family are difficult to identify based on morphological characters alone, especially in the immature stages. Juveniles collected from the field must generally be reared to the adult stage to allow accurate species identification. This process is time consuming and not always successful. Therefore it is essential to develop a quick and reliable method for identification of individual species in this important group of predators.

Multiplex polymerase chain reaction (PCR) simultaneously amplifies several fragments in a single reaction. Under certain conditions, several species can be identified using a single PCR followed by an electrophoretic separation of amplified DNA fragments. So far multiplex PCR has been described for the simultaneous detection of bacterial (Bej et al. 1990, Way et al. 1993, Song et al. 2005), mycobacterial (Bhattacharya et al. 2003), viral (Karlsen et al. 1996) and fungal (Amicucci et al. 2000) pathogens, as well as plankton (Hare et al. 2000), mites (Kumar et al. 1999), insects (Kengne et al. 2001, Hinomoto et al. 2004, Dang et al. 2005), and spiders (Greenstone et al. 2005).

Spiders belong to the most abundant group of predators in agricultural systems, but have received much less attention than insect predators (Whitehouse and Lawrence 2001). A wide diversity of spiders lives in arable fields, of which wolf spiders are one of the most abundant families (Alford 2003). For example, a British wheat field showed densities of up to 76 individuals/m^2 during summer (Workman 1978). With this numerical dominance, they have the potential to consume large numbers of prey. For example, they are one of the most important predators of cereal aphids, *Rhopalosiphum padi,* in Europe (Nyffeler and Benz 1982, Mansour et al. 1992). In a *Festuca-Andropogon* old field system in the United States, 21.1% of the total mortality of herbivorous insects is due to predatory pressure of wolf spiders of the genera *Hogna* and *Rabidosa* (van Hook 1971). Studies on spiders in Australian agroecosystems are scarce, so it is difficult to evaluate the abundance of lycosids relative to other spider families. However, spider families that dominate in the Northern Hemisphere, such as Linyphiidae (Sunderland and Samu 2000), play only minor roles in the Australian fauna (Raven et al. 2002). In Australia, lycosids appear to aggregate in certain agroecosystems (Pearce and Zalucki 2006), and were found to be predators of *Plutella xylostella* on a vegetable farm at Virginia, South Australia (Ma et al. 2005). In Australian cotton fields, wolf spiders are a dominant epigaeic predator, whilst Oxyopidae are the most abundant spider family in higher strata of the vegetation (M.A.E. Whitehouse, personal communication).

Wolf spiders constitute the fourth largest spider family, with ca. 2300 species described in 103 genera (Platnick 2007). Their adult body size ranges from 1 to 30 mm. They pursue an array of different prey capture strategies, from permanently vagrant hunters to permanently burrowing species, and some genera are known to build permanent sheet-

webs (Murphy et al. 2006). The life cycle of wolf spiders, in particular in regions with temperate climates, is generally well synchronised with the season. However, phenology varies among species (Schaefer 1976, Framenau and Elgar 2005). Wolf spiders also differ in their diurnal activity patterns that means that they only forage on insects, which are active at the same time during the day (Marshall et al. 2002). Wolf spiders may also show very specific microhabitat preferences and may be susceptible to changes in habitat structure (Marshall and Rypstra 1999, Jõgar et al. 2004). This ecological diversity may make them suitable for control of a wide variety of insect pests. However, it also means that it is vital to be able to recognise single species to evaluate and support their role in crop management practices.

Family level identification of wolf spiders is easy due to a number of unique characters, such as the eye arrangement, the lack of a retrolateral tibial apophysis on the male pedipalp, and the unique behaviour of females that carry their egg sacs attached to the spinnerets and subsequently their young on the dorsal surface of the abdomen (Dondale 1986, Griswold 1993). In contrast, generic and species level identification is impossible for the non-specialist, as currently no generic key exists in Australia and only one key is available that allows species level identification within a common genus, *Venatrix* (Framenau and Vink 2001, Framenau 2006a), in addition to reviews of some smaller, more cryptic genera (e.g. Framenau 2006b,c, Framenau and Yoo 2006). Species identification of spiders generally requires the examination of male and female genitalia. Hence, the morphologically conservative wolf spiders are impossible to identify accurately as juveniles.

In this chapter a reliable and efficient method is described to identify guilds of predators that are collected in field studies. Firstly DNA markers were developed that identify seven species of wolf spiders that commonly occur in *Brassica* crops in the Adelaide Region of South Australia. Then it was demonstrated that multiplex PCR can be used to identify these species in a single reaction. This approach can be used in similar situations where groups that are difficult to identify are a prominent part of the biota and also for biological control strategies.

2. Materials and methods

2.1. Spider collection

Wolf spiders were collected from Pitchford's broccoli farm at Currency Creek, South Australia at night by using a head lamp (Wallace 1937) about one hour after sunset, when a large number of species appeared to be active. In wolf spiders, light is reflected by the tapeta, a light-reflecting layer of cells in the eyes, and the spider's presence is indicated by a bluish or greenish sparkle (Vink 2002). Spiders were placed in 5 ml vials and transferred to the laboratory. Representative adult wolf spiders were identified to species level and a few immature spiders were maintained at 25°C and provided with moisture and food (*Ephestia* larva) to rear them to maturity for identification.

2.2. DNA extraction

For DNA extraction from wolf spiders, samples of 2 or 3 legs were removed with clean forceps and the rest of the body was kept at -20°C as voucher specimen. DNA from specimens representing sac spiders (Gnaphosidae), another spider family commonly encountered at the study site, and four *Brassica* crop pests (*Plutella xylostella*, *Pieris*

rapae, *Myzus persicae* and *Brevicoryne brassicae*) was extracted in order to test the specificity of the DNA primers. To extract purified DNA from spiders a silica-based method was used (Chapter 2).

2.3. DNA amplification and primer design

The forward primer C1-J-1718, 5′-GGAGGATTTGGAAATTGATTAGTTCC-3′ and the reverse primer C1-N-2191, 5′-CCCGGTAAAATTAAAATATAAACTTC-3′ (Simon et al. 1994) were used to amplify the COI gene of the wolf spider species. PCR was performed in 50 µl total volume of reaction buffer containing 150 µM dNTPs (Fisher scientific Inc., USA), 2 mM $MgCl_2$, 0.4 µM of each primer, 1 U of *Taq* DNA polymerase (Biotech international Ltd., Australia) and 8 µl of DNA template (10 ng/µl). The reaction mix was put into a 0.2 ml PCR tube and amplification was performed in a PTC-200 thermocycler (MJ Research, Massachusetts, USA) with the following profile: 95°C for 2 min followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min. A final strand elongation at 72°C was performed for an addition 5 min.

The desired DNA fragment was isolated and purified and then sequenced (Chapter 2). Sequencing was carried out in both forward and reverse orientations. Sequencing results were analysed for each species separately and edited sequences were aligned (Chapter 2). For each of the seven species, oligonocleotide primer pairs were designed manually for PCR based on four criteria: 1) At least one nucleotide at the 3′ end of the primer must be unique to one target species in the alignment (Kwok et al. 1990); 2) Primers should not show any complementation, as determined by Oligo Calculator (version 3.07; http://www.basic.northwestern.edu/biotools/oligocalc.html); 3) An optimal similar

annealing temperature was determined for all primers; 4) The expected PCR product size was different for each species.

2.4. Specificity tests

The specificity of primers for each species was tested individually and also in multiplex PCR. Attempts were made to amplify DNA using primers from all of the lycosid species, including the target species as a positive control, as well as from the gnaphosid spider and the pests of *Brassica* crops.

2.5. Multiplex PCR

A single multiplex PCR was optimized to amplify all seven specific amplicons. Amplification was performed in 25 µl total volume, containing 4 µl of DNA (10 ng/µl), 1× multiplex PCR master mix (QIAGEN), 1× Q solution (QIAGEN) and 0.2 µM of each of 14 primers. The PCR cycling conditions were 95ºC for 15 min followed by 35 cycles of 94ºC for 30 s, 57.5ºC for 90 s, 72ºC for 60 s and a final cycle of 72ºC for 10 min. PCR products (10 µl) for each species were mixed with 2 µl of loading buffer and run on 2.5% LE Analytical Grade agarose (Promega) containing ethidium bromide for staining. Subsequently electrophoretic separation of PCR fragments was performed at 80 V for 1.5 h.

2.6. Field test

To test the utility of this approach, two separate experiments were carried out against field-collected immature wolf spiders. In the first experiment, a female of *Venator spenceri,* which carried an egg sac, was collected from the field and transferred to the

laboratory. The adult female was maintained at room temperature and provided wet cotton for moisture and diamondback moth larvae as food. After hatching, four spiderlings (1-2 mm in size) were selected. In the second experiment 20 samples of immature wolf spiders from broccoli crops were randomly selected each from among 80 specimens collected at Cudlee Creek, South Australia and from among 90 specimens collected at Currency Creek, South Australia. Selected spiders from both experiments were identified using multiplex PCR.

3. Results

Seven species of wolf spiders in six genera that commonly occur in *Brassica* crops were identified after rearing them to maturity.

Part of the cytochrome oxidase subunit I coding region was amplified for all seven species. These fragments varied in length (*Hogna crispipes* (L. Koch 1877) - 513 bp, *Hogna kuyani* Framenau, Gotch and Austin 2006 - 479 bp, *Lycosa godeffroyi* (L. Koch 1867) - 497 bp, *Trochosa expolita* (L. Koch 1877) - 500 bp, *Venator spenceri* Hogg 1900 - 494 bp, *Venatrix pseudospeciosa* Framenau and Vink, 2001 -500 bp, and an undescribed species, here listed as 'Species A' - 498 bp (description of this new species will be published in an article by V.W. Framenau, (personal communication)). Sequences were submitted to the National Centre for Biotechnology Information GeneBank (http://www.ncbi.nlm.nih.gov; Table 6.1).

The sequences from the seven species were aligned and compared, thereby allowing the design of unique primer pairs for each species (Table 6.1). Specificity tests for each

primer pair showed amplification of fragments only for the DNA of the target species. There was no cross-reactivity among any of the species tested (Fig. 6.1).

To develop a quick and robust molecular technique for identification of common wolf spiders in *Brassica* crops, the seven primers were combined in a multiplex PCR assay. Amplified fragments ranged in size from 59 bp for *L. godeffroyi* to 273 bp for *H. crispipes*. A mixture of seven equimolar primer pairs (Table 6.1) was used for PCR amplification. The optimum annealing temperature for multiplex PCR was determined to be 57.5°C. A DNA ladder (e.g. 20 or 25 bp) was used along with samples to ensure accurate DNA fragment length determination.

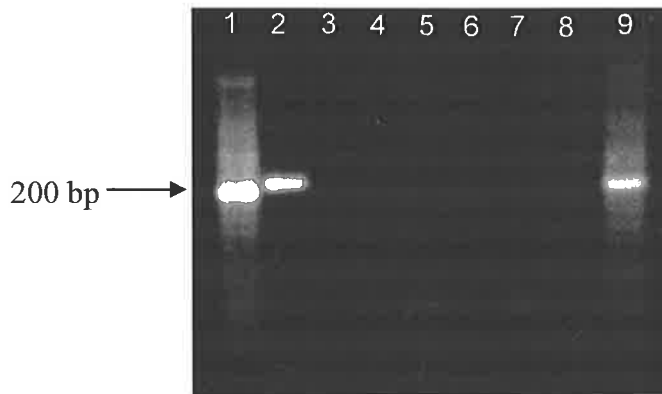

Fig. 6.1. Species-specificity test of wolf spider primers by Multiplex PCR. Agarose-gel (2.5%) in TAE. lane 1,9 molecular-size marker 20bp; lane 2, *Venator spenceri* as positive control, lane 3 *Plutella xylostella*; lane 4, *Brevicoryne brassicae*; lane 5, *Myzus pericae*; lane 6, *Pieris rapae*; lane 7, Gnaphosidae; lane 8, control (water).

Table 6.1. Specific primer sequences and amplified fragment sizes for seven species of Lycosidae.

Species (Genebank accession no)	Primer	Primer sequence	Size (bp)
Lycosa godeffroyi (DQ295872)	TG-F	5′-ATTGTTTGTGTGATCTGTTTTG-3′	59
	TG-R	5′-ACACAGGCAATGAAAGCAAC-3′	
Trochosa expolita (DQ295873)	TE-F	5′-TTTTTCGTTACATTTAGCAGGT-3′	101
	TE-R	5′-TTTTCTCTATTCTTATTCCAAC -3′	
Venatrix pseudospeciosa (DQ295867)	VP-F	5′-CTAGAATAGGTCATACAGGT-3′	139
	VP-R	5′-TTCTCCATTGATATTCCCAAC-3′	
Hogna kuyani (DQ295870)	HK-F	5′-CCTCCTTTAGCATCAAGAGTT-3′	158
	HK-R	5′-GGAACCTTCTCCATAGATATC-3′	
Venator spenceri (DQ295869)	VS-F	5′-CACCTTTAGCGTCAAGAGTG-3′	215
	VS-R	5′-CACAGGTAAAGAAAGTAATAAC-3′	
Species A (DQ295868)	PP-F	5′-ATTATCTATATCTTCAATAGTGG-3′	233
	PP-R	5′-ATACTGACCAAACAAACAACG-3′	
Hogna crispipes (DQ295871)	HC-F	5′-CTATGTCTTCTATGGTAGAGA-3′	273
	HC-R	5′-CCGCTAATACAGGTAACGAA-3′	

Multiplex PCR was shown to be effective in separating all seven species of lycosids (Fig. 6.2). Only one specific band was amplified from the DNA of *H. crispipes*, *L. godeffroyi, T. expolita, V. spenceri* and Species A. Apart from species-specific bands, additional non-specific bands were amplified from the DNA of *H. kuyani* and *V. pseudospeciosa.*

Fig. 6.2. Fragment profile produced with multiplex-PCR of seven target species on 2.5% agarose gel in TBE buffer. Lanes: 1 and 24, DNA marker (20bp); 2-4, *Lycosa godeffroyi* (59bp); 5-7, *Trochosa expolita* (101bp); 8-10, *Venatrix pseudospeciosa* (139bp); 11-13, *Hogna kuyani* (158bp); 14-16, *Venator spenceri* (215bp); 17-19, Species A (233bp); 20-22, *Hogna crispipes* (273bp); 23, control.

Field experiments verified the utility of multiplex PCR for identification of field-collected lycosids. Known specimens of juvenile *V. spenceri* were correctly identified, which is an indication of the sensitivity of this method for identification of minute specimens (Fig. 6.3). Unknown field-collected immature lycosids were readily identified by multiplex PCR. At Cudlee Creek, 95% of immature spiders were *V. pseudospeciosa* and 5% *H. kuyani* (Fig. 6.4A), and at Currency Creek 50% of total specimens were *V. spenceri,* while *V. pseudospeciosa* and *T. expolita* had an equal abundance of 25% (Fig. 6.4B). All of the specimens were identified in this study.

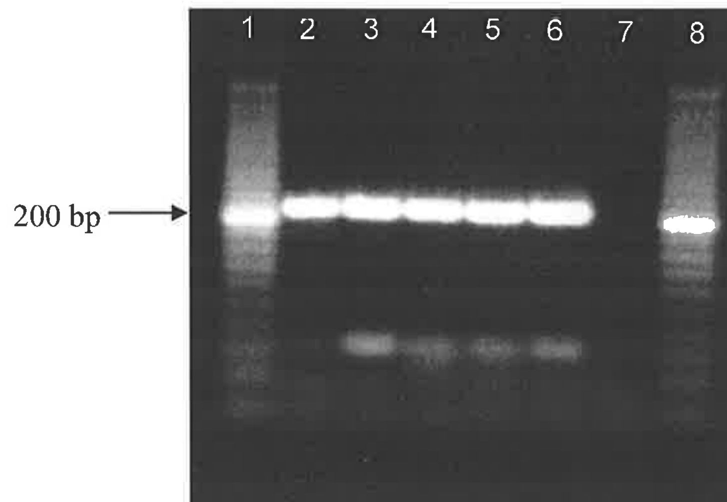

Fig. 6.3. Multiplex PCR of four small size juvenile wolf spiders from an egg sac. Lane 1, 8, DNA marker 20bp; lane 2, Adult of *Venator spenceri*; lanes 3-6, juveniles of *V. spenceri*; lane 7, control.

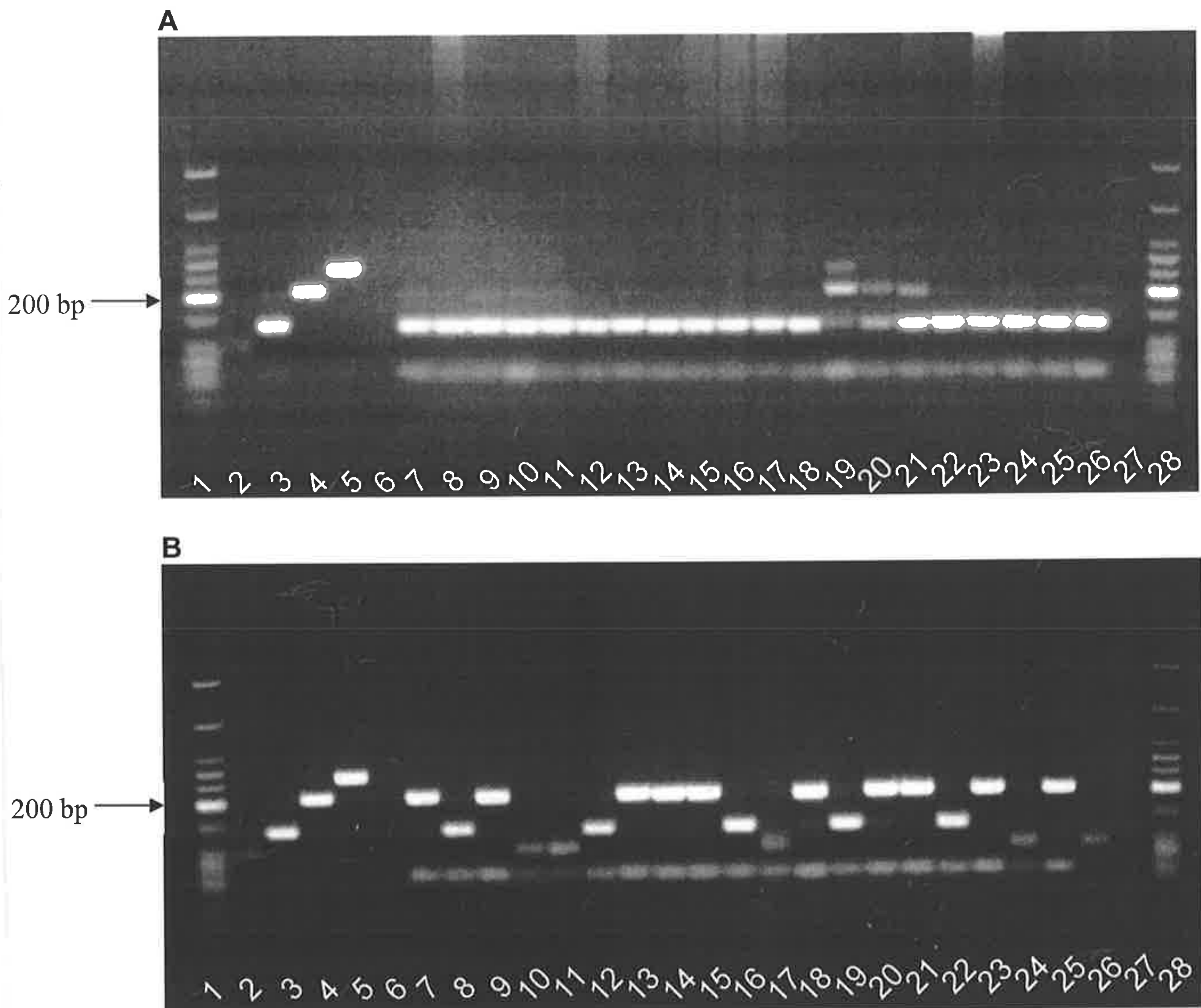

Fig. 6.4. Multiplex PCR with wolf spider primers for 20 immature specimens collected from the field. Agarose gel 2.5% in TAE buffer. (A) Specimens from Cudlee Creek. Lanes 1 and 28, DNA marker (25bp); lanes 2-5 as positive control; 2, *Trochosa expolita*; 3, *Venatrix pseudospeciosa*; 4, *Venator spenceri*; 5, *Hogna crispipes*; lane 6, empty control lane; lanes 7-18 and 20-26, *Venatrix pseudospeciosa*; lane 19, *Hogna kuyani*; 27, Control. (B) Specimens from Currency Creek. Lanes 1 and 28, DNA marker (25bp); lanes 2-5 as positive control; 2, *Trochosa expolita*; 3, *Venatrix pseudospeciosa*; 4, *Venator spenceri*; 5, *Hogna crispipes*; lane 6, empty control lane; lanes 7, 9, 13, 14,

15, 18, 20, 21, 23, 25, *V. spenceri*; lanes 8, 12, 16, 19, 22, *V. pseudospeciosa*; lanes 10, 11, 17, 24, 26, *T. expolita*; lane 27, control.

4. Discussion

Accurate identification of species is critical in studies of insect pests and vectors of diseases in agriculture, natural ecosystems and human health (e.g. Song 2005). Morphological identification of species of bacteria, fungi, and small invertebrates is sometimes difficult because of their microscopic size and/or lack of diagnostic morphological characteristics.

The conventional morphological methods for identification of wolf spiders require specialist knowledge and are time consuming, particularly when the immature stages must be reared to maturity. Currently there is no reliable method for morphological identification of immature wolf spiders found in *Brassica* crops, yet immature spiders are the majority of specimens collected in the field. Therefore molecular identification based on PCR is an important taxonomic advance. DNA-based identification methods are not affected by life stages, size and sex of the specimen. Even newly hatched wolf spiders can be easily identified by this method. In conventional PCR, one pair of primers is used to amplify a specific fragment, while multiplex PCR uses several pairs of primers to potentially amplify many different fragments simultaneously. Multiplex PCR is fast and requires small quantities of DNA.

Recently multiplex PCR has been applied as a powerful tool for identification of insects (Hinomoto et al. 2004, Dang et al. 2005). This technique has proved to be rapid, reproducible and very sensitive. For example, Kumar et al. (1999) used multiplex PCR

based on the ITS-1 gene region for identification of microscopic *Cecidophyopsis* mites, which are difficult to identify with morphological characters. Results in this study also confirmed that multiplex PCR can be applied to identify very tiny spiders lacking any known diagnostic morphological traits. Hinomoto et al. (2004) used multiplex PCR based on the ITS-1 gene region to identify five species of *Orius*. The amplicons varied in size from 190 to 800 bp, which easily separated target species from each other. In the current study coding sequences from the mitochondrial COI gene were used to design specific primers. The overall size of the amplified COI fragment was short (max 513 bp). Dang et al. (2005) used two species-specific primer pairs in a multiplex PCR based on the ITS-2 gene region to distinguish two target species from 12 other *Trichogramma* species. Amplified fragments by designed species-specific primers, which varied in size between 273 bp and 59 bp clearly separated all seven target species. Greenstone et al. (2005) used PCR to distinguish seven species of spiders on the basis of COI sequences, but had to use RFLP analysis for further differentiation as two species produced identical fragment sizes. Results in this study indicate that at least seven species-specific primers can be used in a single multiplex PCR reaction to distinguish species simultaneously. It is not clear how many more different species could be amplified in a single reaction. This will probably be determined in part by the availability of unique primers that produce DNA fragments with distinctly differing lengths.

Primers for multiplex PCR are difficult to develop. All primers must have similar annealing temperatures and minimal complementation. Moreover, particular combinations of primers produce false positive results (e.g. Hare et al. 2000). As the number of primers is increased in a multiplex reaction, there is an increasing probability that two primers in the mix find non-specific annealing sites among genomic DNA

templates from different species. In some cases non-specific faint fragments are also observed (e.g. Manguin et al. 2002, Hinomoto et al. 2004). These non-specific bands are sometimes only produced at relatively low annealing temperatures used in multiplex PCR. In single PCR reactions this problem can usually be solved by increasing the annealing temperature to reduce the non-specific bands, but in multiplex PCR increasing annealing temperatures sometimes reduces detection of other diagnostic fragments. Hinomoto et al. (2004) suggested non-specific bands could be made to disappear by decreasing the concentration of species-specific primers. However, additional non-specific bands do not necessarily reduce the accuracy of identification, and as was found in the current study in some cases they increase the diagnostic power of the method, because they only occur in the presence of a particular species.

Compared to traditional morphological methods of species identification, multiplex PCR is a relatively simple method for non-specialists, and is particularly useful for species identification of immature arthropods. It offers the advantages of speed and simplicity without the need to wait for adult emergence or to have expert knowledge of arthropod morphology. Even partial specimens that lack diagnostic morphological characters can be identified. This study has shown that multiplex PCR can be used for the identification of wolf spiders and other species groups that are difficult to identify.

Wolf spiders are among the most commonly collected soil dwelling predators in *Brassica* fields (Chapter 3). Details of the biology and ecology of these species, such as habitat specificity, prey selection behaviour, and seasonal abundance, are still unclear. However, in this study it was shown that relative abundance among wolf spider species can vary considerably among different localities. One species, *H. crispipes*, occurs

throughout Australia and the Pacific region (Framenau et al. 2006). This species is also the most abundant wolf spider in cotton (M.E.A. Whitehouse, personal communication). Therefore, results of this study should have wider application within the agricultural industries of Australia and allow the identification of wolf spiders in economically important food webs in agricultural ecosystems.

Table 6.2. Alignment of COI sequences from seven species of wolf spiders, species-specific primers of each species are highlighted.

```
V. pseudospeciosa : -------------------A.............................G.................T..G.......... :  62
Species A         : -------------------A..............G..G........................................... :  62
V. spenceri       : -----------------G......................G........................................ :  64
H. kuyani         : -------------------A...............................................T............. :  62
H. crispipes      : ---...............................G..G..G..............G........C..G............. :  78
L. godeffroyi     : -------------------...............G................................G............. :  62
T. expolita       : ------------------.........................A..................................... :  63
                       TGGAGGATTTGGGAAATTGATTAGTTCCTTTAATATTAGGGGCTCCTGATATATCTTTTCCTCGAATAAATAATCTTT

                     *         100         *         120         *         140         *         160
V. pseudospeciosa : ..................................G.....G..........................G..G.....G.... : 143
Species A         : ..............................................A.....G............................ : 143
V. spenceri       : .........................G...........C...........G..G...........A..............A. : 145
H. kuyani         : ................C.................G..........................G..A..G.....T..G..G. : 143
H. crispipes      : ..........G..G....................G.....G........G.....G...........G...........G. : 159
L. godeffroyi     : .........................G...................................G..A........G....... : 143
T. expolita       : .A....................A........G....T...............G.................A..T....... : 144
                    CTTTTTGATTATTACCTCCTTCTTTATTTTTATTATCTATATCTTCTATAGTAGAAATAGGAGTTGGAGCTGGATGAACTG

                     *         180         *         200         *         220         *         240
V. pseudospeciosa : ..........A.....G......A.........C...T........G.................................. : 224
Species A         : ................T.................G.............................................. : 224
V. spenceri       : .A.....A........G..A.....G....................G..........................A....... : 226
H. kuyani         : ...................A.....T........G.........................................G.... : 224
H. crispipes      : .......G.....G....................G..G...........................T.A............. : 240
L. godeffroyi     : .A...........G.....G...A......................G..........................A.....T. : 224
T. expolita       : ................T...............................................GT.A.....A..A..T. : 225
                    TTTATCCTCCTTTAGCATCTAGAGTAGGTCATATAGGAAGTTCTATAGATTTTGCTATTTTTTCTCTTCATTTGGCTGGGG
```

Table 6.2. (Continued)

```
                         *        260         *        280         *        300         *        320
V. pseudospeciosa  : ...................A.................T.................G..G......T............... : 305
Species A          : .......A.............................T.............................T..A..A..A.... : 305
V. spenceri        : ................G..................................................T..A..A....... : 307
H. kuyani          : ................G...................................A........G...T.T............. : 305
H. crispipes       : ................G..A..G..........................G..A............T.G.....A.....A. : 321
L. godeffroyi      : .......A..............A...........................................G............A. : 305
T. expolita        : ....................A...........A....T..............TT..G.T.......G...A.....AA... : 306
                     CTTCTTCTATTATAGGAGCTGTTAATTTTATTTCTACAATTATTAATATACGGATATTAGGAATAACAATGGAGAAGGTTC

                         *        340         *        360         *        380         *        400
V. pseudospeciosa  : ..........A.....A...............A..................................G..A........G. : 386
Species A          : .G..G........G..A..A............A.........................T.....G.....A.......... : 386
V. spenceri        : .............G.......................G....................G..G................... : 388
H. kuyani          : .A........G................................G..............G..G.................G. : 386
H. crispipes       : ....G............................................G..............G..G............C : 402
L. godeffroyi      : .A..G.....G...........G.................G..G.....A..G.....G........G............. : 386
T. expolita        : .......C.....G........T.........A...........T.A..A..............................C : 387
                     CTTTATTTGTTTGATCTGTTTTAATTACTGCTGTTTTATTATTACTTTCTTTACCTGTATTAGCAGGTGCTATTACTATAT

                         *        420         *        440         *        460         *        480
V. pseudospeciosa  : .A..G........T........................................................A.......... : 467
Species A          : .A.................C............................................G................ : 467
V. spenceri        : .............G...................................G..A............................ : 469
H. kuyani          : ....G.............................C..............G..T...........G..C............. : 467
H. crispipes       : .A.....G.........................................G..T............................ : 483
L. godeffroyi      : ....G..G...................................G..A..T....................A.......... : 467
T. expolita        : ........................................G..T..A.....A.................A.......... : 468
                     TGTTAACAGATCGAAATTTTAATACTTCTTTTTTTGATCCTGCAGGTGGAGGGGATCCTATTTTATTTCAGCATTTATTTT
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Table 6.2. (Continued)

```
                   *        500         *        520         *
V. pseudospeciosa : .G........A......................-------------- : 500
Species A         : .G........A...................A---------------- : 498
V. spenceri       : ................G........---------------------- : 494
H. kuyani         : ....C.......----------------------------------- : 479
H. crispipes      : ..........T..................A----------------- : 513
L. godeffroyi     : .............................A----------------- : 497
T. expolita       : ..........T.....................--------------- : 500
                    GATTTTTTGGGCATCCTGAAGTTTATATTTTAT
```

Chapter 7: Predator-prey trophic interactions in *Brassica* crops

1. Introduction

The value of predators in the biological control of insect pests in integrated pest management programs has been highlighted by many investigators (Luff 1983, Booij and Noorlander 1992, Hagler and Durland 1994, Symondson et al. 2002). Identification of trophic relationships between predators and particular pests is one of the first important steps in determining the impact of predators on pest populations. There are inherent difficulties associated with the study of the diet breath of predators in nature because of the relatively small size of both predator and prey, and their often elusive and nocturnal activity (Greenstone 1996, Hoogendoorn and Heimpel 2001). Consequently there is a lack of knowledge about predators' feeding behaviour in the natural environment. Because of this difficulty, little is known about predator-prey trophic interactions and the effects of predators on pests of *Brassica* crops. However to elucidate the role of predators in the control of these pests, some experiments have been done under laboratory conditions or in field cages (e.g. Barry et al. 1984, Schmaedick and Shelton 1999), but these studies do not necessarily accurately simulate field conditions.

So far the most effective and least disruptive method for studying predation has been the development of biochemical diagnostic technologies, especially DNA-based methods (Symondon 2002). PCR-based approaches to detect prey residues in the gut contents of predators collected from the field are a useful tool to help researchers to identify important interactions within an ecosystem. Once they are developed, these

assays can be used to efficiently and sensitively test large numbers of field-collected predators for evidence of feeding on a particular prey species, such as key agricultural pests (Symondon 2002).

In the present study a PCR-based technique was used for the first time to study predator-prey interactions in *Brassica* crops. The aim of research reported in this chapter was to determine the trophic relationships among predators and prey, mainly focusing on the identification of predators of diamondback moth and other common pests.

2. Materials and methods

2.1 Sampling predators

Most predator samples were collected from a broccoli field on a commercial vegetable farm at Currency Creek, South Australia (35° 41′ S, 138° 75′ E). Predators were collected from unsprayed crop residues and regrowth. Phytophagus insects were abundant on these plants. Another sample of wolf spiders only was collected in mixed field of broccoli and cabbage at Cudlee Creek, South Australia (34° 84′ S, 138° 85′ E; Fig. 7.1).

Ground dwelling predators including wolf spiders, *Philonthus* sp. (Coleoptera: Staphylinidae), *Coranus* sp. (Hemiptera: Reduviidae) and *Euander* sp. (Hemiptera: Lygaeidae) were collected at night (7-9 PM) by searching with a headlamp among cultivated rows, under leaves and litter, and on the soil surface around the bases of broccoli and cabbage plants. Collected specimens were put individually into a 5 ml vial. Other predators were collected from vegetation by a vacuum sampler (Makita model

RBL 250, Makita Corporation, Japan), as the investigator walked randomly across the field. All collected samples, including collection bags containing predators that were used in the vacuum sampler, were kept chilled on ice until they were transferred to the laboratory and placed at –80 °C overnight. Afterward predators were separated, identified and kept individually in a 1.5 ml microcentrifuge tube at –80 °C for subsequent molecular assay.

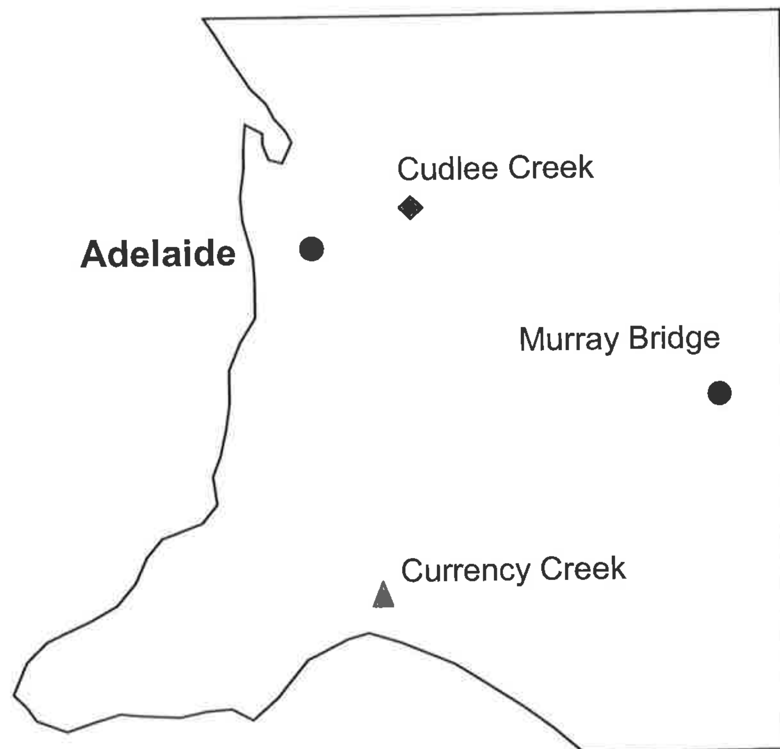

Fig.7.1. Collection sites in South Australia. Cudlee Creek (34° 84′ S, 138° 85′ E), Currency Creek (35° 41′ S, 138° 75′ E).

2.2 DNA extraction

In order to avoid possible external contamination with prey contents, before DNA extraction, each predator specimen individually was washed with washing buffer by the following procedure; 1 ml of washing buffer (Chapter 2) was added to 1.5 ml microcentrifuge containing predator sample and gently vortexed for 5 min. Then the tube was centrifuged for 2 min at 10000 rpm. After centrifugation the supernatant was

removed and washing repeated once more. Finally all collected predators, individually (except for *C. dilutus*) were treated using a DNA extraction method involving silica (Chapter 2). For examination of *C. dilutus,* 5 individuals were pooled in each sample for DNA extraction.

2.3 Molecular assay by PCR

Extracted DNA from each predator was tested separately in PCR with specific primer pairs for detection of six pests of *Brassica* crops (Chapter 4, Table 4.2). PCR conditions were as follows: amplification was performed in a 25 µl reaction volume containing 150 µM dNTPs, 2 mM $MgCl_2$, 2.5 µl of 10× reaction buffer, 1 U of *Taq* DNA polymerase, 0.4 µM each of the respective primer pairs and 4 µl of DNA extract. The thermocycling program consisted of an initial step of 2 min at 95 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec at the specific annealing temperature for each prey specific primer (Chapter 4, Table 4.2), 1 min at 72 °C and a final elongation step of 5 min at 72 °C. PCR products were visualised following electrophoresis on 1.8 % agarose gel in TAE containing 0.5 µl/ml ethidium bromide for DNA staining and then photographed. Each PCR assay had a positive (DNA of relevant species) and a negative (water) control.

3. Results and discussion

Abundant predatory insect species that were collected by the vacuum sampler included *N. kinbergii, O. schellenbergii, M. tasmaniae, H. variegata, C. transversalis,* and *C. dilutus.* Among ground-dwelling predators, wolf spider species were relatively abundant whereas *Coranus* sp., *Euander* sp. and *Philonthus* sp. were less common.

A total of 552 predators belonging to 9 different insect species and 4 wolf spider species were screened for the presence of prey DNA in their guts (Table 7.1). Results showed that it was possible to identify the residues of all potential prey species in the gut contents of the various arthropod predators collected from the field. The specificity of each primer pair was shown previously (Chapter 4), therefore the DNA bands of the size allocated for each candidate species was assumed to be diagnostic for the correct species. Detection of prey was successfully done in various predators with different types of mouthparts. *N. kinbergii, O. schellenbergii, Euander* sp., and *Coranus* sp. have hemipteran piercing-sucking mouthparts and are fluid feeders. *H. variegata, Coccinella transversalis* and *Philonthus* sp. have coleopteran chewing mouthparts. Larval *M. tasmaniae* have piercing-sucking mouthparts and adults have chewing mouthparts. Finally wolf spiders have spiders' specific chewing-sucking mouthparts.

Table 7.1. Summary of prey species detection in the gut contents of field-collected predators.

Predator	No. tested	Prey					
		P. xylostella	*H. hydralis*	*P. rapae*	*H. punctigera*	*B. brassicae*	*M. persicae*
N. kinbergii	130	+	+	+	+	+	+
O. schellenbergii	82	+	+	+	+	+	+
H. variegata	58	+	+	+	-	+	-
C. transversalis	24	+	+	+	-	+	-
M. tasmaniae	49	+	+	+	-	+	-
Lycosidae (4 species)	176	+	+	+	+	+	-
Philonthus sp.	17	+	+	+	+	+	-
Euander sp.	10	+	+	+	+	+	-
C. dilutus *	10	+	+	+	+	+	-
Coranus sp.	6	+	+	+	-	+	-

* Five specimens were pooled in each test; so 50 specimens were tested overall.

3.1 *Nabis kinbergii*

N. kinbergii proved to be a polyphagus predator with a wide range of prey. At least four prey species were found in the gut contents of 54.6% of the tested specimens (Fig. 7.3). Among examined specimens, a high percentage had tested positive for the presence of the DNA of three lepidopteran pests (Fig. 7.2). This species is known as one of the most common and widespread species of Nabidae in all parts of Australia and New Zealand and, like other members of this family of insects, it is thought to feed mostly on small invertebrates (Eickwort 1977, Carroll and Hoyt 1984, Flinn et al. 1985), especially the eggs and larvae of Lepidoptera (Cobbinah 1978, Samson and Blood 1980, Cordingly 1981). In this study, *N. kinbergii* was found as an abundant predator in *Brassica* fields. This potentially important predator also was reported in high numbers in other field and horticultural crops, such as cotton (Stanley 1997, Mensah 1999), soybean (Evans 1985), and lucerne (Awan 1982). They feed voraciously on insect crop pests, such as pea aphids *Acyrthosiphon pisum* (Harris), diamondback moth *Plutella xylostella* L., Australian crop mirid *Sidnia kinbergi* (Stal) (Siddique and Chapman 1987), native bud worm *Helicoverpa punctigera* (Wallengren) (Samson and Blood 1980, Awan 1990), and *P. rapae* (Kapuge et al. 1987). Copper (1981) also demonstrated the role of *N. kinbergii* in reducing larval *H. punctigera* by preying upon them and also by disseminating nuclear polyhedrosis virus. Recently Ma et al. (2005) used a PCR-based method for detection of specific DNA fragments to show that, *N. kinbergii* collected from the field had the remains of *P. xylostella* in their gut contents. In their investigations, 67.6% of examined specimens tested positive for the presence of DNA from *P. xylostella,* while this amount in this study was 93.8%.

Results revealed that *N. kinbergii* could be an important predator of aphids, where 71.5% of specimens tested positive for *B. brassicae* (Fig. 7.2). This clearly shows *N. kinbergii* will vary in its capacity to capture and consume different prey despite it being a polyphagous predator (Propp 1982, Flinn et al. 1985). In a similar study of arthropod predation on aphids in a lucerne crop using serological techniques (Leathwick and Winterbourn 1984), 78% and 75% of adults and nymphs of *N. kinbergii* were found to have fed on aphids.

Overall, the results clearly demonstrated that *N. kinbergii* feeds on diamondback moth and five other pests present in *Brassica* crops.

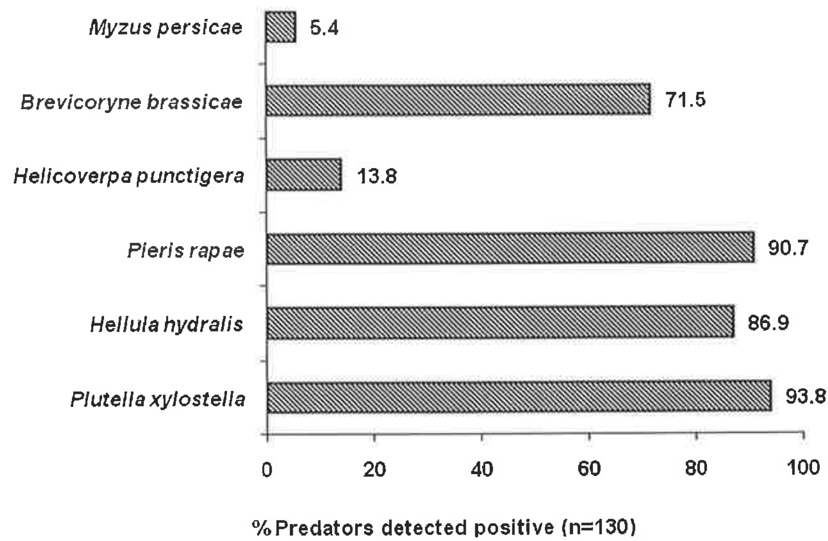

Fig. 7.2. Percentage detection of different prey species in *N. kinbergii*. Numbers beside bars indicate percentages.

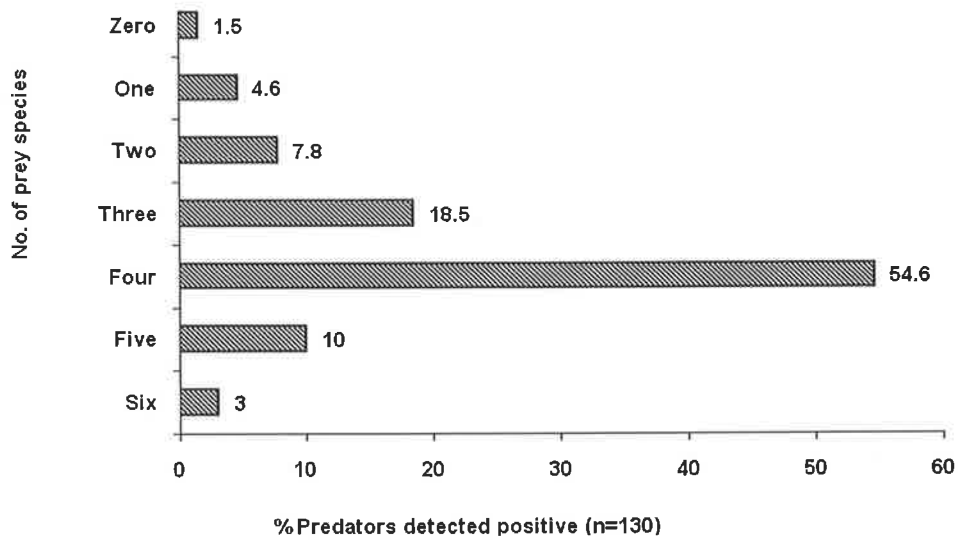

Fig.7.3. Frequency of prey species detected in *N. kinbergii*. Numbers beside bars indicate percentages.

3.2 *Oechalia schellenbergii*

DNA from all six pests was found in the gut contents of *O. schellenbergii*, which indicates that it is a polyphagous predator (Fig. 7.5). This species has previously been reported to feed on a wide range of immature stages of moths, beetles, sawflies and weevils (e.g. Mensah 1999). Molecular analysis of the gut contents of *O. schellenbergii* showed high frequency of positive tests for three lepidopteran species (Fig. 7.4). Moreover, like *N. kinbergii,* aphids are another source of food for *O. schellenbergii*, where 39% of tested specimens were positive to *B. brassicae*. In Australia and New Zealand this species is predator of several lepidopteran (Copper 1981, Cordingly 1981, Awan 1985a,b, 1988, 1990) and coleopteran pests (Edwards and Suckling 1980).

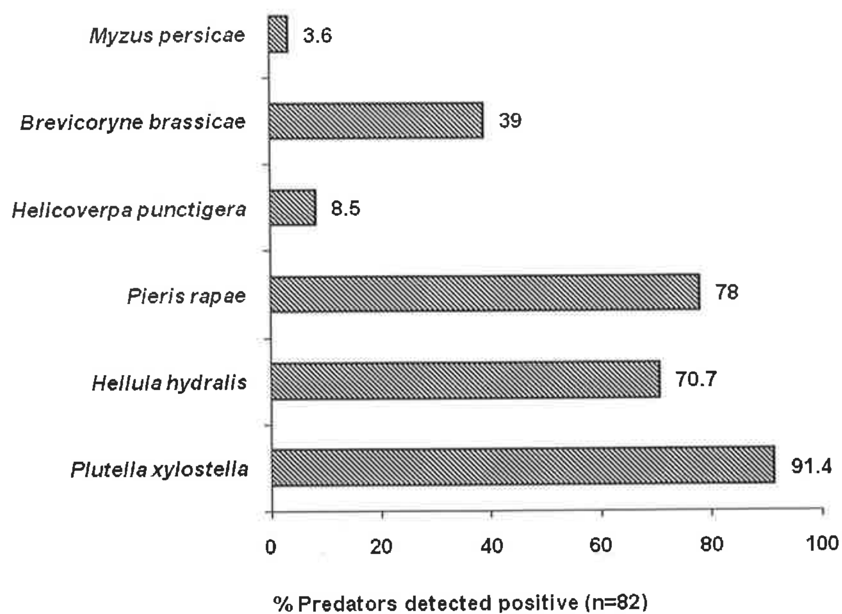

Fig. 7.4. Percentage detection of different prey species in *O. schellenbergii*. Numbers beside bars indicate percentages.

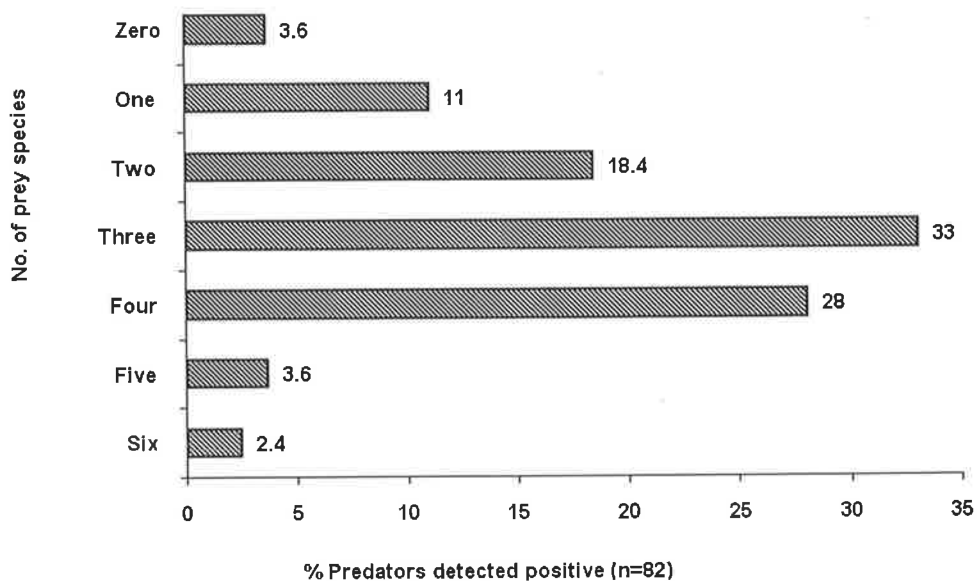

Fig.7.5. Frequency of prey species detected in *O. schellenbergii*. Numbers beside bars indicate percentages.

3.3 *Hippodamia variegata*

H. variegata is a Palaearctic species (Gordon 1987) that is distributed around the world. It was recently introduced to Australia (Franzmann 2002).

Although aphids are often regarded as their preferred prey, this ladybird also proved to be a generalist predator. Among all specimens examined (n=58) 13.8% and 12% tested positive to *P. xylostella* and *H. hydralis,* respectively, while only 1.7 % detected positive for *B. brassicae* (Fig. 7.6). Earlier investigations also confirmed this result. For example, *H. variegata* has been reported not only as predator of *Aphis gossypi* Glover on cotton (Fan and Zhao 1988), *Diuraphis noxia* (Mordviko) and *Schizaphis graminum* (Rondani) on wheat (Aalbersberg et al. 1988, Michels and Flanders 1992), but also as a predator of noctuid larvae (Araya et al. 1997), leafhoppers (Singh et al. 1991), psyllids (Franzmann 2002), insect eggs and thrips (Musser and Shelton 2003), and larvae of Curculionids (Sadeghi and Esmaili 1992).

Results revealed that about three quarters of the total number of *H. variegata* specimens tested in this study were not positive to any of the six potential prey species (Fig 7.7). A possible explanation for this result can be the consumption of non-prey food, such as pollen. Two observations support this hypothesis. Firstly, field observation showed that *H. variegata* was mostly active on flowers and possibly feeding on pollen. For this reason almost all adults of *H. variegata* were collected from older plants in flower. Secondly, entomophagous insects, especially coccinellids are known to feed on non-prey food including nectar, fungal spores, and prey products such as honeydew or pollen (Allen 1979, Alomar and Wiedenmann 1996, Canard 2001, Patt et al. 2003, Lundgren et al. 2005). This strategy may be adopted by some predators when prey is

scarce or during specific life stages for their growth and development. In this instance, prey were not scarce. Within agricultural habitats non-prey food, such as pollen, can influence the feeding behaviour of predators either increasing or decreasing predation on pest populations (Eubank and Denno 2000, Harmon et al. 2000, Coll and Guershon 2002). In the current study it is therefore possible that abundant non-prey food, such as pollen, may actually have reduced the predation rate and consequently this species' impact on pests (Cottrell and Yeargan 1998, Pfannenstiel and Yeargan 2002).

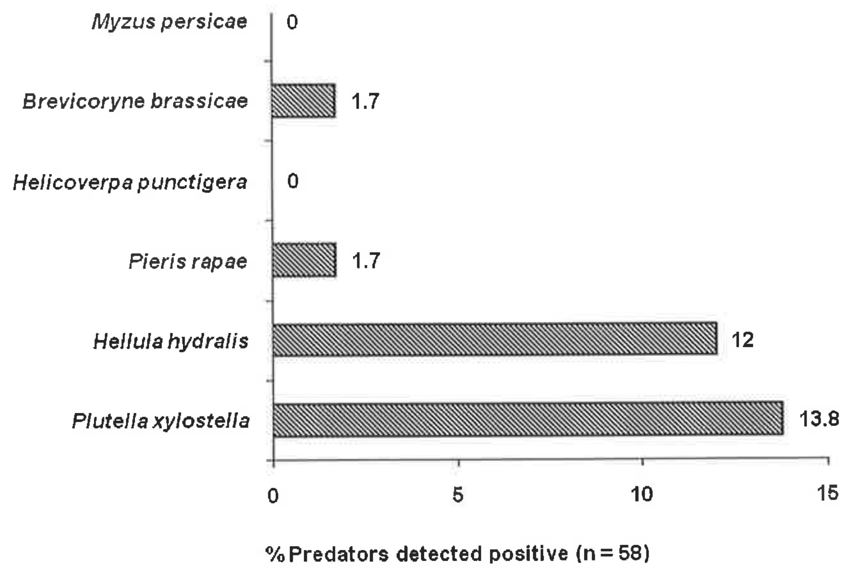

Fig. 7.6. Percentage detection of different prey species in *H. variegata*. Numbers beside bars indicate percentages.

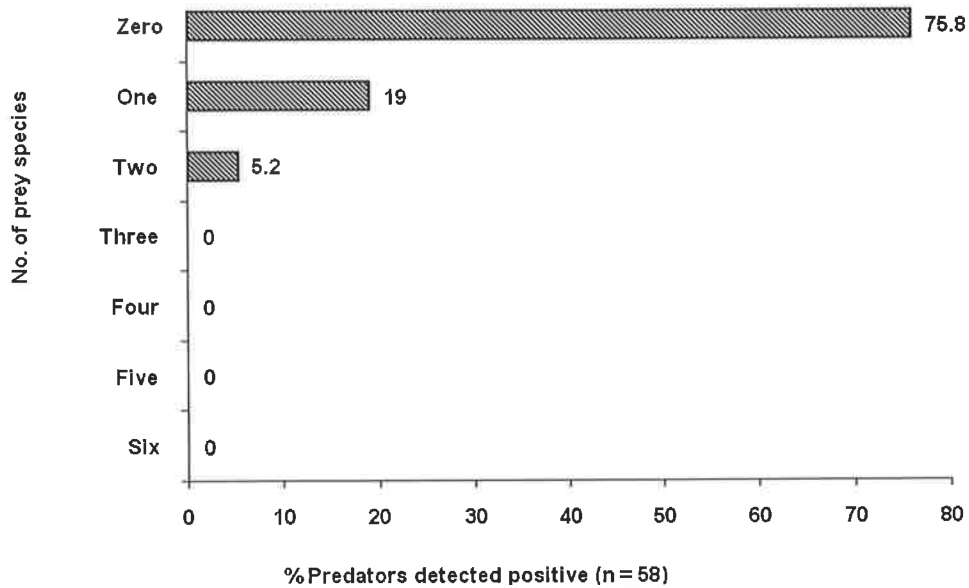

Fig.7.7. Frequency of prey species detected in *H. variegata*. Numbers beside bars indicate percentages.

3.4 *Coccinella transversalis*

Similar to *H. variegata, Coccinella transverslais* seems to be an aphidophagus predator, although almost 20.8% of all examined specimens (n=24) were found positive to *P. xylostella* compared to 8.3% for *B. brassicae* (Fig 7.8). Transverse ladybird is a predaceous insect occurring in every region of Australia and is commonly found primarily feeding on aphids in agricultural and horticultural fields (Bishop and Holtkamp 1982, Agarwala and Ghosh 1988, Pope 1988, Omkar and Bind 1993). The data showed positive signals for three lepidopteran pests. In earlier investigations it was also reported as one of major predators feeding on eggs and young larvae of *Helicoverpa punctigera* Wallengern and *H. armigera* (Hubner) (Lepidoptera, Noctuidae) in Australian cotton fields (Room 1979, Mensah 1997,1999, Ewans 2000) and *Pieris rapae* L. (Kapuge et al. 1987). Similar to *H. variegata,* this ladybird species seems to be a voracious predator to *P. xylostella,* however, it is not clear which developmental stages of prey are preferred by these predators. As has been mentioned before, it is not surprising that *C. transversalis,* like *H. variegata,* feeds on non-prey food, such as cotton nectar (Adjei-Maafo and Wilson 1983). This may account for the observation that more than half of examined specimens (66.7%) were not positive to any of the six prey species (Fig. 7.9).

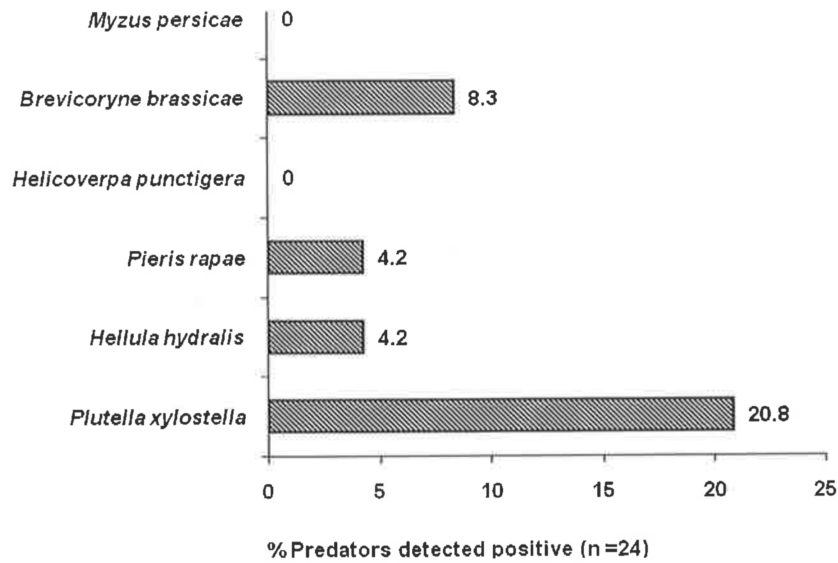

Fig. 7.8. Percentage detection of different prey species in *C. transversalis*. Numbers beside bars indicate percentages.

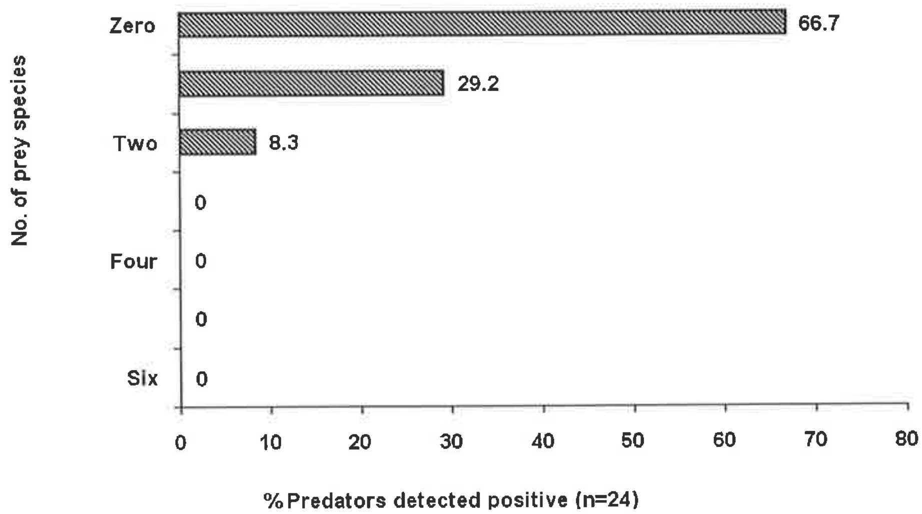

Fig.7.9. Frequency of prey species detected in *C. transversalis*. Numbers beside bars indicate percentages.

3.5 *Micromus tasmaniae*

In this investigation, apart from 6 specimens that were larvae, the rest of examined specimens were adults. Because the number of collected larval was so low, the results were combined. Results interestingly showed more specimens were found positive to *P. xylostella* (44.9%) than aphids (16.3%)(Fig. 7.10). Brown lacewing is native to Australia and New Zealand (Wise 1973) and is known as one of the major predators of aphids (Waters and Dominiak 1978, Hussein 1982, Maelzer 1997). Investigations by Leathwick and Winterbourn (1984) showed 73% of *M. tasmaniae* were positive to aphid specific proteins using serological techniques, which support the view that this species is aphidophagus. However, it is also reported as an important predator of eggs of *Helicoverpa* sp. in cotton (Bishop and Blood 1976, Samson and Blood 1979, Mensah 1999) and *Pieris rapae* (Kapuge et al. 1987). The results reported here clearly show that *M. tasmaniae,* feeds on both lepidoptera and aphids and possibly other arthropods.

Nearly 2/5 of examined specimens show no positive signals for prey DNA (Fig. 7.11), which suggests possible feeding on non-prey food or other species. Compared to other predators, *M. tasmaniae* showed a limited diet as 51% of examined specimens were found feeding on one prey only. As this species is commonly collected in *Brassica* crops, further study aimed at elucidating the diet of this species is warranted.

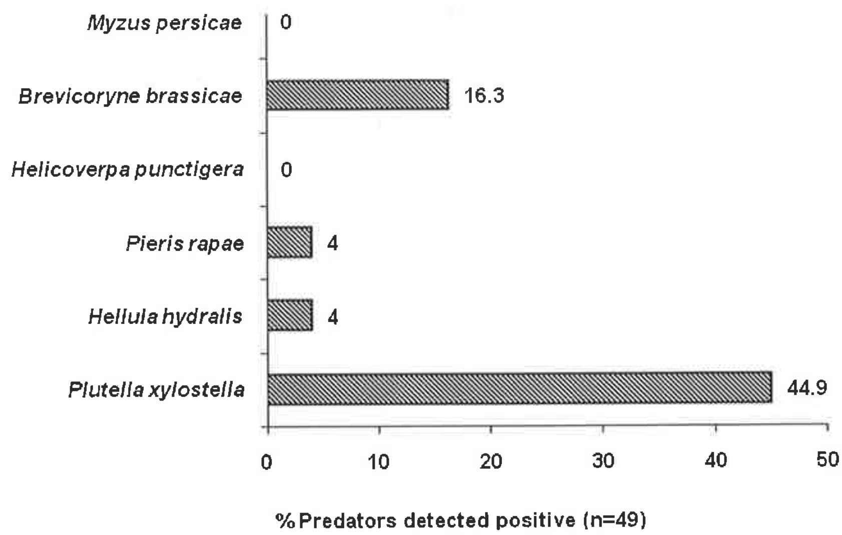

Fig. 7.10. Percentage detection of different prey species in *M. tasmaniae*. Numbers beside bars indicate percentages.

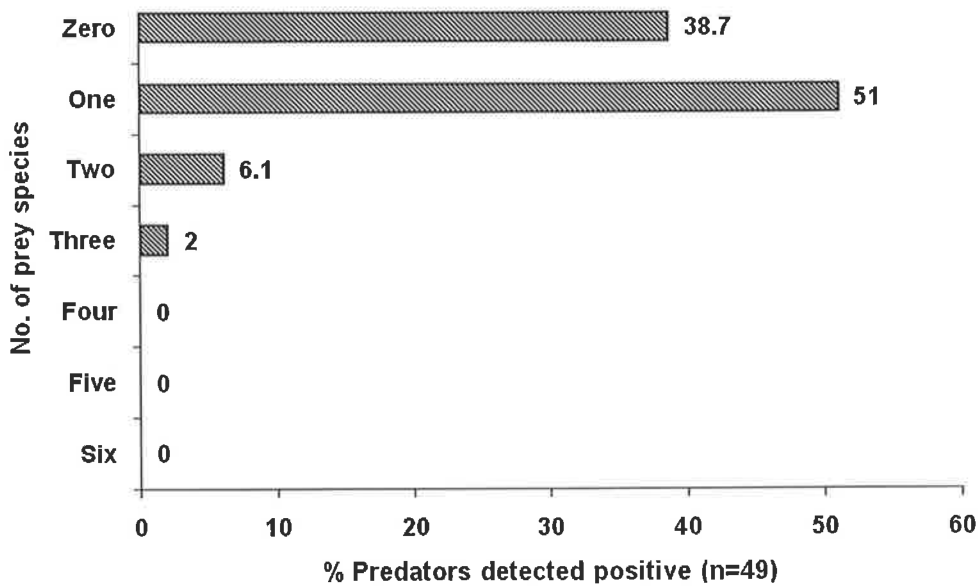

Fig.7.11. Frequency of prey species detected in *M. tasmaniae*. Numbers beside bars indicate percentages.

3.6 Lycosidae (4 species)

Wolf spiders (Lycosids) were commonly found and collected from harvested broccoli crops and within the crops from early to late in the growing season. This suggests they are active throughout the year. The abundance of collected specimens and their diversity at two collection sites showed substantial differences (Fig. 7.12). While *T. expolita* was more common at Currency Creek, *V. pseudospeciosa* was relatively more abundant at Cudlee Creek. It is unclear why species diversity differed between the sites, but soil type may have played a role. The soil at Currency Creek is sandy and at Cudlee Creek it is mostly rocky clay. These ground-dwelling spiders often burrow in the soil (Humphreys 1975, Pyke and Brown 1996), so their ability to burrow at the two sites would have differed. The abundance of wolf spider species varies among habitat types and some species apparently prefer habitats where plant cover is greater and older (Jogar et al. 2004). In some agroecosystems they are most abundant at the start of the season before crop-canopy closure (Agnew and Smith 1989, Pearce and Zalucki 2002). Differences in species between sites may have been affected by the composition of the local plant community.

The result clearly demonstrated that wolf spiders are polyphagous predators (Table 7.2, 7.3). This has been shown in other studies, which indicates that they have this ability to feed on a variety of pests (Nyffeler et al. 1994a,b). Pearce et al (2004) showed wolf spiders feed on *H. armigera* (Hubner). Bishop (1978) observed lycosids feeding on cotton looper larvae and rough bollworm larvae in cotton fields, and Kapuge et al. (1987) found 33.3% of tested field-collected lycosids were positive to *P. rapae* by

immunological assays. Agnew and Smith (1989) estimated that almost 8% of diets of lycosids in Texan peanut fields consisted of lepidoptera.

Despite wolf spiders being ground active predators, surprisingly a noticeable number of specimens, 25.5% at Currency Creek and 18.3% at Cudlee Creek, tested positive to aphid residues (Table 7.2). In a study of arthropod predation on aphids in lucerne crop 25% of *Lycosa* sp. tested gave positive precipitin reactions using serological methods (Leathwick and Winterbourn 1984). In a similar result, adults and immature stages of lycosids significantely caused 52% and 42% reduction of aphid populations under laboratory conditions (Mansour and Heimbach 1993). Another investigation also showed 20-24% of the diet of lycosids consisted of aphids (Sunderlands 1988b). Results reported here and other investigations clearly demonstrated that lycosid spiders could be considered as predators of aphids.

Results in this study showed that 23.4% of lycosids collected from Currency Creek were found positive to *H. punctigera* compare with only 1.21% in the examined lycosids collected from Cudlee Creek. This difference in detection may have been due to differences in the population density of *H. punctigera* in the two different locations. As long as we do not know the density or even occurrence of all pest species in the field, the ranking of predators for frequency of feeding on particular pests is impossible. Laboratory observations showed that wolf spiders are highly aggressive predators and easily feed on other predators and even show cannibalism (Chapter 5). Other studies also confirmed this result (Yeargan 1975, Nyffeler et al. 1994 a,b, Wagner and Wise 1996). Therefore some of the positive signals could be due to cannibalism (wolf spiders consuming target prey, which in turn were consumed by a bigger wolf spider) or

intraguild predation (non-wolf spider predators consumed target prey, which in turn were consumed by a wolf spider).

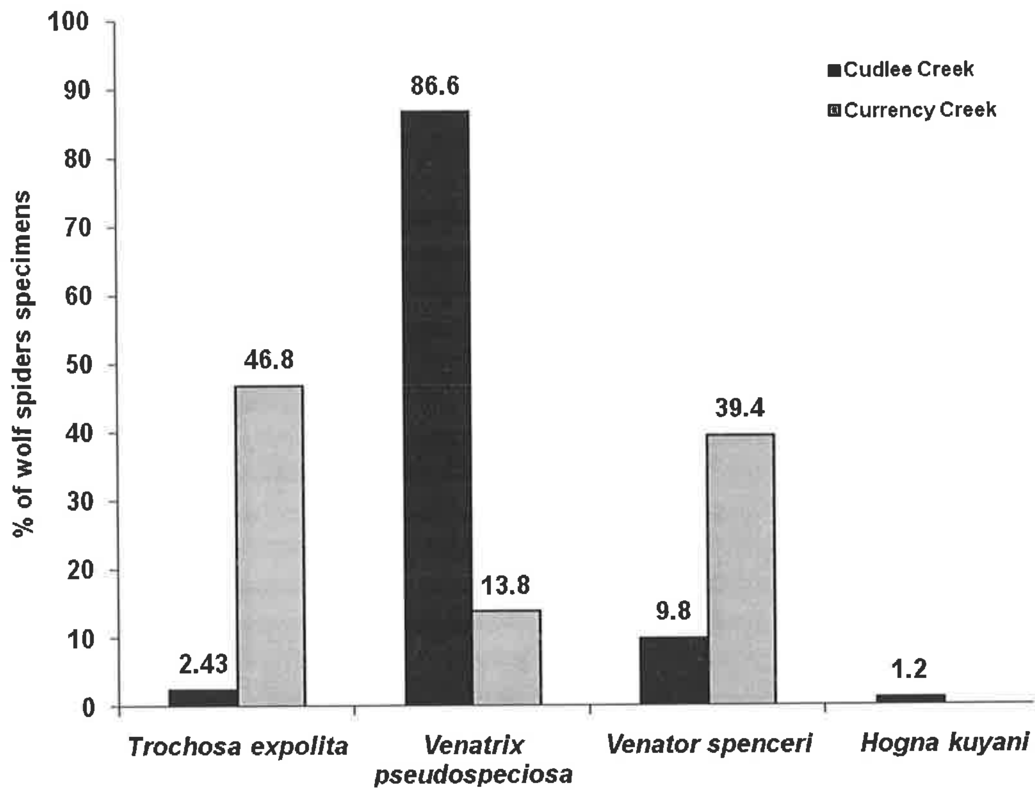

Fig. 7.12. Comparison of wolf spider species abundance in two collection sites. Numbers beside bars indicate percentages.

Table 7.2. Frequency of prey species detection rate in different wolf spider species collected from two fields (Numbers in table indicate percentage).

	Trochosa expolita		*Venator spenceri*		*Venatrix pseudospeciosa*		*Hogna kuyani*	
Prey species	Field 1 (n=44)	Field 2 (n=2)	Field 1 (n=37)	Field 2 (n=8)	Field 1 (n=13)	Field 2 (n=71)	Field 1 (n=N/A)	Field 2 (n=1)
P. xylostella	63.6	100	59.4	87.5	38.4	35.2	-	0
H. hydralis	31.8	0	16.2	0	23	14	-	100
P. rapae	56.8	100	64.8	100	69.2	59.1	-	100
H.punctigera	38.6	0	10.8	0	7.7	1.4	-	0
B.brassicae	20.4	0	29.7	25	30.7	18.3	-	0
M. persicae	0	0	0	0	0	0	-	0

Table 7.3. Frequency of prey species detected in different wolf spider species collected from two fields.

	Trochosa expolita		*Venator spenceri*		*Venatrix pseudospeciosa*		*Hogna kuyani*	
Number of prey	Field 1 (n=44)	Field 2 (n=2)	Field 1 (n=37)	Field 2 (n=8)	Field 1 (n=13)	Field 2 (n=71)	Field 1 (n=N/A)	Field 2 (n=1)
Six	0	0	0	0	0	0	-	0
Five	1	0	0	0	0	0	-	0
Four	7	0	2	0	2	0	-	0
Three	12	0	8	1	0	7	-	0
Two	7	2	13	5	4	19	-	1
One	10	0	9	2	6	27	-	0
Zero	7	0	5	0	1	18	-	0

Field 1 = Currency Creek, Field 2 = Cudlee Creek

3.7 *Philonthus* sp. (Staphylinidae)

Philonthus sp. specimens were collected from the soil surface under litter and cabbage leaves at late evening where they were found to be the most active. With some exceptions, adults of most species of Staphylinidae family are either nocturnal or generally avoid direct contact with light and prefer dark moist habitats (Klimaszewski 1996). Despite a small sample size (n=17), the use of PCR-based assays indicated that *Philonthus* sp. is a polyphagous predator and commonly feeds on aphids (Fig. 7.13, 7.14). Over 94.1% of all specimens tested gave positive signals for *B. brassicae,* indicating the presence of residues of this aphid in the gut contents of this predator. Moreover, DNA of *H. hydralis*, *P. rapae,* and *P. xylostella* and even *H. punctigera* were found in the predator's gut contents. Similarly other investigators reported that *Philonthus* sp. is a polyphagous predator feeding on aphids (Potts and Vickerman 1974, Sunderland and Vickerman 1980, Sopp and Wratten 1986, Chiverton 1987, Wratten and Powell 1991), immature stages of flies (Coaker and Williams 1963, Andersen et al. 1983, Hu and Frank 1998), and eggs, larvae and pupae of lepidopterans (Frank 1967, Tuwfik et al. 1976, Johansen 1997, Frank and Shrewsbury 2004) and mites, small arthropods and nematodes (Mank 1923, Voris 1934 in Klimaszewski 1996, Shimoda et al. 1997).

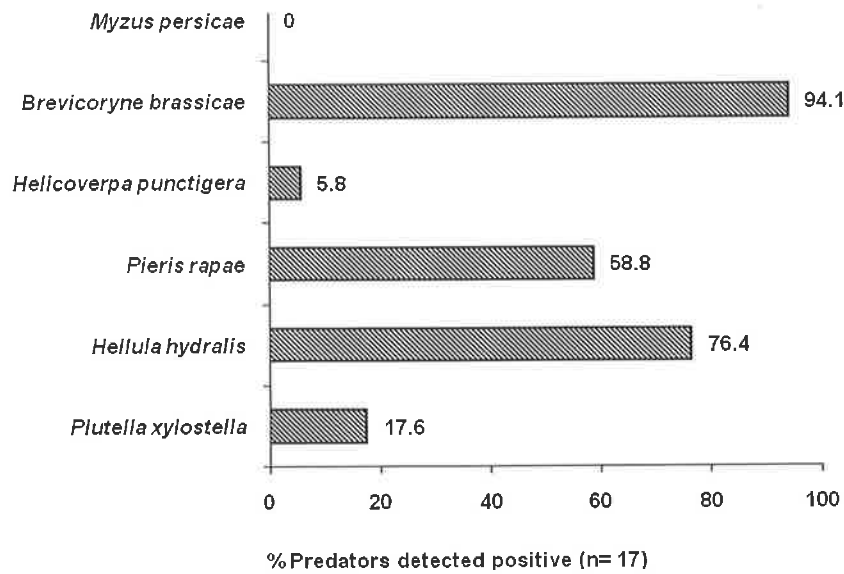

Fig. 7.13. Percentage detection of different prey species in *Philonthus sp.* Numbers beside bars indicate percentages.

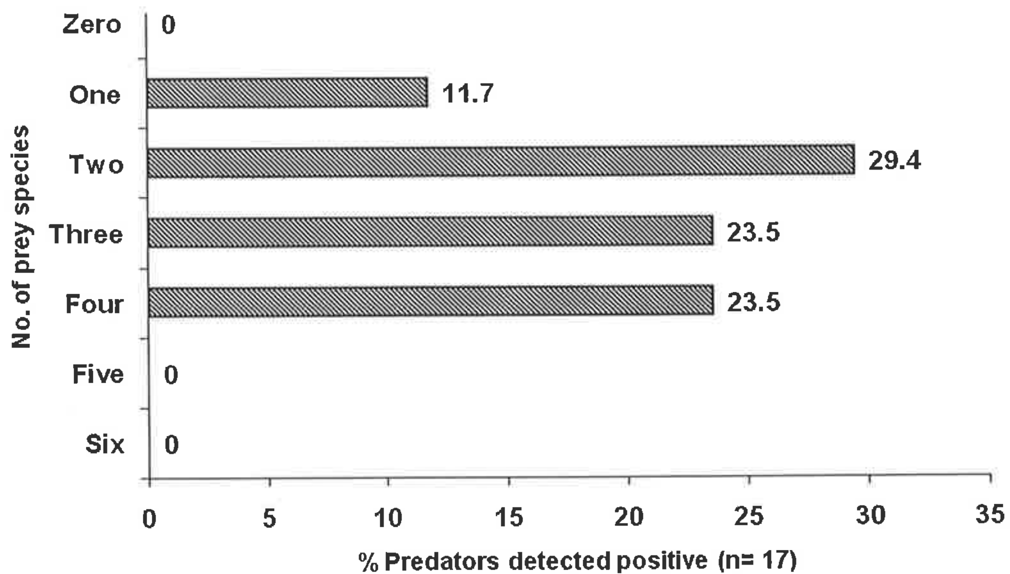

Fig.7.14. Frequency of prey species detected in *Philonthus sp.* Numbers beside bars indicate percentages.

3.8 *Euander* sp. (Lygaeidae)

This lygaeid species is believed to be a ground dwelling granivore or herbivore found under leaves or litters (Evans 1939) on different host plants, especially strawberries (Australian Biological Resources Study (http://www.environment.gov.au/, Sweet 2000). So far there is no report of this species as a pest in *Brassica* crops. Small sample size (n=10) in this species was a limitation because it was a relatively rare species (Chapter 3). However, surprisingly in 80% of total specimens examined, residues of three species (*P. xylostella, H. hydralis* and *P. rapae*) were found, while all specimens tested positive to *B. brassicae* (Fig. 7.15, 7.16). More investigations are needed to elucidate the role of this species in *Brassica* crops.

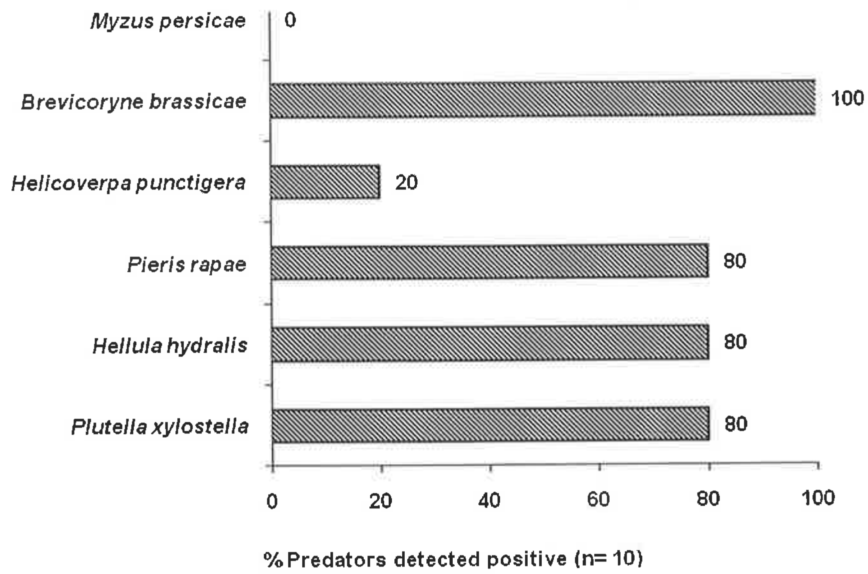

Fig. 7.15. Percentage detection of different prey species in *Euander* sp. Numbers beside bars indicate percentages.

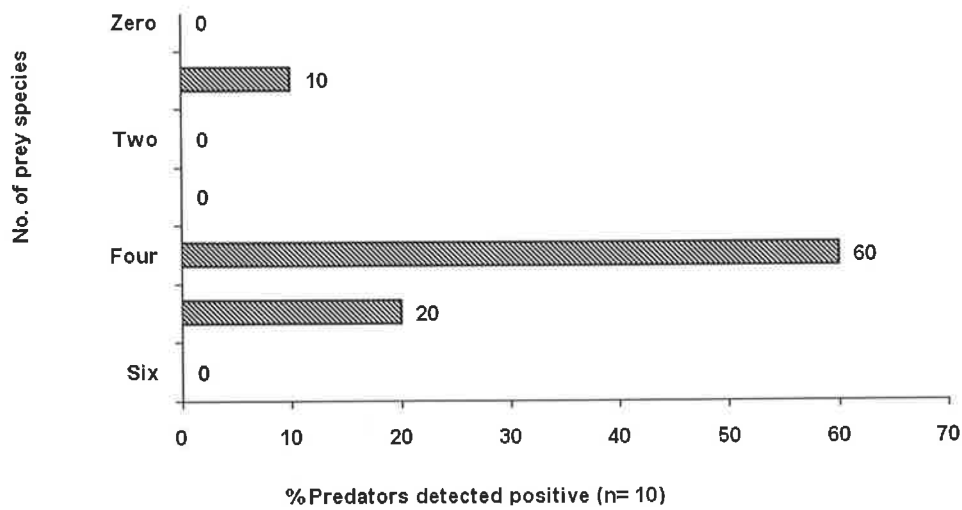

Fig.7.16. Frequency of prey species detected in *Euander* sp. Numbers beside bars indicate percentages.

3.9 *Creontiades dilutus* (Miridae)

Gut content analysis of green mirid bugs interestingly revealed that this species could be a polyphagous predator (Fig. 7.18). However, this mirid bug is known to be a sap-feeding herbivore that has been reported as a pest on different agricultural crops in Australian (Woodward et al. 1970), including grape, potato, lucerne and cotton (Mensah and Khan 1997, Grundy 2004), passionfruit, peach and nectarine (Waterhouse and Sands 2001), but there is no report that this species is a pest in *Brassica* crops.

Samples examined were positive to all prey species present in the field, which means that at least one of individuals in the mixture of specimens had eaten the relevant prey (Fig. 7.17). Another mirid bug, example *Lygus lineolaris,* is known to be an herbivorous mirid, but it also feeds on *Helicoverpa zea* eggs (Pfannenstiel and Yeargan 2002), eggs and larvae of *Heliothis virescens* (Cleveland 1987), and other soft bodied arthropods (Lindquist and Sorenson 1970, Wheeler 1976). Results presented here show that green mirid it also a predator (Fig. 7.17). The predatory behaviour of green mirid on spotted alfalfa aphid (*Therioaphis trifolii* (Monell) *f. maculata*) was reported by Hori and Miles (1993). Molecular assay of this species showed the predatory behaviour, although further investigations are needed to elucidate the role of this insect in *Brassica* fields.

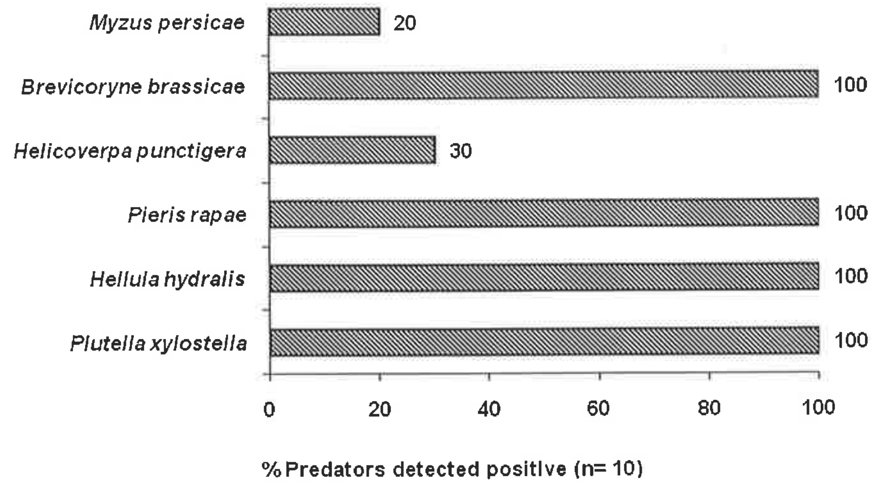

Fig. 7.17. Percentage detection of different prey species in *C. dilutus*. Numbers beside bars indicate percentages.

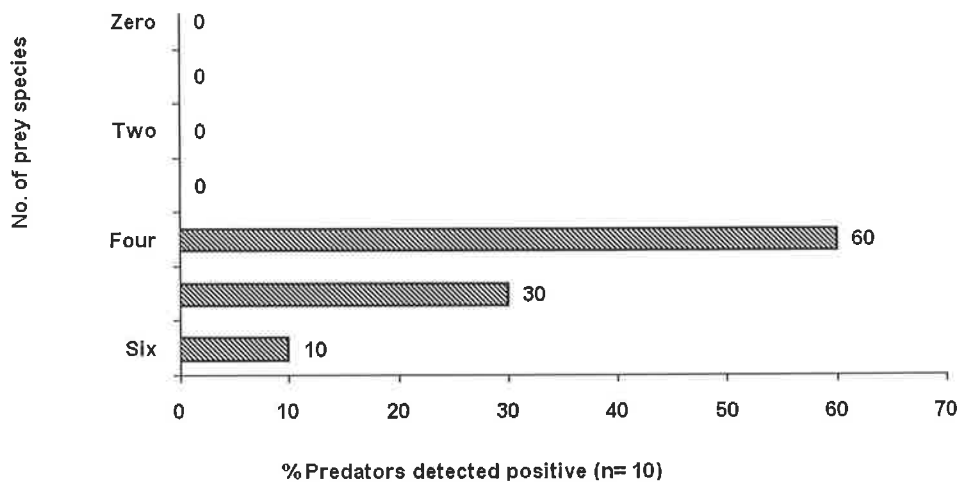

Fig.7.18. Frequency of prey species detected in *C. dilutus*. Numbers beside bars indicate percentages.

3.10 *Coranus* sp. (Reduviidae)

Compared with other species, this predator is relatively rare in the field, which is why only 6 specimens were collected and examined in the molecular assay. Results showed *Coranus* sp. is a polyphagous predator (Fig. 7.19). Four prey species were found in the gut contents of tested specimens (Fig. 7.20). Members of the Reduviidae are known as predators of other arthropods. It has been reported that *Coranus* sp. is one of the potential predators feeding on *Aphis gossypii* (Glover) (El-Sebaey and El-Wahab 2003) and other aphids (Faragall 2004), cicadellid pests (Singh 1993), *Spodoptera litura* (Bosid.) (Sitaramaiah and Ramaprasad 1982, Ren 1984, El-Sebaey and El-Wahab 2003), *Agrotis ipsilon* (El-Sebaey 2001), termites and grasshoppers (Ambrose and Livingstone 1985, Ambrose and Alexander 1989).

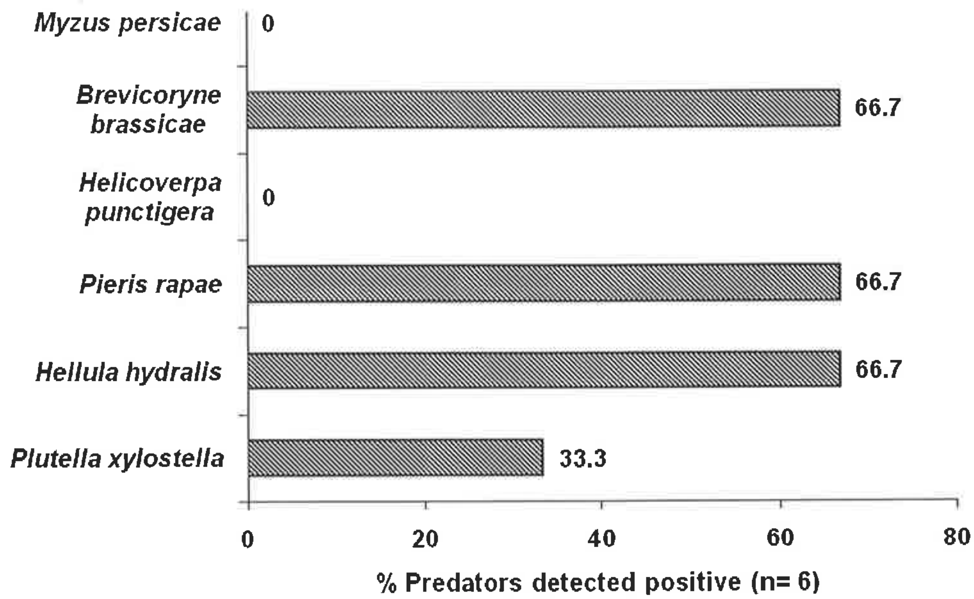

Fig. 7.19. Percentage detection of different prey species in *Coranus sp*. Numbers beside bars indicate percentages.

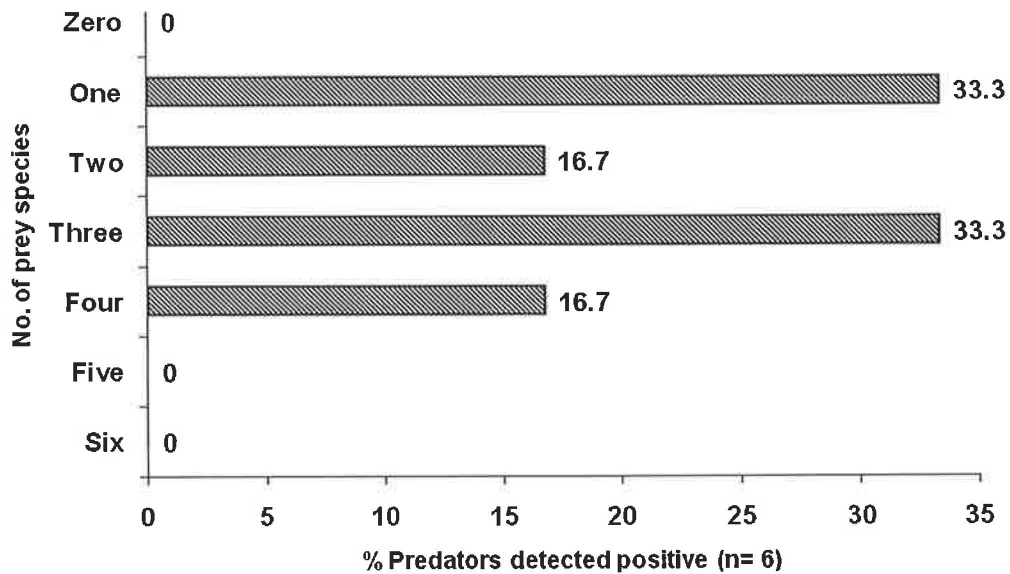

Fig.7.20. Frequency of prey species detected in *Coranus sp*. Numbers beside bars indicate percentages.

4. Conclusion

In conclusion, this study of trophic relationships of selected predators and their prey in *Brassica* fields demonstrated the potential of DNA-based techniques to screen predator communities and to identify their prey. In this investigation all examined predators showed some extent of polyphagy. All tested positive for *P. xylostella* hence be considered as predators of this pest. Although many of the records of DNA from the guts of predators probably represent instances of real predation, it is possible that some of the records result from carrion feeding or secondary predation. For example, some species of Miridae (Wheeler 1974), Coccinellidae (Wheeler 1977) and even wolf spiders (Knost and Rovner 1975) have been reported to scavenge on dead insects. Also secondary predation was demonstrated to be a potential source of error (Chapter 5). However, pilot laboratory observations showed that four of the examined species of wolf spiders, *N. kinbergii* and *H. variegata* reject dead prey, so most of the positive results recorded here for these predators are probably not due to scavenging. Nevertheless, this issue is not resolved under natural conditions and at different levels of hunger. Therefore to get an accurate indication of the likelihood of predation, scavenging and secondary predation behaviour should be investigated in the laboratory and under natural conditions. These experiments have not been done for all predators in the current study because it was outside the scope of this work. Moreover, in this study a lack of data for population densities of prey was a limitation in interpretating predation data. As an example, a lack of positive signals to *M. persicae* in most of the predators is ambiguous. It is not clear if this was due to low aphid density or non-preference of predators to this prey.

Chapter 8: General discussion

The current study demonstrated the usefulness of PCR-based techniques towards development of a better understanding of predator-prey ecology in *Brassica* crop ecosystems. In this investigation for the first time large numbers of field-collected predators were examined for residues of six pests of *Brassica* crops and the results of the study provided information on the predators' diet ranges, especially *P. xylostella*. Data on the comparative frequency of different prey species in the gut contents of predators were obtained. Moreover, for identification of a difficult-to-identify group of predators (wolf spiders), an accurate and reliable identification method was developed based on multiplex PCR. This provided a tool for identification of a number of immature field-collected wolf spiders without any necessity to consult a specialist. Study of predator-prey trophic interactions in *Brassica* crops indicated that *N. kinbergii*, *O. schellenbergii* and Lycosids are abundant and most of selected prey species were found in their diets. Consequently these species are likely to be relatively important as biological control agents in *Brassica* crops. However, ranking of predators in their ability to suppress particular pests is incomplete, because differences among predator species in detection or being positive to different prey species such as *P. xylostella*, could be influenced by many factors such as 1) temperature, 2) time since feeding, 3) prey species, 4) prey size and developmental stage, 5) prey availability or prey population density, 6) predator's species, behaviour, level of hunger and digestion rate, 7) partial prey consumption or killing without consumption, 8) scavenging, 9) secondary predation, and 10) factors like season, crop varieties, location, weather and many others.

It is noteworthy to consider that molecular techniques do not take in account the behaviour of predators and, as mentioned before, all interpretations are based only on the percentage of positive signals for a particular pest.

The frequency of consumption of prey is likely to depend on the abundance of each species in the field. Since no data were collected for the densities of pests, differences in the frequency of consumption of different prey species could be due either to variable pest abundance or to preferences of the predators. As an example, with the exception of a few positive tests for the presence of DNA from *M. persicae* in *N. kinbergii* and *O. schellenbergii* (Chapter 7), most predators, even aphidophagous predators, did not test positive for this aphid. As long as we do not know the density or even presence of this pest in the field, PCR-based detection alone could lead to a misinterpretation of predation frequency for this prey.

Nocturnal or diurnal activity of predators is another important factor, which should be considered for each predator. For example, Fewkes (1961) found more Nabidae in sweep net samples taken from grassland at night. Therefore samples taken during the day are unlikely to be fully representative, and comparisons of data obtained at different times of day may be of limited value (Leatwick and Winterbourn 1984).

Among predators, climbing or vegetation dwelling predators maybe able to eat or kill more pests compared to ground dwelling predators because more prey populations are available for them, whilst ground dwelling predators only rely on prey that have fallen or walked away from the crop (Losey and Denno 1998). Therefore it is impossible to compare the rate of impact of these two groups of predators with each other. Moreover,

as mentioned previously (Chapter 5), predators have the opportunity to scavenge or consume other predators, which in turn may have consumed a prey (Sheppard et al. 2005). Thus a predator testing positive in a DNA-based assay does not necessarily indicate the role of it in pest population control.

For comparison of predators, the half-life of detection (Chapter 5)(the time of which half of the predators tested positive) may be used in an initial ranking of predators. Half-life is an effective indicator of the detection of a certain prey species, which in fact depends on the predator's digestion rate. For example, if more positive signals were observed for *P. xylostella* in wolf spiders and *N. kinbergii* compared to *H. variegata* (Chapter 7), it could be attributed to the longer retention time after feeding the target prey, because the half-life was longer for wolf spiders and *N. kinbergii* compared to *H. variegata* (Chapter 5). Therefore, if this value was available for all predators, then comparison of the relative importance of confirmed predators may become easier. However, this kind of comparison is only an indicator and not an absolute method for ranking of the impact of predators.

Despite a great deal of progress that has been made in the development of new technologies, the exact measurement of predation and ranking predators for a particular pest are still not easily done. Molecular approaches used in post mortem gut analysis usually estimate the consumption rate of prey by predators rather than the predation rate (Sunderland 1996). It should be considered that consumption rate and predation rate are two different concepts. Study of consumption rate is relevant for the energetic relationships of trophic interactions among predators and prey, which has been investigated in this study, but predation rate is essential and applicable to pest control by

predators. Currently, with the available technologies, it is impossible to distinguish between large meals eaten a long time ago by a predator and small meals eaten more recently, because both of these situations can provide the same signal. Therefore PCR-based techniques are basically qualitative. Then cannot distinguish among developmental stages of prey or even number of consumed prey, and all efforts to quantify raw data obtained by molecular techniques can only be regarded as a first estimation.

Because of limitations of molecular techniques in the interpretation of predation data obtained from the field, it is necessary to get support from other predator assessment techniques to fill the information gap. Once trophic relationships are determined between a predator and its prey, other factors can be examined. For example, research could examine the variation of attack rate of an individual predator with regard to prey density (functional response)(e.g. Schenk and Bacher 2002), or which prey and its developmental stages and sizes are preferred by a predator species (prey preference)(e.g. Lucas et al. 1997). Other aspects worthy of study are a predator's voracity to a particular prey. For example, small wolf spiders feed on small prey like aphids while big wolf spiders prefer to feed on bigger prey (Nyffeler et al. 1994a).

Community level interactions are also important. Whether a guild of predators or a specific predator has the potential to control a pest population (e.g. Frazer et al. 1981) is affected by the intensity of synergistic interactions within the community. Some predators can influence prey behaviour and thus affect prey susceptibility to other predators (Charnov et al. 1976). Others can neutralize each other's actions through cannibalism and intraguild predation (Polis et al. 1989, Rosenheim et al. 1993).

Predators can also influence the foraging range of other predator species resulting in antagonistic interactions (Jeffries 1990, Lima and Dill 1990, Rosenheim et al. 1993). For example, data presented in this thesis suggests that the consumption rates of *P. xylostella* by *H. variegata* and *C. transversalis* were lower than other predators. These predators are foliar-foraging predators that can stimulate larval *P. xylostella* to fall down to make them available for ground-foraging predators like wolf spiders (Losey and Denno 1998). Therefore despite their direct effect on the pest being low, they may have positive interactions with other predators, which increase their predatory value and consequently lead to a greater overall impact on this pest.

Results obtained from this study were essential in the determination of trophic interactions of predators and prey, but alone not sufficient to allow interpretation of predation data from the field. Overall, we are still a long way from having reliable techniques for studying predator-prey food webs and to get a complete view of their complex relationships in agroecosystems. Each available technique has its own advantages and disadvantages. Therefore it is advisable to combine results obtained by using more than one method of study. In investigations of predator-prey interactions, at least one method should be used that has the least disruption of predators' behaviour, which could be the identification of prey in the guts of predators.

8.1 Future research

The research undertaken in this thesis succeeded in identifying the most abundant predators in *Brassica* fields (Chapter 3), developing and evaluating primers for the assessment of field-collected predators (Chapter 4), and clarifying some important

issues involved in detection of prey in the gut contents of predators (Chapter 5). However, many issues remain unexplored. For this reason the following suggestions could be helpful for future research:

Determination of the seasonal abundance of predaceous arthropods is an important requirement for development of biological control strategies for *Brassica* crops. In this study, predator density was investigated in a limited period. It would be valuable to identify the major arthropods and their seasonal fluctuations throughout the year and to determine the role of dominant predators, which were not investigated in this study. For example carabid beetles have been frequently reported to account for significant larval mortality in *P. xylostella* and *P. rapae* (Yamada 1985, Sivapragasm et al. 1988, Schellhorn and Sork 1997, Suenaga and Hamamura 1998, 2001), but were not prominent in the results reported here.

Future studies could aim to develop group-specific markers to study the prey diversity found in the gut contents of predators. For example, the design and evaluation of dipteran, homopteran, coleopteran, etc, specific primers would make it possible to study the frequency of these insect groups in the diets of predators (e.g. Jarman et al. 2004).

Multiplex PCR can be a powerful and quick tool for the simultaneous identification of prey species (Chapter 4 and 6). Thus in future more investigation could be focused on the establishment of sensitive and reliable multiplexing methods for predation studies.

Currently used methods are not able to quantify the number or amount of prey consumed by predators. The development and evaluation a method based on qRT-PCR

(Nejstgaard et al. 2007) to quantify prey consumption might open a new window on the investigation of predation.

Overall, this study clearly has revealed the effectiveness of the DNA-based method for establishment of trophic interactions of predators and their prey in *Brassica* ecosystems. The results will enable future researchers to confidently identify predators that attack six pests of *Brassica* crops. This will open the way for future studies on the assessment of predators as biological control agents.

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