THE ROLE OF WOLF SPIDERS (ARANEAE: LYCOSIDAE) ON THE BIOLOGICAL CONTROL OF THE BOLLWORM *HELICOVERPA* SPP. (LEPIDOPTERA: NOCTUIDAE) IN COTTON CROPS

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STATEMENT OF CANDIDATE

I certify that the work in this thesis entitled "**THE ROLE OF WOLF SPIDERS (ARANEAE: LYCOSIDAE) ON THE BIOLOGICAL CONTROL OF THE BOLLWORM *HELICOVERPA* SPP. (LEPIDOPTERA: NOCTUIDAE) IN COTTON CROPS"** has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

The research presented in this thesis did not require Macquarie University Ethics Review Committee approval, since all the experiments presented here were done with arthropods.

Signature

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THESIS ABSTRACT

Cotton bollworm larvae (Lepidoptera: Noctuidae, *Helicoverpa* spp.) that survive on genetically modified 'Bt cotton' contribute to the risk of widespread resistance to Bt toxins. A resistance management technique in cotton fields involves deep tilling of the soil to kill overwintering pupae ('pupae busting'), which is incompatible with the agronomic practice of minimum tillage. As a biological control alternative in minimum-tillage cotton fields, ground predators can kill *Helicoverpa* spp. Larvae as they descend from the plant to pupate in the soil, or moths emerging from underground. In this thesis, I examine the impact of biological control from wolf spiders (Araneae: Lycosidae) on ground-dwelling stages of *Helicoverpa* spp., as a strategy for Bt resistance management in minimum tillage fields. Wolf spider diversity was higher in complex minimum-tillage cotton plots compared to simple tilled cotton plots. Predation events of general prey were rare to observe in the field, and gut-content tests revealed that a low proportion of wolf spiders (2.1%) potentially killed IgG-marked *Helicoverpa* spp. larvae, but this is likely due to the low rate of spider recapture in cotton plots due to migration. In enclosed containers, the three largest and abundant species of wolf spiders *Tasmanicosa leuckartii, Hogna crispipes,* and *Hogna kuyani* all kill high proportions of 5th instar *Helicoverpa* spp. larvae on the soil. *Tasmanicosa* killed *Helicoverpa* before and after pupation; in glasshouse enclosures, a single *Tasmanicosa* can reduce by 38% the number of larvae surviving to pupation, and by 66% the number of larvae surviving to moth emergence. The increase in abundance (one or two *Tasmanicosa* or *Hogna* individuals) in glasshouse enclosures did not increase *Helicoverpa* mortality. Increasing spider abundance and diversity (*Tasmanicosa* + *Hogna*) in glasshouse enclosures reduced *Helicoverpa* survival compared to one *Tasmanicosa* only, but this effect was not additive, suggesting that antagonistic intraguild interactions between wolf spiders can limit biological control on *Helicoverpa.* In the presence of the ground cricket *Teleogryllus commodus* (a prey commonly observed in the field), *Tasmanicosa* still killed high proportions of *Helicoverpa* larvae in

laboratory containers. However, transient acquired toxicity by *Teleogryllus* which let to spider mortality can disrupt biological control of *Helicoverpa.* In addition to consumptive effects of direct predation, wolf spiders also exerted non-consumptive effects on *Helicoverpa;* in laboratory containers, *Helicoverpa* larvae spent less time on a cotton boll and more time on the soil in the presence of a spider. Additionally, increased loss of cotton boll mass likely reflects changes in *Helicoverpa* foraging behaviour induced by the presence of spiders. Considering the setting (laboratory, glashouse, field), and the interactions with intraguild predators and alternative prey, wolf spiders showed various strengths and limitations in their capacity to control *Helicoverpa*. Given the high diversity and abundance of wolf spiders in cotton fields throughout the cropping season, and the high proportion of *Helicoverpa* spp. larvae and moths that spiders kill even in the presence of alternative prey, wolf spiders should be considered important biological control agents when implementing pest and Bt resistance management strategies.

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I would like to give an unusual acknowledgement to all the things that went wrong! Like the times I got bogged in the flooded fields and had to be rescued....or that time when I put diesel in a petrol car...or when the roof of my glasshouse got ripped off in a storm....or when the air conditioning broke and all my animals and cotton plants got baked at 60C, therefore losing an entire trial....or when my videorecording system stopped working because I mixed up the power adaptors...or when I killed a whole batch of pupae by accidentally washing them with ethanol instead of bleach. This taught me that I will never stop learning and still have so many more mistakes to make, but regardless of them, I can still keep on going...And it also made me jump with joy whenever things went smoothly!

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TABLE OF CONTENTS

STATEMENT OF CANDIDATE ..... **i**

THESIS ABSTRACT ..... **iii**

ACKNOWLEDGEMENTS ..... **v**

LIST OF FIGURES ..... **ix**

LIST OF TABLES ..... **xiii**

INTRODUCTION **1**

CHAPTER 1: Influence of crop management and environmental factors on wolf spider assemblages (Araneae: Lycosidae) in an Australian cotton cropping system. **17**

Abstract 18

Introduction 19

Materials and Methods 22

Results 26

Discussion 36

Acknowledgements 40

References 41

CHAPTER 2: Ecological and molecular approaches for assessing common prey and *Helicoverpa* larva consumption by wolf spiders in a Bt-cotton field **51**

Abstract 52

Introduction 53

Materials and methods 59

Results 69

Discussion 75

Acknowledgements 80

References 80

CHAPTER 3: Consumptive and non-consumptive effects of wolf spiders on cotton bollworms. **89**

Abstract 90

Introduction 91

Materials and methods 95

Results 102

Discussion 111

Acknowledgments 119

References 119

CHAPTER 4: Intraguild interactions between two wolf spider species lead to non-additive effects on the biological control of the cotton bollworm.....127
Abstract.....128
Introduction.....129
Materials and methods.....132
Results.....137
Discussion.....144
Acknowledgments.....151
References.....152
CHAPTER 5: Prey encounter, prey vulnerability and nutritional content in a ground arthropod trophic web in cotton crops.....159
Abstract.....160
Introduction.....161
Materials and methods.....164
Results.....169
Discussion.....175
Acknowledgements.....180
References.....181
CHAPTER 6: A killer killed? Wolf spider mortality after feeding on ground crickets 187
Abstract.....188
Introduction.....189
Materials and methods.....190
Results.....194
Discussion.....197
Acknowledgments.....200
References.....200
GENERAL DISCUSSION.....205
APPENDIX I: Wolf spiders (Araneae: Lycosidae) found at the Australian Cotton Research Institute (Narrabri, NSW).....231
APPENDIX II: Author permission to reproduce tables and figures.....255

LIST OF FIGURES

CHAPTER 1: Influence of crop management and environmental factors on wolf spider assemblages (Araneae: Lycosidae) in an Australian cotton cropping system

Figures 1a-1c. Picture of *Venatrix konei* (adult male), *Hogna crispipes* (adult female), and *Tasmanicosa leuckartii* (adult female), the three most common species of wolf spiders in the cotton fields.....27

Figure 2. Histogram of overall abundance of wolf spider species in cotton fields.....28

Figure 3. Distribution of adult males and females of the three most dominant species in simple and complex plots over the cotton season. Error bars represent 1 SE. 29

Figure 4. Distribution of wolf spider juveniles and spiderlings on simple and complex plots over the cotton season. Error bars represent 1 SE. 30

Figure 5. Abundance and species richness in simple and complex plots through the cotton stages.....32

Figure 6. Species-accumulation curve of adult wolf spiders caught in simple and complex plots.....33

Figure 7. Biodiversity (Shannon-Weaver and Simpson's index) of wolf spiders in simple and complex plots through the cotton stages. Letters indicate differences between cotton stages (but not plots) according to Least Square Differences. Error bars represent 1 SE. 34

Figure 8. Direct ordination analysis (CCA) of spider species in relation to field treatment and environmental variables (three most common species are in a square; abbreviations: Vf = *Venatrix fontis*, Aw= *Artoriopsis whitehousease*, Ae= *Artoriopsis expolita*, Hk= *Hogna kuyani*, Ao= *Anomalosa oz*, A sp= *Artoria sp*., H sp= *Hogna sp*., Av= *Artoria victoriensis*, Vs= *Venatrix speciosa*).....35

CHAPTER 2: Ecological and molecular approaches for assessing common prey and *Helicoverpa* larva consumption by wolf spiders in a Bt-cotton field

Figure 1. a) Focal plot surveyed for gut-content analysis and mark-recapture. b) Larger cotton field adjacent to focal plot surveyed for wolf spider predation, RRF= roundup ready Flex cotton, which means it contains genetic coding for resistance to glyphosate (Roundup ™), a herbicide; BRF = Bt Roundup Ready Flex cotton, which means the cotton also contains the gene to produce Bt toxins. 68

Figure 2. Mean absorbances of gut content of spiders after feeding on *H. armigera* at 25°C. Numbers on top of bars represent percentage of samples scored positive for presence of IgG. *For "spider exposed to marked *Helicoverpa*" treatment, only positive absorbances are graphed. Bars represent 95% confidence intervals.....69

Figure 3. Cephalothorax width of wolf spiders in field containers and *Helicoverpa* spp. predation outcome.....71

Figure 4. Predation outcomes of wolf spider males, females and juveniles of wolf spiders on *Helicoverpa* spp. in field containers. 71

Figure 5. Mark-recapture survey in the focal plot across multiple nights. Days with no spiders represent nights where surveys were not possible due to inclement weather.....73

Figure 6. Total mark-recapture data from wolf spider survey in focal plot. Numbers indicate how many spiders of each species were found in total with or without a mark and their location, pooling all nights. 73

CHAPTER 3: Consumptive and non-consumptive effects of wolf spiders on cotton bollworms

Figure 1. Average proportion of time *Helicoverpa armigera* larvae spent on plant (cotton boll), on soil, or underground in laboratory trials with no spider or a risk spider.....105

Figure 2. Mass loss (%) by cotton boll in laboratory trials with no spider and risk spider. Asterisk on top of bars represents significantly different treatments (ANCOVA, $p < 0.05$). Horizontal line represents median, box length represents interquartile range, whiskers length are upper and lower quartiles, dots represent suspect outliers in untransformed data (values greater than 1.5 box lengths), light grey stars represent extreme outliers in untransformed data (values greater than 3 box lengths).....106

Figure 3. Number of moths emerged from enclosures with no spider, predation spider, and risk spider. Asterisk represents significantly different treatments. (ANOVA, $p < 0.05$). Horizontal line represents median, box length represents interquartile range, whiskers length are upper and lower quartiles, and dots represent suspect outliers in untransformed data (values greater than 1.5 box lengths).....107

Figure 4. Average proportion of live *Helicoverpa armigera* larvae in each of three locations in cotton plant enclosures with no spider, predation spider, and risk spider. Bar size represents average proportion of larvae observed in each location per enclosure.....109

Figure 5. Average proportion of live *Helicoverpa armigera* larvae performing each of four behaviors inside cotton plant glasshouse enclosures with no spider, predation spider, and risk spider. Bar size represents average proportion of larvae observed performing each behavior per enclosure.....110

Figure 6. Number of visible *Helicoverpa armigera* through different life stages in glasshouse enclosures by day, with no spider and risk spider. Bars represent 1 SE.....111

CHAPTER 4: Intraguild interactions between two wolf spider species lead to non-additive effects on the biological control of the cotton bollworm

Figure 1. Number of surviving *Helicoverpa armigera* pupae in glasshouse enclosures in each spider treatment. Different letters represent significantly different treatments. Horizontal line represents median, box length represents interquartile range, whiskers length are upper and lower quartiles. 139

Figure 2. Proportion of attacks by either *Tasmanicosa leuckartii* or *Hogna crispipes* on *Helicoverpa armigera* or each other when tested in isolation or paired together with the other spider species in laboratory arenas. Males, females and juveniles were pooled together as the analysis failed to find a relationship between spider sex and intraguild predation. The second bar for each spider represents the same trials in treatment 3, but analyzed separately for each spider species. 140

Figure 3. Spider size difference (percent relative to *Hogna crispipes* cephalothorax weight) and the occurrence of attack between *Tasmanicosa leuckartii* and *Hogna crispipes* paired together in laboratory arenas. Horizontal line represents median, box length represents interquartile range, whiskers length are upper and lower quartiles. 142

CHAPTER 5: Prey encounter, prey vulnerability and nutritional content in a ground arthropod trophic web in cotton crops

Figure 1. Percentages of first encounter (dashed arrows) and first attack (solid arrows) of *Tasmanicosa* in a 3-way videorecorded trophic web arena (experiment 2). Direction of arrows point to the predator that made the encounter/attack. Percentages indicate cases in which prey was encountered first (out of n = 27), or attacked first (out of n = 27). 171

Figure 2. Percentages of first encounter (dashed arrows) and first attack (solid arrows) of Tasmanicosa and Hogna in a 4-way videorecorded trophic web arena (experiment 2). Direction of arrows point to the predator that made the encounter/attack. Percentages indicate cases in which prey was encountered first (out of n = 23), or attacked first (out of n = 23). .172

Figure 3. 4-way trophic web between *Tasmanicosa, Hogna, Teleogryllus* and *Helicoverpa* (multiple predation trials; experiment 3). Direction of arrows point to the predator that made the attack (energy flow). Percentages indicate cases in which the prey was attacked by the predator (out of n= 31). Black arrows indicate *Tasmanicosa* predation, grey arrows indicate *Hogna* predation. Arrow width represents proportion of predation from total trials. 173

Figure 4. Percent body content of protein and lipid in four arthropods, caught near cotton fields, reared on cotton plant material, or reared on soy and agar diet. 175

CHAPTER 6: A killer killed? Wolf spider mortality after feeding on ground crickets

Figure 1. Predation sequence of *Tasmanicosa* paired with *Helicoverpa* and *Teleogryllus* (videorecorded trials). Direction of arrows indicates predation sequence. Percentages indicate how many spiders out of the original n = 27 followed the predation sequence indicated by the arrow. 196

LIST OF TABLES

INTRODUCTION

Table 1: Questions to assess the impact of a predator on a pest prey, and the chapters in this thesis that address the corresponding question (Macfadyen *et al.*, 2015). 6

CHAPTER 2: Ecological and molecular approaches for assessing common prey and *Helicoverpa* larva consumption by wolf spiders in a Bt-cotton field

Table 1: Reported prey of wolf spiders using direct observation and gut-content methods. Studies include only spiders freely captured in the field, and not used in feeding experiments in enclosures. 57

Table 2. Wolf spider prey observed in visual surveys around edges of cotton fields. 74

CHAPTER 4: Intraguild interactions between two wolf spider species lead to non-additive effects on the biological control of the cotton bollworm

Table 1. One-way logistic regression relationships between six predictors and predation outcomes in laboratory trials where *Tasmanicosa*, *Hogna* and *Helicoverpa* are paired together. 143

GENERAL DISCUSSION

Table 1: Questions to assess the impact of a predator on a pest prey, and the chapters in this thesis that address the corresponding question (adapted from Macfadyen *et al.*, 2015). 206

Table 2. Benefits and challenges associated with measuring different components of the ecology and impact of a predator on a pest prey (copied with permission; Macfadyen *et al.*, 2015). 219

INTRODUCTION

The use of natural predators to control pest populations (biological control) has become a key element of Integrated Pest Management (IPM) in agricultural systems (Naranjo *et al.*, 2015; Zalucki *et al.*, 2015). Habitat management to enhance predator populations, and thereby improve biological control ('conservation biological control'), has become a significant area of research and has been proposed as a method to increase IPM sustainability through enhanced biodiversity (Landis *et al.*, 2000). In annual crops, fallow fields between growing seasons offer few resources for native predators, forcing them to overwinter in vegetation outside of the fields. Increased complexity in vegetation within or near crops can provide native predators a stable shelter for overwintering and reproduction. Techniques for increasing habitat diversity within crops include intercropping, weed strips, mulching, minimum tillage and incorporated stubble (Sunderland & Samu, 2000). Habitat diversity can help reduce fluctuations in the abundance of native predators by maintaining a continuous and diverse community (Brevault *et al.*, 2007; House & Parmelee, 1985; Stinner & House, 1990). High densities of predators early in the crop growing season can suppress emerging pests and keep them under a threshold throughout the year. Farming practices that can enhance native predator populations and contribute to conservation biological control should be considered when designing IPM strategies.

In the Namoi Valley (northern New South Wales [NSW]) in Australia, cotton is an annual summer crop, planted in October and harvested in May. Two of the main pests of cotton are the cotton bollworm, *Helicoverpa armigera* (Hübner) and its close relative the native budworm *Helicoverpa punctigera* Wallengren (= *Heliothis,* Fitt, 1989; Zalucki *et al.*, 1994; hereafter referred together as *Helicoverpa* spp.). *Helicoverpa armigera* is widely distributed throughout Australia, Asia, South America Africa and southern Europe, whereas *H. punctigera* is endemic to Australia (Fitt, 1989). *Helicoverpa* spp. moths lay eggs on the

leaves, buds and squares of plants, and larvae feed on cotton plant structures during five instars, before descending from the plant to pupate in the soil. *Helicoverpa* spp. has 4-6 generations during the cotton growing season in Northern NSW (Baker *et al.*, 2011; Zalucki *et al.*, 1986). After cotton harvest, *H. punctigera* migrates from cropping areas, while *H. armigera* goes into diapause and overwinters underground as pupae in cotton fields (Duffield, 2004; Fitt, 1989; Zalucki *et al.*, 1986). In the Namoi Valley, *H. armigera* moths emerge from winter diapause from mid-October to mid-November. Diapause in pupae is triggered by day length less than 12h and temperatures below 23°C; *H. armigera* pupae enter diapause between April and May (Wilson *et al.*, 1979). Together, these two *Helicoverpa* species pose a serious threat for cotton production in Australia.

Historically, *Helicoverpa* spp was controlled by sprayed insecticides (pyrethroids, endosulfan, carbamates and organophosphates), but the emergence of resistance to sprayed insecticides, by *H. armigera,* led to an economically and environmentally unsustainable situation, in which cotton growers had to spray crops every few days, in some years more than 20 times a season (Forrester, 1989; Forrester *et al.*, 1993; Hearn, 1975). Additionally, the continuous application of sprayed insecticides decreased populations of predators that could exert biological control of pests in cotton fields. Growers not only faced the problem of controlling pests during the growing season and keeping them below an economic threshold, but also needed to implement strategies to prevent emergence of insecticide resistance. In 1983, as a complement to IPM, the Australian cotton industry developed an Insecticide Resistance Management Strategy (IRMS), which focused mainly on insecticide rotation, application restriction, and post-harvest field cultivation (Forrester, 1989; Forrester & Bird, 1996).

In 1996, genetically modified Bt cotton was introduced and widely adopted to control *Helicoverpa* spp (Whitehouse *et al.*, 2009b; Wilson *et al.*, 2013). Bt cotton contains genes of

Bacillus thuringiensis that code for a protein (Bt toxin) that destroys the gut lining of specific Lepidoptera larvae (Van Rie, 2000). Following the introduction of Bt cotton, pesticide use decreased markedly (Knox *et al.*, 2006), resulting in an increased abundance and diversity of native predators, and correspondingly increasing their impact and importance in IPM (Johnson *et al.*, 2000; Whitehouse *et al.*, 2005). While the introduction of Bt cotton revolutionized cotton IPM, the rapid development of resistance to sprayed insecticides in the past remained a concern, as *Helicoverpa* spp. could similarly develop resistance to the toxins expressed by Bt cotton (Bird & Downes, 2014; Deguine *et al.*, 2008). This led to the development of a Resistance Management Plan (RMP), an adaptation of IRMS for genetically modified cotton to prevent risk of resistance to Bt toxins (Wilson *et al.*, 2013). The main practices for Bt resistance management involve planting refuge crops and "pupae busting". Refuges are conventional (non-Bt) crops from where Bt-susceptible *Helicoverpa* moths emerge and mate with Bt-resistant moths, which reduces the likelihood of transferring Bt-resistant genes (Baker & Tann, 2014), while 'pupae busting' involves cultivating the soil to a depth of 10cm to mechanically destroy surviving pupae(Lloyd *et al.*, 2008; Wilson *et al.*, 2013).

Pupae busting is an issue particularly for dryland crops, where conservation of soil moisture is a priority. In minimum-tillage cotton fields, the crop is planted in soil beds retained intact from previous years, with shallow soil disturbance limited to deepening the furrows. Traditionally, minimum-tillage has been used as an agronomic practice to reduce soil erosion, retain soil moisture, and enhance nutrient cycling (Hulugalle *et al.*, 1997); however, minimum-tillage is not compatible with soil-disruptive practices such as 'pupae busting'. From this conflict arises the need for sustainable biological control strategies to destroy surviving pupae and delay the emergence of Bt resistance in *Helicoverpa* spp. populations.

In minimum-tillage fields with a winter (cover) crop rotation (for example, wheat or vetch), all the plant stubble remains intact on the soil. The combination of cover crops and minimum-tillage leaves a structurally complex ground, which may harbour a higher diversity of native ground predators before and during the cotton cropping season (House & Stinner, 1983; Rypstra *et al.*, 1999; Stinner & House, 1990). Therefore, despite its conflict with pupae busting, minimum-tillage has the potential to contribute to IPM by enhancing conservation biological control from ground predators. This strategy aligns with Australian cotton IPM programs, in which the conservation of native beneficial arthropods has been favoured over introduction of foreign predators (Wilson *et al.*, 2004).

Studies of predation on *Helicoverpa* spp. usually focus on eggs and early instar larvae on the plant foliage (Bahar *et al.*, 2013; Johnson *et al.*, 2007; Mansfield *et al.*, 2003; Perez-Guerrero *et al.*, 2014; Sansone & Smith, 2001; Seagraves & Yeargan, 2009; Van Den Berg & Cock, 1995), yet little is known about *Helicoverpa* spp. predation after larvae have descended from the plant. There are records of mice, earwigs (*Labidura*) (Room, 1979; Wilson, 1983), and beetle larvae (Lesiewicz *et al.*, 1982; Murray & Zalucki, 1994) feeding on *Helicoverpa* spp. pupae in the field, but most of the evidence concerning predators that attack the ground-dwelling stages of *Helicoverpa* spp. is anecdotal.

Spiders are among the most abundant and diverse predators in agroecosystems, offering great potential for biological control of pests (Greenstone, 1999; Nyffeler & Benz, 1987; Sunderland, 1999; Symondson *et al.*, 2002). Consideration of spiders as predators usually emphasizes species that hunt on the cotton canopy and greatly reduce the most plant-damaging stages of *Helicoverpa* spp. Previous studies have shown that foliage hunting spiders such as Clubionidae, Pisauridae, Thomisidae, Oxyopidae, Salticidae, (Johnson *et al.*, 2000; Pearce *et al.*, 2004; Perez-Guerrero *et al.*, 2013; Room, 1979), Philodromidae , Miturgidae (Perez-Guerrero *et al.*, 2013) and Anyphaenidae (Pfannenstiel, 2008) consume *Helicoverpa*

spp. first instar larvae or eggs, yet there is very limited information on the interactions between spiders and ground-dwelling stages of *Helicoverpa* spp.

Among ground-hunting spiders, wolf spiders (Araneae: Lycosidae) are abundant in many agroecosystems (Kuusk & Ekbom, 2012; Marshall & Rypstra, 1999; Oberg & Ekbom, 2006), including cotton (Bishop, 1980; Hayes & Lockley, 1990; Whitehouse *et al.*, 2009a). Wolf spiders are generally under-represented in predator surveys because they are rarely observed on the plant canopy, remaining on the soil most of the time. Peer-reviewed literature on wolf spider phenology, taxonomy, and behaviour in Australian cotton fields is very scarce (for main studies, see Bishop, 1980; Bishop & Blood, 1981; Pearce & Zalucki, 2002; Whitehouse *et al.*, 2009a), and the limited information on *Helicoverpa* spp. predation by wolf spiders is based on theses (e.g., Bishop, 1978) and cooperative research centre reports (Pyke & Brown, 1996; Johnson *et al.*, 2000; Williams *et al,.* 2011). No study has measured the impact of wolf spiders as potential biological control agents of *Helicoverpa* spp. Because wolf spiders encounter *Helicoverpa* spp. stages that have already caused plant damage, they are often overlooked as contributors to biological control.

The importance of wolf spiders in biological control of *Helicoverpa* spp. lies not only in their immediate impact on crop yield by reducing larval densities, but also in their role in long-term plant protection by inhibiting emergence of Bt-resistance. There is growing evidence that predators in agroecosystems can help delay the evolution of Bt resistance (Liu *et al.*, 2014; Lundgren *et al.*, 2009). To address the gap in information about biological control potential of wolf spiders, this thesis aims to assess the impact of wolf spiders as predators of ground-dwelling stages of *Helicoverpa* spp. in an Australian cotton crop. Assessing the impact of a native predator on overall biological control of a pest is challenging, and often confounded by multiple environmental variables. A means of reducing

this complexity and quantifying the impact of predators is to ask targeted questions (Table 1; adapted from Macfadyen *et al.*, 2015):

Table 1: Questions to assess the impact of a predator on a pest prey, and the chapters in this thesis that address the corresponding question (Macfadyen *et al.*, 2015).

Question	**Chapter**
Q1: Does the predator species kill the pest species?	2, 3
Q2: How many pests does the predator kill and how quickly?	2, 3, 4, 5
Q3: How do predator populations respond to changes in prey density?	1, 2
Q4: How does the predator search for prey?	2, 3, 5
Q5: What are the indirect impacts of predator behaviours on pest populations and herbivory?	3, 4
Q6: Are predator species present in the crop at the same time as pest species?	1, 2
Q7: Do other species in the community impact the predator-prey interaction?	4, 5, 6
Q8: Do other abiotic factors impact the predator-prey interaction?	1

The research for this thesis was carried out at the Australian Cotton Institute, in Narrabri NSW (Namoi Valley). The detailed description of field sites and cotton crops is included in each chapter. This thesis is divided into two chapters where data was collected in the field, and four chapters carried out as controlled experimental settings in the laboratory and glasshouse. These chapters address complementary components of the ecology and behaviour of wolf spiders. Together, these thesis chapters aim to answer the general question "Can wolf spiders contribute to biological control of ground-dwelling stages of *Helicoverpa* spp. in cotton fields?"

Chapter 1: Influence of Crop Management and Environmental Factors on Wolf Spider Assemblages (Araneae: Lycosidae) in an Australian Cotton Cropping System. The aim of this chapter is to evaluate how two different crop rotation and tillage systems in a cotton field influence wolf spider abundance, richness and diversity. From the results of this study, I also choose a species of wolf spider to use in laboratory and glasshouse experiments. This chapter is coauthored by Dr. Phil Taylor, Dr. Mary Whitehouse, and Dr. Nilantha Hulugalle (NSW Department of Primary Industries). I collected and analysed the data and wrote the manuscript. P.W. Taylor, M.E.A. Whitehouse and N.R. Hulugalle contributed to experimental design, and revisions on manuscript drafts. N.R. Hulugalle gave me access to his experimental plots for collection. This chapter has been published in *Environmental Entomology*.

Chapter 2: Ecological and molecular approaches for assessing common prey and *Helicoverpa* larva consumption by wolf spiders in a Bt-cotton field. This chapter aims to describe the behaviour, mobility and common prey of wolf spiders in a Bt cotton field. Additionally, we simulate a scenario in which Bt resistant *Helicoverpa* spp. larvae descend to the soil, and using molecular gut-content analysis (ELISA), we determine if wolf spiders present in this plot hunt 5th instar *Helicoverpa* spp. larvae on the soil. This chapter is coauthored by Dr. Phil Taylor, Dr. Mary Whitehouse, and Dr. James Hagler (United States Department of Agriculture). I designed the surveys, collected and analysed the data and wrote the manuscript. P.W. Taylor, M.E.A. Whitehouse contributed to experimental design, and revisions on manuscript drafts. Dr. James Hagler developed and taught me the ELISA method for gut-content analysis.

The subsequent chapters of this thesis are developed under controlled experimental environments, in enclosed cotton microcosm and laboratory arenas. Here I explore the predator-prey interactions between wolf spiders and *Helicoverpa armigera* (hereafter referred

to as '*Helicoverpa'),* and how additional elements of a ground food web (intraguild predators, alternative prey) can affect *Helicoverpa* predation. The models for wolf spider species, alternative prey and predators were chosen based on the results observed in the first two chapters.

Chapter 3: Consumptive and nonconsumptive effects of wolf spiders on cotton bollworms. This chapter describes the predator-prey interactions between the wolf spider *Tasmanicosa leuckartii* and *Helicoverpa* when they share the same microhabitat, such as when 5th instar larvae go to the ground to pupate, or when adults are recently emerging from their underground pupal chamber. This chapter explores two main topics: consumptive effects and nonconsumptive effects. For consumptive effects, I determine whether *Tasmanicosa* kills *Helicoverpa* 5th instar larvae, pupae or emerging moths in small laboratory arenas, and how many *Helicoverpa* does *Tasmanicosa* kill in glasshouse enclosures. Additionally, as nonconsumptive effects, I evaluate the effect that *Tasmanicosa* has on *Helicoverpa* behavior and development, and whether this in turn influences cotton damage by herbivory (trophic cascades). This chapter is coauthored by Dr. Phil Taylor and Dr. Mary Whitehouse. I designed the experiments, collected and analysed the data, and wrote the manuscript. P.W. Taylor, M.E.A. Whitehouse contributed to experimental design, and revisions on manuscript drafts. This chapter has been submitted for publication in *Entomologia Experimentalis et Applicata.*

Chapter 4: Intraguild interactions between two wolf spider species lead to non-additive effects on the biological control of the cotton bollworm. This chapter examines predator-prey interactions between *Helicoverpa* 5th instar larvae, and two coexisting wolf spiders, *Tasmanicosa leuckartii* and *Hogna crispipes*. Here I assess how the presence of an intraguild predator affects *Helicoverpa* predation by *Tasmanicosa* or *Hogna*, and whether the presence of these two spiders has an additive, neutral or disruptive effect on *Helicoverpa*

mortality in glasshouse enclosures. This chapter is coauthored by Dr. Phil Taylor and Dr. Mary Whitehouse. I designed the experiments, collected and analysed the data, and wrote the manuscript. P.W. Taylor, M.E.A. Whitehouse contributed to experimental design, and revisions on manuscript drafts. This chapter is formatted for publication in *Agricultural and Forest Entomology.*

Chapter 5: Prey encounter, prey vulnerability and nutritional content in a ground arthropod trophic web in cotton crops. This chapter examines the predator-prey interactions between *Helicoverpa* 5th instar larvae, *Tasmanicosa* and *Hogna* in the presence of an additional prey species, the abundant ground cricket *Teleogryllus commodus.* I here assess how the presence of alternative prey affects *Helicoverpa* predation by *Tasmanicosa* or *Hogna* in laboratory arenas. Additionally, I describe variables of the food web that influence predation outcomes, such as prey encounters, prey vulnerability, and prey nutrient content. This chapter is coauthored by Dr. Phil Taylor, Dr. Mary Whitehouse and Dr. Shawn Wilder (Oklahoma State University). S.M. Wilder and I designed the experiments. S.M. Wilder taught me the methodology for estimating lipid and protein content in arthropods. I collected and analysed the data, and wrote the manuscript. P.W. Taylor, M.E.A. Whitehouse contributed to experimental design, and revisions on manuscript drafts.

Chapter 6: A killer killed? Wolf spider mortality after feeding on ground crickets. This chapter arose from an unexpected observation in Chapter 5, whereby some wolf spiders died after eating *Teleogryllus,* suggesting that *Teleogryllus* can be toxic to wolf spiders. As there are no records of Orthoptera toxicity to spiders, I here assess whether *Teleogryllus* toxicity is endogenous or environmentally acquired, and whether this toxic effect is transient or can be observed across years. This chapter is coauthored by Dr. Phil Taylor and Dr. Mary Whitehouse. I designed the experiments, collected and analysed the data, and wrote the manuscript. P.W. Taylor, M.E.A. Whitehouse contributed to experimental design, and

revisions on manuscript drafts. This chapter is formatted as a short communication for *Journal of Applied Entomology.*

In the thesis discussion, I assess how the key findings of each chapter address the questions posed in Table 1, and discuss the impact of wolf spiders on biological control on *Helicoverpa* spp., as well as the limitations and future directions from each methodology.

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CHAPTER 1: Influence of crop management and environmental factors on wolf spider assemblages (Araneae: Lycosidae) in an Australian cotton cropping system.

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Abstract

Wolf spiders (Lycosidae) are the most abundant ground hunting spiders in Australian cotton (*Gossypium hirsutum* L.) agroecosystems. These spiders have the potential to control pest bollworms, *Helicoverpa* spp. (Lepidoptera: Noctuidae) in minimum-tilled fields. A study was carried out during a wet (2011/2012) growing season in Narrabri, New South Wales, Australia, to determine how different crop rotations and tillage affect wolf spider assemblages in cotton fields. Spider abundance and species richness did not differ significantly between simple plots (no winter crop) and complex plots (cotton-wheat *Triticum aestivum* L.-vetch *Vicia benghalensis* L. rotation). However, wolf spider biodiversity, as expressed by the Shannon-Weaver and Simpson's indices, was significantly higher in complex plots. Higher biodiversity reflected a more even distribution of the most dominant species (*Venatrix konei* Berland, *Hogna crispipes* Koch, *Tasmanicosa leuckartii* Thorell) and the presence of more rare species in complex plots. *Tasmanicosa leuckartii* was more abundant in complex plots and appears to be sensitive to farming disturbances, whereas *V. konei* and *H. crispipes* were similarly abundant in the two plot types, suggesting higher resilience or recolonizing abilities. The demographic structure of these three species varied through the season, but not between plot types. Environmental variables had a significant effect on spider assemblage, but effects of environment and plot treatment were overshadowed by the seasonal progression of cotton stages. Maintaining a high density and even distribution of wolf spiders that prey on *Helicoverpa* spp. should be considered as a conservation biological control element when implementing agronomic and pest-management strategies.

KEY WORDS: Agroecosystem, abundance, richness, minimum-tillage, biological control.

Introduction

With recent increased interest in environmentally benign agricultural production systems, conservation biological control has arisen as a sustainable component of integrated pest management (IPM). Conservation biological control involves manipulating the environment to enhance the survival, fecundity, longevity and behavior of natural enemies to increase their effectiveness in pest control (Landis et al. 2000). In annual crops such as cotton, fallow fields without vegetation between growing seasons offer few resources for beneficial arthropods, forcing them to overwinter in vegetation outside of the fields. Moreover, intensive tillage and flood irrigation in cotton cropping systems can make the ground strata of cotton fields a hazardous area for colonization by beneficial arthropods due to repeated disturbance (Bishop and Blood 1980, Oraze et al. 1988, Sharley et al. 2008). Arthropod diversity, including natural enemies, can be enhanced by crop rotation and minimum-tillage practices that were originally implemented for agronomic benefits (Nkem et al. 2002). Farming practices that reduce disturbance could contribute to conservation biological control and therefore should be considered when designing IPM strategies.

In cotton systems, two practices are becoming widespread: minimum-tillage and crop rotation. Minimum-tillage involves the maintenance of permanent beds, with only occasional furrow delving to build up the ridges (NSW Department of Primary Industries 1998). In cotton systems, crop rotations may include planting wheat or legumes during the winter between summer cotton-growing seasons, or in alternating years during the cotton-growing season. Although crop rotation and minimum-tillage were originally implemented for agronomic benefits (House and Stinner 1983, House and Parmelee 1985, Hulugalle et al. 1997, Nkem et al. 2002), these practices can provide natural enemies with a stable refuge for overwintering and reproduction, and complementary food sources within the fields (Glueck and Ingrisch 1990, Sunderland and Samu 2000, Ishijima et al. 2004, Oberg and Ekbom 2006,

Rencken 2006). This continuous vegetation cover can protect ground–dwelling arthropods from extreme variations in temperature, humidity and radiation and can thereby increase arthropod biodiversity between cropping cycles (House and Parmelee 1985, Tillman et al. 2004, Brevault et al. 2007). This is important because high densities of natural enemies early in the crop growing season can suppress emerging pests and then restrict pest abundance throughout the growing season (Bishop and Blood 1981, Johnson et al. 2000, Marasas et al. 2001). However, minimum-tillage practices may conflict with pest management practices that require deep mechanical disturbance of the soil beds.

Larvae of the noctuid moths *Helicoverpa armigera (*Hübner) and *Helicoverpa punctigera* (Wallengren) are major pests of Australian cotton crops, historically costing millions of dollars annually in crop loss and operations (Fitt 1989). In 1996, genetically modified 'Bt cotton' was introduced specifically to target *Helicoverpa* spp. (Bt cotton contains genes of *Bacillus thuringiensis* bacteria that code for a protein that perforates the gut lining of *Helicoverpa* larvae; Van Rie 2000). Following the introduction of Bt cotton, pesticide use decreased markedly, resulting in an increase in the abundance and diversity of beneficial natural enemies, correspondingly increasing their impact and importance in IPM (Johnson et al. 2000, Lloyd et al. 2008).

To inhibit the emergence of resistance to the Bt toxins in *Helicoverpa*, growers must comply with a Resistance Management Program (RMP). This includes 'pupae busting' which involves cultivating the top 10 cm of soil in the plant line and furrows after harvest to destroy the escape tunnels of overwintering pupae that could carry Bt resistance genes to the next season (Rourke 2002, Duffield 2004, Lloyd et al. 2008, Whitehouse et al. 2009b, Maas 2013). However, minimum-tillage may conflict with pupae busting because it may not provide enough disturbance to effectively destroy pupae or their escape tunnels. Therefore, practices

that enhance predator diversity or abundance through conservation biological control could offer an alternative means of targeting Bt-resistant *Helicoverpa* in minimum tillage systems.

Spiders are important predators in many agroecosystems (Nyffeler and Benz 1987, 1988) and make up a large proportion of the ground predator assemblage in cotton crops (Bishop and Blood 1980, Whitehouse et al. 2005). Minimum tillage could enhance spider abundance by enhancing their habitat (Sunderland and Samu 2000). Additionally, vegetation cover and composition can influence biodiversity of ground spiders (Rypstra et al. 1999, Glueck and Ingrisch 1990, Ishijima et al. 2004, Motobayashi et al. 2006, Rypstra and Marshall 2005, Bowden and Buddle 2010). Plant stubble that rises above the soil level also serves as a refuge that allows spiders to avoid flooding by climbing up into vegetation (Lambeets et al. 2008). Because spiders are abundant as well as taxonomically and morphologically diverse predators of *Helicoverpa* (Johnson et al. 2000) it is important to understand the effects of crop management systems on spider biodiversity and life history (Bowden and Buddle 2010).

Wolf spiders (Lycosidae) are the most abundant family of ground hunting spiders in Australian cotton fields throughout the year (Pearce et al. 2004, Whitehouse et al. 2009a). Wolf spiders do not build webs, and are the largest ground-hunting spiders normally found in Australian cotton fields (Whitehouse et al. unpublished data). As such, they are more likely than other smaller spiders to attack and subdue the large final instar *Helicoverpa* larvae that descend from the plant to pupate under the soil (Nentwig and Wissel 1986). Wolf spiders are known to attack *Helicoverpa* larvae (Johnson et al. 2000) and they have also been observed to attack emerging moths (Rendon, unpublished data).

To better understand the links between agronomic practices in cotton fields and wolf spider populations, we here investigate: 1) differences in abundance, richness, and overall biodiversity of wolf spiders in fields with different crop rotations and tillage systems; 2)

which environmental variables best explain variation in wolf spider assemblages; and 3) the life history of wolf spiders in the cotton agroecosystem across the cropping season.

Materials and Methods

Field site and crop management

The study plots were located within a long-term experiment that has been running since 2005 at the Australian Cotton Research Institute (ACRI) near Narrabri, New South Wales, Australia (30ºS, 149ºE). Narrabri has a sub-tropical semi-arid climate (Kottek et al. 2006) and experiences four distinct seasons with a mild winter and a hot summer. The hottest month is January (mean daily maximum of 35ºC and minimum of 19ºC) and July the coldest (mean daily maximum of 18ºC and minimum of 3ºC). Cotton is usually planted in October, and harvested April/May. Mean annual rainfall is 593 mm (Hulugalle et al. 2012a), although the year that this study was conducted was wetter than normal (annual rainfall for 2011= 757.40 mm, 2012= 619.40 mm).

The long-term experiment was organized as a randomized complete block design with three replicates of six treatments. Individual plots were 165 m long and 20 rows wide. The rows (beds) were spaced at 1-m intervals with vehicular traffic restricted to the furrows. We sampled from two cropping systems, viz. 'simple plots' (summer cotton - winter fallow), and 'complex plots' (summer cotton / winter wheat – vetch rotation / with retained wheat stubble). The soil beds of the simple plots remained bare until cotton sowing (late October), and were cultivated between rows before planting, and once again in the middle of the growing season (January). Simple plots received nitrogen as urea broadcast after sowing cotton. In the complex plot, stubble from the winter wheat was left standing all summer, enabling the winter vetch to be planted directly into the stubble. Vetch, which is a prostrate, leguminous crop, was killed during or just prior to flowering through a combination of mowing and contact

herbicides (Hulugalle et al. 2012a). Remains of the vetch were retained *in situ* as mulch into which the cotton was sown. All plots were furrow irrigated at a rate of 1 ML/ha (= 100 mm) of water when rainfall was insufficient to meet evaporative demand.

Specimen collection

Pitfall traps were used in this study to sample ground spider populations. Although they have their limitations (Adis 1979, Standen 2000, Topping and Sunderland 1992) pitfall traps provide continuous sampling across all sites, catch a high number of spiders, are not subject to observer bias (Brennan et al. 1999, Cheli & Chorley 2010) and enable direct comparisons to other studies of spider communities in cotton (Dippenaar-Schoeman et al 1999, Whitehouse et al. 2009b). To minimize potential edge effects, the outer 4 rows on each side of each plot, and the outer 2 m at the ends of the plots were excluded from sampling, such that only the inner 12 rows were sampled. In each plot, 20 pitfall traps were randomly distributed by dividing the fields into a grid with each cell measuring 10 m x 1 m, and then assigning pitfall trap locations using a random numbers table. A total of 120 pitfall traps were used, distributed across three replicates of the two field treatments. Each pitfall trap consisted of a clear plastic cup fitted inside a PVC pipe that measured 9 cm internal diameter by 12 cm height, covered by a 15 cm diameter white plastic plate positioned with toothpicks 4 cm above the trap to prevent rain and debris from entering. Each trap was filled with 50 ml of 70% ethanol, and was left open for six nights; after the third night the trap was refilled with ethanol. At the end of the sampling period, the contents of each trap were collected, sorted, and preserved in 70% ethanol for identification in the laboratory. The wolf spiders collected from the 20 pitfall traps in each plot were combined in the analysis because of sparse data in some traps. Adult wolf spiders were identified to species according to Framenau and Vink (2001), Framenau (2002, 2005, 2006a, 2006b, 2007, 2010), Framenau et al. (2006), and Yoo and Framenau (2006). Since juveniles lack developed genitalia and accessory structures used

in species identification, they were only classified to family (Lycosidae). Juveniles with cephalothorax width less than 1 mm were classified as spiderlings.

There were nine sampling dates in the 2011/2012 growing season, scheduled according to degree days (Constable 1988) and cotton phenology: winter crop (22 July), pre-planting (10 October), cotton emergence (10 November), 5th leaf (5 December), 1st square (20 December), 1st flower (12 January), peak flower (22 February), open boll (12 March), and 60% bolls open (22 April).

Analysis

Three indicators were analyzed: abundance, richness, and biodiversity. Abundance was calculated as the total number of individual spiders collected in simple and complex plots. Richness was calculated using two parameters: the total number of spider families collected, and species-accumulation curves. Species-accumulation curves depict the rate at which additional species are encountered as more individuals are collected. Species-accumulation curves were calculated using EstimateS, using a general binomial mixture model, according to Colwell et al. (2004). Alpha biodiversity, which describes the overall diversity within a habitat, was estimated using Shannon-Weaver ('H') and Simpson indices ('D') (Magurran 2004). Indices vary in their sensitivity to rare and abundant species. The Shannon-Weaver and Simpson's indices are from different ends of the Renyi diversity index family, where the Shannon-Weaver index is more sensitive to rare species (richness), and Simpson's index is more sensitive to common species (evenness) (Tothmeresz 1995). The Shannon-Weaver index is defined by the formula $H' = - \Sigma p_i * \ln p_i$, where p_i is the proportion of individuals found in the *i*th species (Shannon and Weaver 1949). Simpson's index is defined by the formula $D = \Sigma n_i(n_i-1)/N(N-1)$, where n represents the number of individuals of the *i*th species, and N the total number of individuals (Simpson 1949). The values for Simpson's index were modified (1- D), so that the units increase with an increase in diversity.

The biodiversity indices were calculated using EstimateS (Colwell 2006), and all spiders that could not be identified to species were excluded from the biodiversity index analysis. A repeated-measures ANOVA and post-hoc LSD with field treatment as a between-subjects factor was used to test whether abundance of spiders, species richness, Shannon-Weaver index, or Simpson's index differed between simple and complex plots across the cotton growing season (referred to hereafter as 'cotton stage'). Data that violated the sphericity assumption were analyzed using a Greenhouse-Geisser transformation (Tabachnick and Fidell 2007). The sphericity assumption states that the variances of the different values in a repeated-measures test are equal across the groups, and it is equivalent to the homogeneity of variance assumption in independent-samples ANOVA; the Greenhouse-Geisser transformation adjusts the degrees of freedom to decrease the chance of a Type-I error (Greenhouse and Geisser 1959). A redundancy analysis (RDA) was performed to test for differences between the field treatments in spider community assemblages. Redundancy analysis is a repeated measures multivariate method designed to test treatment effects that change over time (Leps and Smilauer 2003). A log-linear multiway frequency analysis was performed to test for differences in the relative abundance of adult males and females of the most dominant species, and juveniles and spiderlings that could not be identified to species between field treatments over time.

Local environmental data were obtained from the ACRI weather query database (http://www.weather.cottassist.com.au//) for each of the sampling dates. The following variables were selected: Maximum temperature (◦C), total radiation (MJ/m^2), maximum relative humidity (%), and cumulative rainfall in the past 10 days (mm). A canonical correspondence analysis (CCA) was performed to analyze the effects of these environmental factors, field treatment, and cotton stage on the species assemblage. The ordination analyses (RDA and CCA) were calculated using CANOCO (Ter Braak and Smilauer 2009). All other statistical analyses were carried out using SPSS version 20 (IBM 2011).

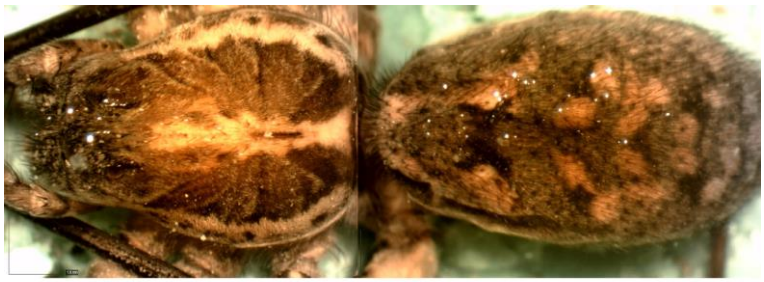
Results

Spider assemblage

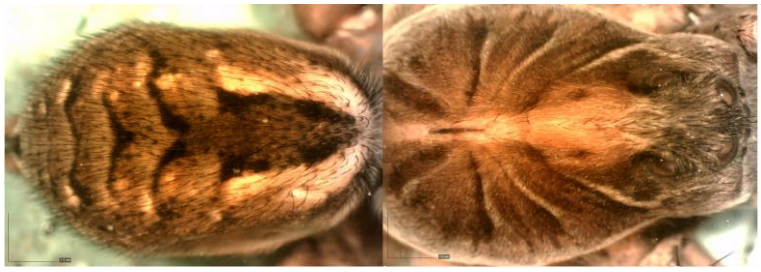
The three most common wolf spider species found were *Venatrix konei* Berland, *Hogna crispipes* Koch and *Tasmanicosa leuckartii* Thorell (Figures. 1a, b, c, Figure 2). There was no significant difference in the abundance of *Venatrix konei* or *Hogna crispipes* between simple and complex plots (*V. konei*: mean abundance ± S.D, simple plots = 81.00 ± 14.17, which was 41.9% of the wolf spider community; complex plots = 95.67 ± 17.21, 43% of spider community; ANOVA: F = 2.89 df = 1, 53; P = 0.098. *H. crispipes* : mean abundance ± S.D, simple plots = 69.66 ± 11.67, 36% of spider community; complex plots = 73.67 ± 7.57, 33.1% of spider community; ANOVA: F =0.21; df = 1, 53; P = 0.649). Abundance of *Tasmanicosa leuckartii* was significantly higher in complex plots than in simple plots (mean abundance, simple plots = 20.00 ± 4.58, 10.3% of spider community; complex plots = 29.33 ± 4.72, 13.2% of spider community; ANOVA: F = 5.93; df = 1, 53; P = 0.020). One species, an undescribed species of *Hogna*, was unique to the simple plots, and three species, *Venatrix speciosa* Koch, *Artoria victoriensis* Framenau, and an undescribed species of *Artoria*, were unique to the complex plots (Figure 2). All the wolf spiders that could not be identified to species (N = 950) were juveniles and classified as "Lycosidae sp". Although there was a significant trend towards higher association of *T. leuckartii* with complex plots and a suggestive trend for *V. konei* to be associated with complex plots, redundancy analysis detected only a non-significant trend in differences between the spider community assemblages of simple and complex plots (Monte Carlo tests, F = 3.72; P = 0.082).

a)

b)



c)



Figures 1a-1c. Picture of *Venatrix konei* (adult male), *Hogna crispipes* (adult female), and *Tasmanicosa leuckartii* (adult female), the three most common species of wolf spiders in the cotton fields.

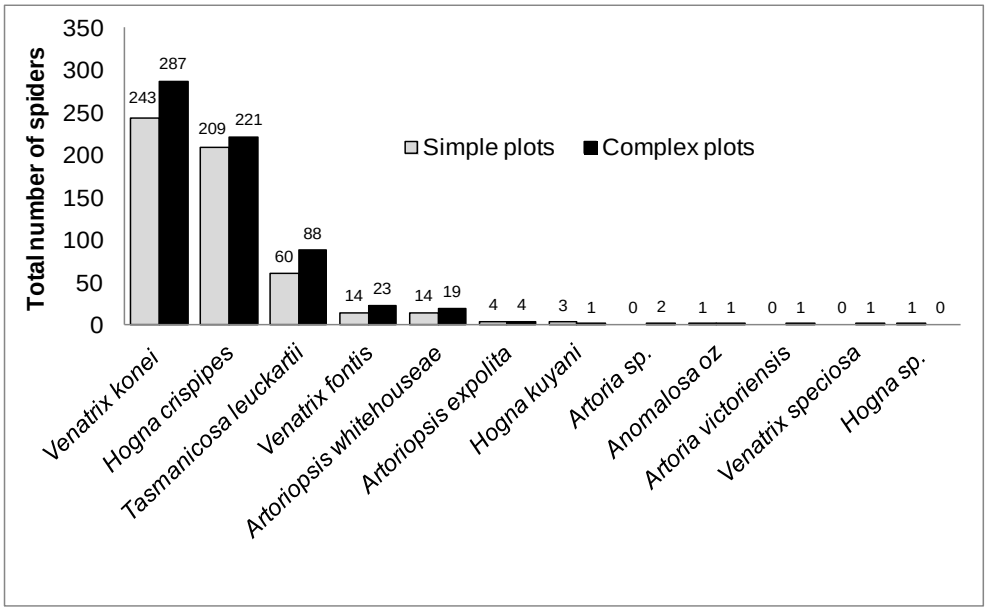


Figure 2. Histogram of overall abundance of wolf spider species in cotton fields.

Phenology

Since it is difficult to identify juveniles and spiderlings to species, the distribution of juvenile wolf spiders was analyzed separately from adult males and females of the most dominant species (Figures 3, 4). For *V. konei*, distribution of sexes varied significantly through the season, but not between simple and complex plots (log-linear analysis, Sex*Cotton stage interaction: F = 2.35; df = 8, 68; P= 0.026; Sex*Plot treatment interaction: F= 0.01; df = 1, 68; P= 0.92). Males tended to increase in abundance more rapidly in complex plots after cotton emergence. The abundance of males decreased in the middle of the season to a similar extent in both plot treatments. Female abundance increased after cotton emergence, and remained uniform across the season in both plot treatments. For *H. crispipes*, distribution of sexes varied significantly through the season, but not between plot treatments (log-linear analysis, Sex*Cotton stage interaction: F= 2.96; df = 8, 76; P = 0.006; Sex* Plot treatment interaction: F= 1.09; df = 1, 76; P = 0.30). Male abundance remained lower than female abundance early in the season, and increased rapidly at peak flower. Likewise, for *T.*

leuckartii, distribution of sexes varied significantly through the season, but not between plot treatments (log-linear analysis, Sex*Cotton stage interaction: F= 2.35; df = 8, 80; P = 0.0026; Sex* Plot treatment interaction: F = 0.09; df = 1, 80; P= 0.76). Female abundance peaked at cotton emergence, whereas male abundance remained more uniform through the season. Distribution of juvenile and spiderling wolf spiders varied significantly through the season, and there was a non-significant trend for spiderlings to be more abundant in complex plots (log-linear analysis, Spider stage*Cotton stage: F = 11.94; df = 8, 76; P = 0.001; Spider stage* Plot treatment: F = 3.53; df = 1, 76; P = 0.06). In both plot treatments, juveniles were more abundant early in the season than spiderlings, and spiderling abundance increased rapidly after first flower (Figure 4).

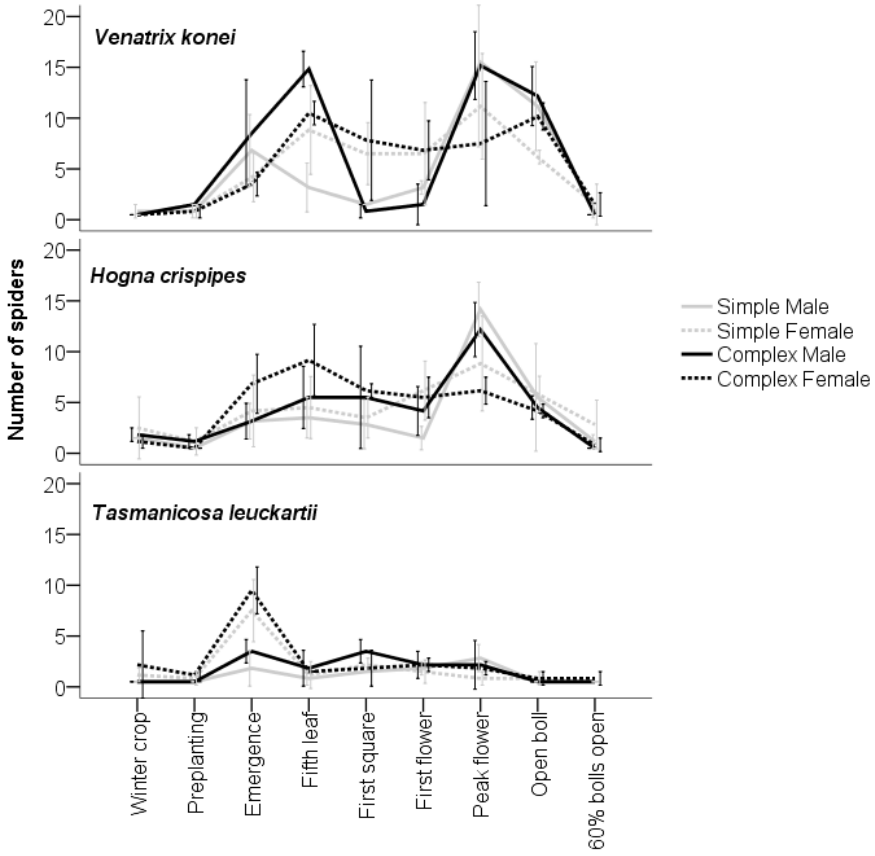


Figure 3. Distribution of adult males and females of the three most dominant species in simple and complex plots over the cotton season. Error bars represent 1 SE.

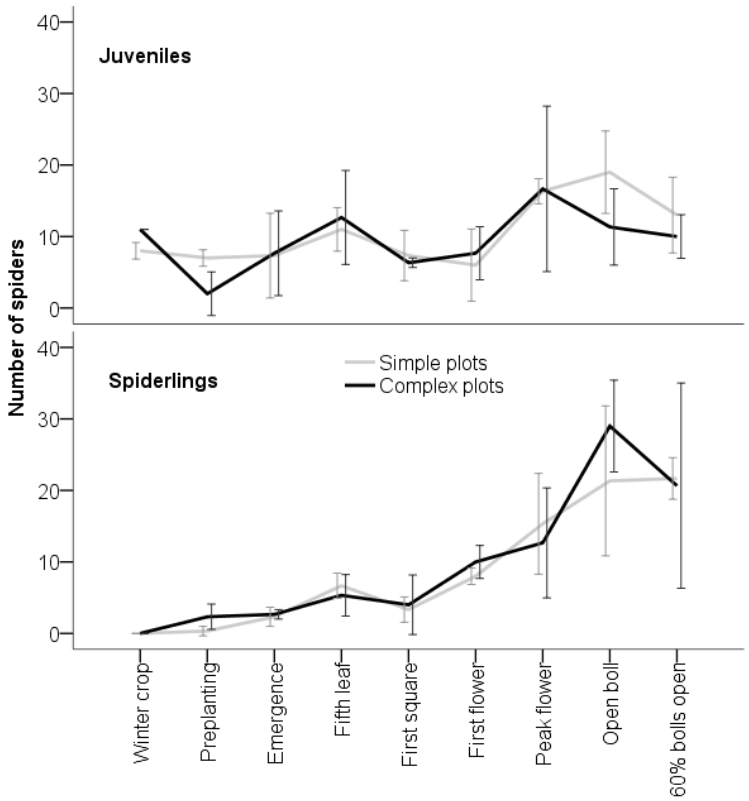


Figure 4. Distribution of wolf spider juveniles and spiderlings on simple and complex plots over the cotton season. Error bars represent 1 SE.

Variations in abundance, richness and biodiversity indicators

Over the cotton stages, a total of 1051 wolf spiders of 10 species were collected in the simple plots, and 1145 spiders of 12 species were collected in the complex plots. Simple and complex plots did not differ significantly in spider abundance (number of spiders) (repeated-measures ANOVA, $F = 0.86$; $df = 1, 4$; $P = 0.405$), and there was no significant interaction between plot treatment and cotton stage ($F = 1.77$; $df = 8, 32$; $P = 0.119$). Spider abundance did vary significantly with cotton stage ($F = 40.51$; $df = 1.9, 7.8$; $P < 0.001$). Mean abundance of spiders in both plot treatments was highest at peak flower (simple plots = 86.66 ± 10.01, complex plots = 75.00 ± 27.22; mean ± S.D) and lowest at pre-planting (simple plots = 9.00 ± 2.64, complex plots = 8.00 ± 1.00; mean ± S.D; Figure 5).

Simple and complex plots did not differ in species richness (number of species) ($F = 1.50$; $df = 1, 4$; $P = 0.218$). Species richness did however vary with cotton stage ($F = 7.60$; $df = 2.3, 9.3$; $P = 0.009$) although these changes followed the same trajectory in the two plot treatments ($F = 2.15$; $df = 2.3, 9.3$; $P = 0.166$). Species-accumulation curves (Figure 6) indicate that complex plots contain more spider species than simple plots at any given sample size.

Both biodiversity indices found complex plots to be significantly more diverse overall than simple plots ($D_{simple\ plots} = 0.56 \pm 0.01$, $D_{complex\ plots} = 0.68 \pm 0.013$; $F = 41.53$; $df = 1, 4$; $P = 0.03$; $H_{simple\ plots} = 0.98 \pm 0.2$, $H_{complex\ plots} = 1.11 \pm 0.2$, $F = 14.86$; $df = 1, 4$; $P = 0.018$). The Shannon-Weaver index also found that biodiversity also changed through the cotton stages (Shannon-Weaver index $F = 3.34$; $df = 8, 32$; $P = 0.007$; Simpson's index $F = 1.91$; $df = 8, 32$; $P = 0.092$) although interactions between cotton stage and plot treatment indicate that the effects of cotton stage on biodiversity differed in the two plot treatments (Simpson's index $F = 4.02$; $df = 8, 32$; $P = 0.020$; Shannon-Weaver index $F = 2.15$; $df = 8, 32$; $P = 0.059$). Biodiversity in simple plots was significantly lowest at 'pre-planting' according to Shannon-Weaver index, and at 'pre-planting' and 'winter crop' according to Simpson's index (LSD all $p < 0.05$; Figure 7). Biodiversity in complex plots did not show a significant peak in any cotton stage according to Shannon-Weaver index, but biodiversity in complex plots is significantly higher in 'pre-planting' according to Simpson's index. The greatest difference in diversity between simple and complex plots, using both indices, occurred between pre-planting and emergence, when diversity was much higher in complex plots (T-test, Simpson's index $F= 5.741$; $df = 4$; $p= 0.017$; Shannon-Weaver index $F = 9.413$; $df = 4$; $p = 0.007$; Figure 7).

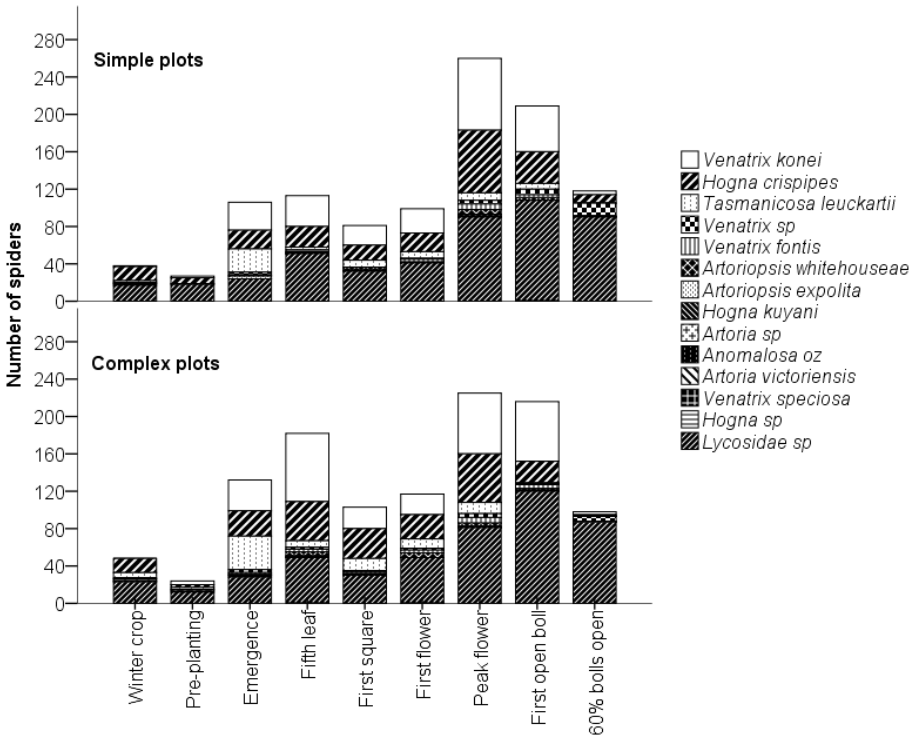


Figure 5. Abundance and species richness in simple and complex plots through the cotton stages.

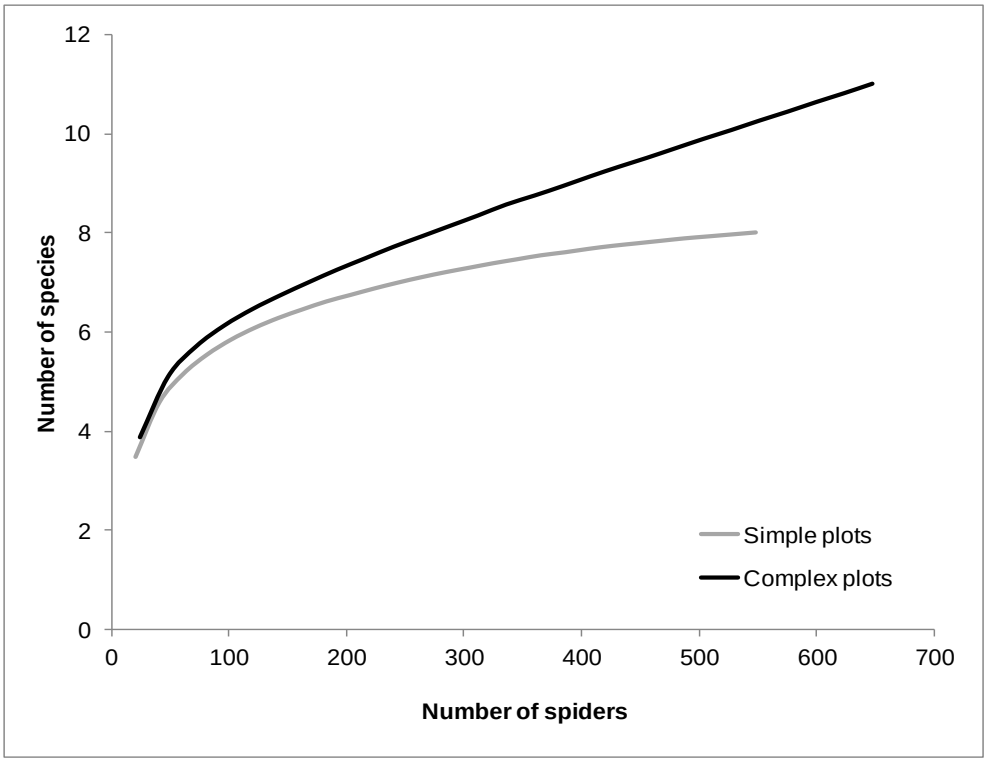


Figure 6. Species-accumulation curve of adult wolf spiders caught in simple and complex plots.

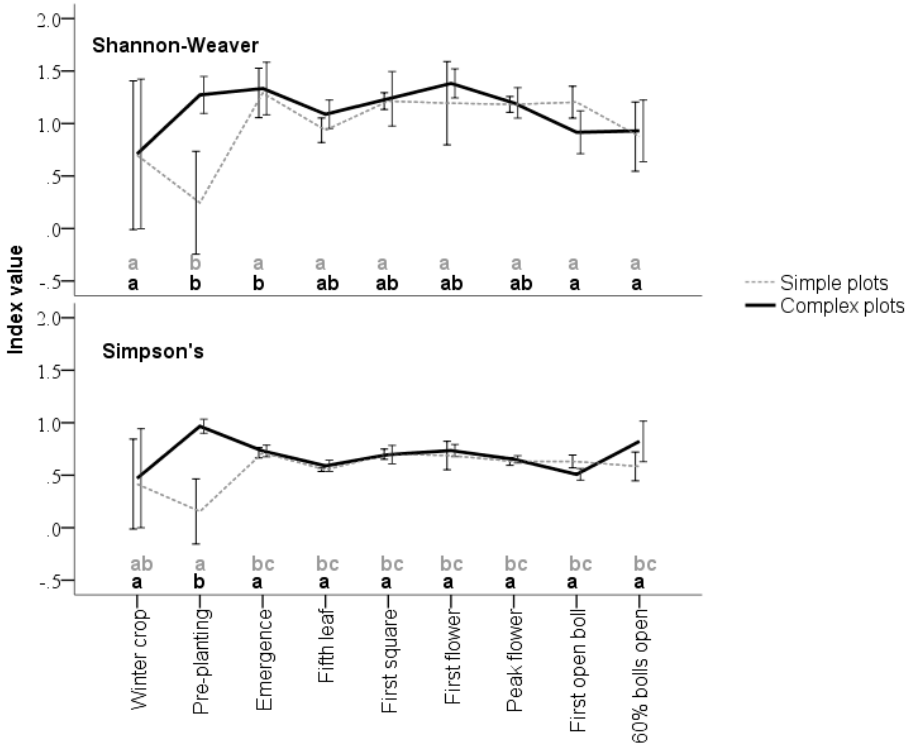


Figure 7. Biodiversity (Shannon-Weaver and Simpson's index) of wolf spiders in simple and complex plots through the cotton stages. Letters indicate differences between cotton stages (but not plots) according to Least Square Differences. Error bars represent 1 SE.

Environmental factors

A canonical correspondence analysis (CCA) showed that all the environmental variables combined (maximum temperature (◦C), total radiation (MJ/m^2), maximum relative humidity (%), cumulative rainfall in past 10 days (mm), cotton stage, and plot treatment) explained 16% of the variance in spider assemblage (Monte Carlo test, F = 9.26; P = 0.002; Figure 8). 'Cotton stage' had the strongest effect on spider assemblages (as it is most strongly aligned with the x-axis) overshadowing variance explained by plot treatment. 'Humidity' was more closely associated with simple plot assemblages than those of complex plots, while 'rainfall' was more closely associated with complex plots, although these relationships are weak. As these variables are most closely aligned with the y-axis, 'maximum temperature'

and 'radiation' had similar affects on the species assemblages in both simple and complex plots. The 2011/2012 cotton-growing season had higher rainfall than previous seasons (total rainfall 2008/2009 = 449.59 mm, 2009/2010 = 520.60 mm, 2010/2011 = 542.39 mm, 2011/2012 = 782.59 mm). The distributions of *H. crispipes*, *V. fontis*, and *A. whitehouseae* were not strongly influenced by any of the environmental variables tested, while *A. victoriensis* was most closely associated with 'maximum temperature' and 'radiation' (but this could be partially due to its scarcity). *Artoriopsis expolita* and *T. leuckartii* were more closely associated with complex plots than simple plots.

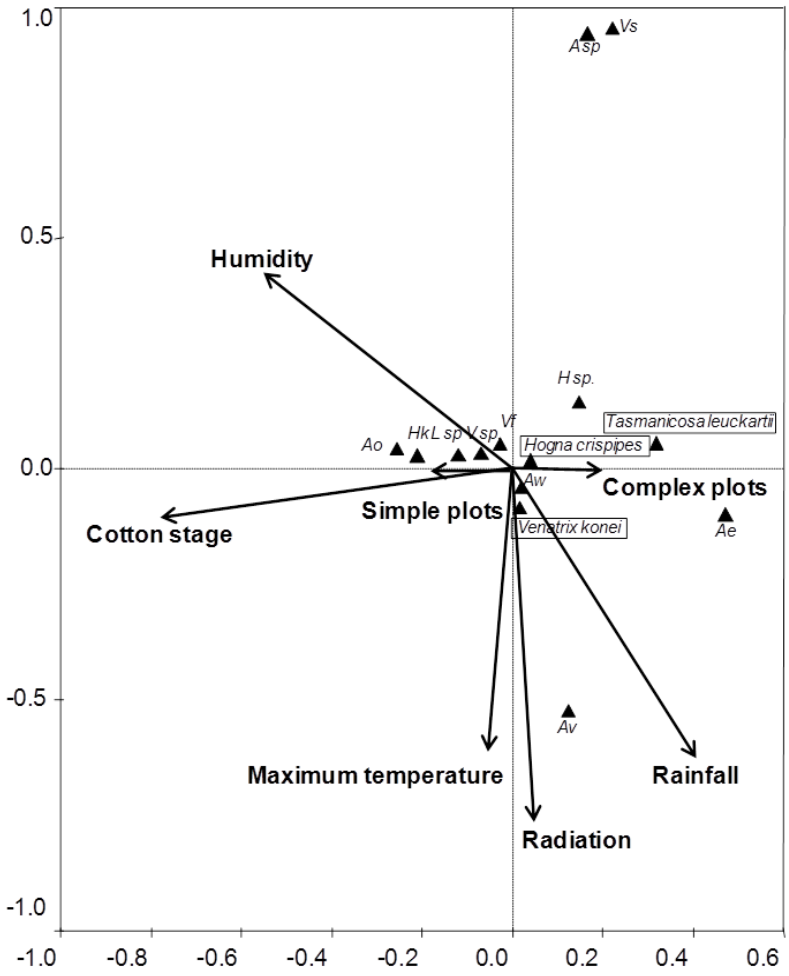


Figure 8. Direct ordination analysis (CCA) of spider species in relation to field treatment and environmental variables (three most common species are in a square; abbreviations: Vf = *Venatrix fontis*, Aw= *Artoriopsis whitehousease*, Ae= *Artoriopsis expolita*, Hk= *Hogna kuyani*, Ao= *Anomalosa oz*, A sp= *Artoria sp*., H sp= *Hogna sp*., Av= *Artoria victoriensis*, Vs= *Venatrix speciosa*).

Discussion

Wolf spider diversity patterns

In Australian cotton agroecosystems, moisture retention is enhanced in untilled fields (Hulugalle et al. 2012b). As the 2011/12 season was more wet than usual, the greater moisture retention capacity of complex plots would have been largely redundant, and therefore have less effect on the invertebrate community. Nevertheless, while the absence of tillage and incorporation of winter crops did not affect the overall abundance (total number of individuals) or richness (total number of species) of wolf spiders through the cotton-growing season, it did affect overall wolf spider biodiversity. Biodiversity was higher in complex plots, where common species were more evenly distributed (as indicated by the Simpson index) and contained more rare species (as indicated by the Shannon Weaver index) than simple plots.

While the present study did not find wolf spiders to be significantly more abundant in untilled plots than in tilled plots (as has been reported in rice paddies; Ishijima et al. 2004, Motobayashi et al. 2006), it was in accord with another study reporting that spider populations are more even in minimum-tilled fields (Glueck and Ingrisch 1990). Likewise, in the same experimental plots, invertebrate abundance did not vary between tilled and untilled plots (Hulugalle et al. 1997). Spider diversity tends to increase with extent of plant cover and bushes compared to bare soil crust, presumably because vegetation provides higher structural complexity and multiple microhabitats to sustain a more even spider community (Whitehouse et al. 2002). Since dominant species are more evenly distributed, it is possible that complex plots had more niches to support a more even community of wolf spiders.

The steep increase in biodiversity of complex plots between winter crop and pre-planting reflects a higher number of species present, despite overall abundance being similar.

Even though there was not a significant difference in species richness between plot treatments, the lack of asymptote in the species-accumulation curve in the complex plots (Figure 6) suggests that there are more species present that have not been collected yet. In addition, new species begin to appear in simple plots later in the season than in complex plots. The results suggest that complex plots have a more even distribution of spider species because they provide winter refuges.

Phenology of dominant species

While overall wolf spider abundance did not differ significantly between simple and complex plots, one species of wolf spider, *Tasmanicosa leuckartii* (formerly *Lycosa*), was more abundant in complex plots. Since *T. leuckartii* is the largest wolf spider in these fields (mean ± se cephalothorax width of adult males is 0.77 ± 0.06 cm, and of females is 0.81 ± 0.07 cm; Rendon et al., unpublished data), it might act as a stabilizing species, preying on and outcompeting smaller wolf spider species and preventing the dominance of a single species. *Tasmanicosa leuckartii* may be more abundant in the complex plots because they inhabit permanent burrows in the soil (Humphreys 1978) and these burrows would be destroyed by the mechanical action of tilling in the simple plots.

Venatrix konei (= *V. goyderi*) is commonly associated with wetlands, riparian areas, irrigation ditches and springs that have been exposed to significant disturbance such as grazing, dredging or flooding, and it tolerates a wide climatic range (Framenau et al. 2006). Most adults of this species in museum collections around Australia have been collected between September and January (Framenau and Vink 2001) but in this study there was a peak of abundance for this species during February (peak flower) just after flooding. Abundance of males varied more than abundance of females through the season, suggesting that male activity patterns are more seasonal (for example when they search for mates) or that males are more sensitive than females to environmental variability. The fact that *V. konei* was the

dominant species in both simple and complex plots, and that there were no significant differences in abundance between the plot types, suggests that this species has a high colonizing ability, and disperses quickly through diverse habitats.

Hogna crispipes is usually found on the edges of springs and in the ephemeral wet zone beyond the permanent vegetated wetland (Framenau et al. 2006). Like *V. konei*, the highest abundance of *H. crispipes* was during peak flower. Both species have a high dispersal ability and widespread distribution in Australia, and recover quickly from disturbances (Framenau et al. 2006). Coexistence of these two species in cotton fields suggests resource partitioning (Schoener 1974); these two species may target different prey, or have different daily activity patterns that reduce competition (Marshall et al. 2002). The lack of a strong response to the plot treatments by *H. crispipes* and *V. konei* may also indicate that their home ranges could be larger than the width of the plots, and the small size of the plots themselves may have acted as a source-and-sink for one another (Perovic 2009).

Information on the prey preferences of the common species in this study could provide insights on the community structure that would best control *Helicoverpa*. For example, the prey preferences of *V. konei* are unknown, but due to its size (mean cephalothorax width = 2.1mm; Framenau 2006b) it probably targets prey that is smaller than final instar *Helicoverpa* larvae. However, *H. crispipes*, *T. leuckartii*, and *H. kuyani* do attack late instar *Helicoverpa* larvae (Rendon et al., unpublished data). So rather than aiming for a community with greater species richness or overall abundance, a community that maintains an even abundance of these species could be more effective for controlling *Helicoverpa*.

All life stages of wolf spiders were found in cotton fields throughout the season, even when the ground of the simple plots was bare. Early in the season there was a low abundance of spiderlings and adults, while later in the season there was a high abundance of spiderlings and juveniles and a low abundance of adults. The low densities of adults after the cotton

season and during winter and a single peak of spiderlings, suggests that wolf spiders in this ecosystem are univoltine. Juveniles and spiderlings were similarly abundant in simple and complex plot treatments early in the season, but adults of more species (richness) were present in complex plots, suggesting that juveniles of more species use complex fields as refuges between cropping cycles. Difficulty in identifying juveniles to species limited the description of wolf spider phenology in this study. That is, it was not possible to determine if phenology of spiderlings overlapped between species, or if species were staggered across the season. Regardless, a combination of juveniles and adults throughout the cotton season maximizes the potential of the overall spider community to target a range of pests.

Environmental factors

A combination of all the environmental variables significantly explained variance in spider assemblage. According to Mutshinda et al. (2009) environmental stochasticity is a major driver for biodiversity patterns to the extent that species assemblages can be more influenced by environmental variables than by associations and interactions between species. Progression through the cropping season (cotton stage) had the strongest influence on spider assemblage, overshadowing the effects of farming practices.

The results of this study coincide with a season of higher than average rainfall. Rainfall had a greater influence on spider assemblages in complex plots than simple plots (Figure 8). Overall spider abundance decreased mid-season at first square and first flower (Figure 5), which coincided with a period of heavy flooding in the cotton fields, but there was a trend for spider abundance to be higher in complex plots during this period (between the fifth leaf and first flower stage). Spiders use plant stubble to climb above the soil when the ground is flooded (Lambeets et al. 2008). Complex plots have more vegetation that stands above flooded grounds, while simple plots get completely flooded. During periods of heavy rainfall spiders may leave simple plots, while spiders can remain on complex plots by

climbing above the ground onto plant stubble, thereby maintaining the spider assemblages of complex plots during surface flooding. Future studies can focus on examining how the patterns of abundance, richness and biodiversity change on drier years in minimum-tillage cotton fields.

Implications for pest control

Before cotton was planted, complex plots with reduced tillage contained a more diverse wolf spider community than simple plots, characterized by more evenness among dominant species and more rare species. The most likely reason for this difference is the structural complexity added by stubble retained in the complex plots providing a more stable and diverse microhabitat. With a more diverse range of predators established early in the season, complex plots are more likely to control a greater range of pest species as they colonize the crop, including *Helicoverpa* moths emerging from overwintering.

The ability of predator assemblages to control specific pests, such as *Helicoverpa*, depends on interactions within the community's food web (Snyder and Ives 2003). Results here suggest that complex plots provide more microhabitats, thereby facilitating tolerance between the predators that are expected to be most effective for control of *Helicoverpa*.

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CHAPTER 2: Ecological and molecular approaches for assessing common prey and *Helicoverpa* larva consumption by wolf spiders in a Bt-cotton field

Abstract

Helicoverpa spp. (Lepidoptera: Noctuidae) are major pests of cotton, and have been largely controlled since the introduction of genetically modified 'Bt cotton' that contains insecticidal toxins from *Bacillus thuringiensis* (Bt) bacteria. However, the potential for evolution of resistance to Bt toxins remains a constant threat that can be managed by eradicating those few *Helicoverpa* larvae that survive foraging on Bt cotton. Wolf spiders (Araneae: Lycosidae) are abundant predators that inhabit the soil surface of cotton fields in Australia, and have the potential to inhibit proliferation of Bt resistance by hunting *Helicoverpa* larvae when they descend from the plant to pupate underground. By direct observation, we identified prey that can sustain wolf spider populations in Bt cotton fields. Additionally, we assessed wolf spider predation on *Helicoverpa* larvae released in a Bt cotton field, by using a molecular gut-content analysis method (ELISA) that detects the presence of antigens (IgG) used to mark *Helicoverpa* larvae. From direct observations, 3% of wolf spiders were found with prey, including an abundant ground cricket *Teleogryllus commodus,* and a Lepidoptera larva and adult. Although mark-recapture surveys revealed that spiders encountered IgG-marked *Helicoverpa* larvae released along field edges, gut-content analysis indicated that a low proportion (2.1%) of wolf spiders collected in cotton fields tested positive for the presence of IgG from marked *Helicoverpa* larvae. This might reflect that spiders do not kill *Helicoverpa* in high proportions in a field setting; however, because predation trials of spiders placed in containers in the field with *Helicoverpa* larvae revealed that the three spider species studied (*Tasmanicosa leuckartii, Hogna crispipes, Hogna kuyani*) do kill *Helicoverpa* larvae, low likelihood of spider recapture is a likely explanation for the low proportion of field-collected spiders testing positive for IgG in gut-content analysis.

Introduction

The cotton bollworms (*Helicoverpa armigera* [Hübner] and *Helicoverpa punctigera* [Wallengren], together referred to as '*Helicoverpa* spp.') are historically important pests in cotton crops *Gossypium hirsutum* L. (Malvaceae), in Australia (Fitt *et al.*, 2009), now largely controlled following the introduction of genetically modified 'Bt cotton' in 1996 (Whitehouse *et al.*, 2009b; Wilson *et al.*, 2013). Bt cotton contains genes from the bacteria *Bacillus thuringiensis* that produce proteins toxic to some Lepidoptera larvae ("Bt toxins", Van Rie, 2000). As *Helicoverpa armigera* has developed resistance to sprayed insecticides, there is ongoing concern about the potential for an increase in the frequency of alleles that confer *Helicoverpa* spp. resistance to Bt toxins (Downes & Mahon, 2012; Wilson *et al.*, 2013). To inhibit emergence and proliferation of Bt resistance, larvae, pupae and moths that have succeeded in foraging and developing in Bt cotton must be eradicated. Natural enemies are an important component of integrated pest management in cotton crops (Johnson *et al.*, 2000; Naranjo *et al.*, 2015), and may contribute to inhibiting emergence and proliferation of Bt resistance (Liu *et al.*, 2014). *Helicoverpa* spp. larvae that have survived foraging on Bt-cotton descend from the plant to pupate underground, where they are exposed to a guild of ground predators. Wolf spiders comprise an abundant group of ground predators in cotton crops (Rendon *et al.*, 2015; Whitehouse *et al.*, 2009a), and have been reported to feed on Lepidoptera larvae in cotton crop systems (Bishop, 1978; Hayes & Lockley, 1990; Johnson *et al.*, 2000). Therefore, wolf spiders in cotton crops can contribute to eradicate ground-dwelling stages of Bt-resistant *Helicoverpa* spp.

It is challenging to assess the contribution of hunting spiders to biological control of crop pests. Many methods have been employed to examine prey specificity, frequency, and timing of hunting spider predation in the field. Most studies aim to determine which and how many individual prey are killed by spiders. This is usually achieved by manipulating spider

and prey densities in confined spaces, and then estimating prey mortality by counting survivors (Greenstone, 1999). However, more reliable data of prey identity and predation rates can be obtained from extensive field observation (Tahir & Butt, 2009), or through gut-content analysis (Sunderland, 1988).

Direct field observations can assess the whole spectrum of wolf spider prey in a field setting, and allow for determination of which pest prey and alternative prey are eaten by spiders. Even though direct field observations are the most natural way to assess predatory behaviour of spiders, wolf spider predation events are rarely observed in the field. Previous studies making field observations of wolf spider predation have reported predation in 106 (4.2%) out of 2499 (Nyffeler & Benz, 1988), three (1.8%) out of 162 (Edgar, 1969), 13 (12.2%) out of 106 (Samu *et al.*, 2003), and 4.4% of 3704 (Hayes & Lockley, 1990) observations. The most commonly observed prey of wolf spiders are other spiders and insects, including Hemiptera, Diptera, Coleoptera and Collembola (Table 1). Since alternative prey can sustain high and continuous spider populations in crop fields during periods when pest densities are low (Harwood & Obrycki, 2005), a diverse range of potential prey can stabilize and improve biological control by generalist predators.

To estimate predation on specific target pests, gut-content analysis offers a more time-effective method that complements direct field observations. Molecular gut-content assays that detect prey-specific DNA fragments or specific proteins are the most commonly used methods (Greenstone, 1999; Sheppard & Harwood, 2005). However, unlike direct observations, molecular gut-content analysis only allows screening for specific target taxa, and does not enable assessment of the whole spectrum of potential prey. To determine what spiders eat (whole prey spectrum) and assess predation on *Helicoverpa* spp. (specific target pest) in a Bt-cotton field, both direct field observations and molecular gut-content analysis

should be employed, as these two complementary techniques allow to develop a broader overview of the prey consumed by spiders in a specific agroecosystem,

One technique for molecular gut-content analysis is detecting specific antigens (proteins) through enzyme-linked immunosorbent assays (ELISA). These antigens can be endogenous (i.e., naturally present in the species to be analysed, detected using monoclonal antibodies [Mab]), or exogenous. Using exogenous antigens, multiple prey can be marked ("immunomarking") with different antigens (Immunoglobulin G, IgG) internally or externally, which can then be detected in the gut content of predators using specific antibodies in ELISA. Another commonly used method for molecular gut-content analysis is polymerase chain reaction (PCR) detection of species-specific strands of DNA. Most field studies where wolf spiders were collected from field settings for gut-content analysis (i.e., not considering field cage experiments) have used PCR (Ekbom et al., 2014; Furlong et al., 2014; Hagler and Blackmer, 2013; Kobayashi et al., 2011; Kuusk and Ekbom, 2010; Kuusk and Ekbom, 2012; Kuusk et al., 2008; Monzo et al., 2010) or Mab ELISA (Fournier et al., 2008; Hagler and Naranjo, 2005; Mansfield et al., 2008). ELISA has a longer detection time (for comparison, see (Agusti et al., 1999a; Agusti et al., 1999b) and is more reproducible (Hagler et al 2015) than PCR, and IgG ELISA is cheaper and more sensitive than monoclonal endogenous antibody (Mab) ELISA (Furlong, 2015; Mansfield et al., 2008). IgG-specific ELISA has been used to detect predation in controlled enclosures (Hagler, 2006a; Hagler, 2006b; Hagler, 2011), but there are very few studies in which predators are collected after the release of marked prey from open field settings (Kelly et al., 2014; Mansfield et al., 2008). Given that IgG ELISA has multiple advantages over PCR and Mab ELISA, it is surprising that this method has been rarely been used to assess predation in field settings. IgG ELISA is a suitable, yet unexplored, technique for assessing wolf spider predation of *Helicoverpa* spp. in cotton fields.

Because Bt cotton fields require fewer insecticide spray applications than conventional cotton, they can harbour a diverse and sizeable arthropod community (Whitehouse *et al.*, 2005). These arthropods may serve as prey to sustain wolf spider populations in Bt cotton fields. Cotton fields in northern NSW, Australia, can harbour up to 12 different species of wolf spiders. Of these, the wolf spiders *Tasmanicosa leuckartii* and *Hogna crispipes* are abundant (Rendon *et al.*, 2015), and will readily prey on 5th instar *H. armigera* larvae in laboratory arenas and glasshouse plant enclosures (Rendon *et al., in press*). Another species present in these same fields, *Hogna kuyani,* has a body size large enough to kill 5th instar *Helicoverpa* spp. larvae (cephalothorax width 4.7-5.4mm; (Framenau *et al.*, 2006). *Helicoverpa* spp. larvae are rarely encountered in Bt cotton crops (Whitehouse *et al.*, 2005), and as a consequence, predation on *Helicoverpa* spp. larvae by wolf spiders has not yet been observed or quantified in cotton fields. In this study, we simulate a field scenario in which *Helicoverpa* spp. larvae survive foraging on Bt-cotton and descend to the soil. This study aims to determine whether wolf spiders prey on *Helicoverpa* spp. 5th instar larvae using a sandwich ELISA gut-content analysis. Additionally, we describe the prey of wolf spiders naturally occurring in a Bt-cotton field through direct observations. Furthermore, the movement and densities of wolf spiders in a cotton plot are examined as an important element to assess likelihood of spiders encountering *Helicoverpa* spp. larvae in the field.

Table 1: Reported prey of wolf spiders using direct observation and gut-content methods. Studies include only spiders freely captured in the field, and not used in feeding experiments in enclosures.

Prey item	Wolf spider species	Ecosystem	Method	Source
Hemiptera (unspecified family)	*Pardosa pseudoannulata* *Pardosa subpiraticus*	Rice paddies (*Oryza sativa*)	Direct observation	(Ishijima *et al.*, 2006)
	Lycosa terrestris *Pardosa birmanica*			(Tahir & Butt, 2009)
	Lycosa antelucana	Cotton (*Gossypium hirsutum*)	Direct observation	(Hayes & Lockley, 1990)
- **Aphididae**				
○ ***Rhopalosiphum padi***	*Pardosa agrestis*	Winter wheat (*Triticum aestivium*)	Direct observation	(Nyffeler & Benz, 1988)
	Pardosa agrestis	Alfalfa (lucerne, *Medicago sativa*)	Direct observation	(Samu *et al.*, 2003)
	Pardosa sp.	Barley (*Hordeum vulgare*)/ wheat (*Triticum aestivium*) rotation	Gut-content PCR	(Kuusk *et al.*, 2008)
	Pardosa sp.	Mixed cereals (*Trifolium*, *Festuca*, *Phleum*)	Gut-content PCR	(Kuusk & Ekbom, 2012)
- **Miridae**				
○ ***Anthocoris nemorum***	*Lycosa lugubris*	Oak forest	Direct observation	(Edgar, 1969)
○ ***Lygus lineolaris***	*Lycosa antelucana*	Cotton (*Gossypium hirsutum*)	Direct observation	(Hayes & Lockley, 1990)
○ **Sorghum plant bug *Stenotus rubrovittacus***	*Pardosa subpiraticus*	Rice paddies (*Oryza sativa*)	Gut-content PCR	(Kobayashi *et al.*, 2011)
○ ***Metapholophium* sp.**	*Pardosa amentata* *Pardosa palustris*	Winter wheat (*Triticum aestivium*)	Direct observation	(Nyffeler & Benz, 1988)
- **Cicadellidae**				
○ **Green rice leafhopper *Nephotettix cincticeps***	*Pardosa pseudoannulata*	Rice paddies (*Oryza sativa*)	Direct observation	(Ishijima *et al.*, 2006; Kiritani *et al.*, 1972)
- **Delphacidae**				
○ **Brown planthopper *Nilaparvata lugens***	*Lycosa (=Pardosa) pseudoannulata*	Rice paddies (*Oryza sativa*)	Direct observation	(Visarto *et al*, 2001; Kiritani *et al.*, 1972)
- **Aleyrodidae**				
○ **Sweet potato whitefly *Bemisia tabaci***	*Hogna* sp.	Cotton (*Gossypium hirsutum*)	Gut-content PCR	(Hagler & Blackmer, 2013)
- **Lygaeidae**				
○ **Big-eyed bug *Geocoris* sp.**	*Hogna sp.*	Cotton (*Gossypium hirsutum*)	Gut-content PCR	(Hagler & Blackmer, 2013)
Diptera (unspecified family)	*Lycosa terrestris* *Pardosa birmanica*	Rice paddies (*Oryza sativa*)	Direct observation	(Tahir & Butt, 2009)
	Lycosa lugubris	Oak forest	Direct observation	(Edgar, 1969)
	Pardosa sp.	Winter wheat (*Triticum aestivium*)	Direct observation	(Nyffeler & Benz, 1988)
	Pardosa sp.	Mixed cereals (*Trifolium*, *Festuca*, *Phleum*)	Gut-content PCR	(Kuusk & Ekbom, 2010)
	Pardosa milvina	Corn (*Zea mays*)/ soybean (*Glycine max*) rotation	Gut-content Mab ELISA	(Schmidt *et al.*, 2012)
- **Chironomidae**	*Pardosa pseudoannulata* *Pardosa subpiraticus*	Rice paddies (*Oryza sativa*)	Direct observation	(Ishijima *et al.*, 2006)
	Pardosa agrestis	Alfalfa (lucerne, *Medicago sativa*)	Direct observation	(Samu *et al.*, 2003)
- **Culicidae**	*Lycosa antelucana* *Pardosa milvina*	Cotton (*Gossypium hirsutum*)	Direct observation	(Hayes & Lockley, 1990)

Prey item	Wolf spider species	Ecosystem	Method	Source
- **Dolichopodidae**	*Pardosa palustris*	Winter wheat (*Triticum aestivium*)	Direct observation	(Nyffeler & Benz, 1988)
- **Tephritidae** o **Mediterranean fruit fly (*Ceratitis capitata*)**	*Pardosa cribata*	Citrus orchards	Gut-content PCR	(Monzo *et al.*, 2010)
Araneae (unspecified family)	*Lycosa antelucana* *Pardosa milvina*	Rice paddies (*Oryza sativa*)	Direct observation	(Hayes & Lockley, 1990; Kiritani *et al.*, 1972)
	Lycosa pseudoannulata			(Kiritani *et al.*, 1972)
	Lycosa terrestris *Pardosa Birmanica*			(Tahir & Butt, 2009)
	Lycosa lugubris	Oak forest	Direct observation	(Edgar, 1969)
	Pardosa sp.	Winter wheat (*Triticum aestivium*)	Direct observation	(Nyffeler & Benz, 1988)
	Pardosa agrestis	Alfalfa (lucerne, *Medicago sativa*)	Direct observation	(Samu *et al.*, 2003)
Collembola (unspecified family)	*Lycosa lugubris*	Oak forest	Direct observation	(Edgar, 1969)
	Pardosa agrestis	Winter wheat (*Triticum aestivium*)	Direct observation	(Nyffeler & Benz, 1988)
	Pardosa agrestis	Alfalfa (lucerne, *Medicago sativa*)	Direct observation	(Samu *et al.*, 2003)
	Pardosa sp.	Mixed cereals (*Trifolium*, *Festuca*, *Phleum*)	Gut-content PCR	(Kuusk & Ekbom, 2010; Kuusk & Ekbom, 2012)
Lepidoptera (unspecified family)	*Pardosa terrestris* *Pardosa birmanica*	Rice paddies (*Oryza sativa*)	Direct observation	(Tahir & Butt, 2009)
	Lycosa lugubris	Oak forest	Direct observation	(Edgar, 1969)
- **Noctuidae**	*Lycosa antelucana*	Cotton (*Gossypium hirsutum*)	Direct observation	(Hayes & Lockley, 1990)
o **Cotton looper (*Anomis flava*)** o **Cotton bollworm (*Heliothis* sp. = *Helicovepa*)** o **Rough bollworm (*Earias huegeliana*)**	*Lycosa* sp.	Cotton (*Gossypium hirsutum*)	Direct observation	(Bishop, 1978)
- **Crambidae** o ***Crocidolomia pavonata***	Lycosidae	Cabbage(*Brassica oleracea*)	Gut-content PCR	(Furlong *et al.*, 2014)
- **Plutellidae** o ***Plutella xylostella***	Lycosidae	Cabbage(*Brassica oleracea*)	Gut-content PCR	(Furlong *et al.*, 2014)
Coleoptera (unspedicified family)	*Lycosa antelucana*	Cotton (*Gossypium hirsutum*)	Direct observation	(Hayes & Lockley, 1990)
	Pardosa agrestis *Pardosa amentata*	Winter wheat (*Triticum aestivium*)	Direct observation	(Nyffeler & Benz, 1988)
	Pardosa agrestis	Alfalfa (lucerne, *Medicago sativa*)	Direct observation	(Samu *et al.*, 2003)
- **Chrysomelidae** o **Flea beetle *Phyllotreta* sp.**	*Pardosa* sp.	Oilseed rape (*Brassica napus*)	Gut-content PCR	(Ekbom *et al.*, 2014)
Hymenoptera (unspecified family)	*Lycosa lugubris*	Oak forest	Direct observation	(Edgar, 1969)
	Pardosa sp.	Winter wheat (*Triticum aestivium*)	Direct observation	(Nyffeler & Benz, 1988)
- **Formicidae**	*Lycosa antelucana*	Cotton (*Gossypium hirsutum*)	Direct observation	(Hayes & Lockley, 1990)
Orthoptera (unspecified family)	*Pardosa terrestris* *Pardosa birmanica*	Rice paddies (*Oryza sativa*)	Direct observation	(Tahir & Butt, 2009)

Prey item	Wolf spider species	Ecosystem	Method	Source
- **Acrididae** o **Spur-throated locust *Austracris guttulosa***	*Lycosa* sp.	Cotton (*Gossypium hirsutum*)	Direct observation	(Bishop, 1978)

Materials and methods

Protocol standardization for IgG protein marking and ELISA gut-content analysis.

Spider collection

This experiment aimed to standardize a protocol for IgG marking and gut-content analysis using ELISA, and was carried out at the Australian Cotton Research Institute (ACRI; 33ºS, 149ºE), near Narrabri, New South Wales, Australia during October and November 2014. Males of the wolf spider *Tasmanicosa leuckartii* were found by visual search after sunset in and around cotton fields using a headlamp (Petzl Tikka, 140 lumens), and collected manually in clear 70 ml cylindrical plastic containers. After collecting, all spiders were housed individually in clear plastic containers ('spider container', 228 mm height x 238 mm length x 238 mm width, 8.5 L, Décor Tellfresh superstorer®) containing 2L of moist soil and kept in a controlled environment room (24.4 ± 0.5 ºC, mean ± SD) with a L14:D10 photoperiod. Spiders were kept in the controlled environment room for 2-4 days before being used in experiments; during this period each container was sprayed with water daily and no prey was offered.

IgG larval mark

Helicoverpa armigera larvae were reared in individual wells in trays with unmarked soy and agar diet (Downes *et al.*, 2009; Teakle & Jensen, 1985) and were maintained in a controlled environment room (24.4 ± 0.5 ºC, mean ± SD) with a L14:D10 photoperiod, until they reached 5th instar. An IgG enriched diet was prepared by placing 2mL of a soy and agar diet in individual wells in trays, allowing it to set solid. Using a fine paintbrush, a layer of

rabbit IgG solution (1mg technical grade rabbit IgG / 1mL ultrapure water, Sigma-Aldrich #I5006) was spread over the surface of the diet on each well (2cm x 2cm area). One 5^{th} instar larva was immediately placed in each well of IgG marked diet, and an additional layer of IgG solution (approximately 1uL) was spread over the body of the larvae using a fine paintbrush. This ensured that (1) the concentration of IgG in the diet was high enough to be detectable, and (2) that larvae were marked both internally and externally. Spiders consume their prey by injecting venom containing enzymes that liquefy tissues, and then suck the prey contents leaving behind a dry exoskeleton. Hence, it was possible that an external topical IgG mark would not be eaten by the spider, but it could leave traces on the spider's chelicerae and legs during prey manipulation. Additionally, the IgG protein in the gut of the larvae could be denatured or expelled after the larvae fed on plant material. Therefore, we wanted to maximize the chances of the spider picking up the IgG while eating larvae by marking larvae both internally and externally. Larvae fed on the IgG diet for 24hrs, and then were transferred to individual wells containing unmarked (without IgG) conventional (non-Bt) cotton plant material (a mixture of leaves and green bolls), and allowed to feed for another 24hrs. This simulated field scenarios in which marked larvae are placed on the soil near cotton plants, where larvae could still feed on nearby stubble or weeds before encountering spiders.

After larvae had spent 24hrs feeding on IgG-marked diet, and 24hrs on cotton plant material, each larva was weighed to the nearest 0.01g using a digital scale (Sartorius model A200S), and then randomly assigned to a spider feeding treatment. Larvae were placed inside the spider's container 30-60 min after the dark phase began in the controlled room, and spiders were allowed to hunt freely.

Gut-content analysis using ELISA.

To test persistence of the IgG mark in the gut of the spiders after feeding on *H. armigera* larvae, spiders were randomly assigned to time treatments, and frozen at -20°C 3h,

12h, 24h, 48h, and 72h after killing and eating an IgG-marked larva (N=10 for each time treatment). Spiders in the negative control group were frozen 12h after killing one unmarked (without IgG) larvae. To test for the possibility of spiders picking up traces of IgG on the soil or by contact (false positives), spiders that did not kill *H. armigera* after 24h were frozen at -20°C (N=11).

A sandwich ELISA was performed on the whole body of each spider, using a protocol modified from Hagler (2004). All spiders were killed by freezing. Spiders heavier than 0.40g were sliced in smaller pieces using a razor blade, and then crushed in a centrifuge tube in 1000uL of tris buffered saline (TBS, pH 7.4; Sigma-Aldrich T1503). Spiders lighter than 0.40g were crushed in a centrifuge tube in 500uL of TBS. Each of the wells of a 96 microtiter plate (Cellstar® #655-180, Greiner Bio-one) was coated with 100uL of goat anti-rabbit IgG (1mg/mL stock solution diluted 1:500 in TBS; Sigma-Aldrich #R2004), and incubated overnight at 4°C. Antibody was discarded, and each well then was coated with 300uL of protein-blocker for 30 minutes at room temperature; blocking solution was prepared by diluting 1000uL of whole milk in 100 mL ultrapure H_2O. Blocking solution was discarded, and 100uL aliquot of spider-TBS sample was added in each well and incubated for 1h at room temperature. Sample solution was discarded, and wells were washed three times with 300uL of TBS-Tween 20 (0.05%). A 50uL aliquot of peroxidase conjugate goat anti-rabbit IgG (Sigma-Aldrich #A6154) diluted to 1:1000 in the milk protein-blocking solution was added in each well and incubated for 1h at room temperature. Plates were washed again three times as described above, and 50uL of TMB substrate (Sigma-Aldrich #T0440) was added to each plate. After 10min, absorbance of each plate was measured using a microplate reader (Biotek EL808) set at 655nm. Samples were scored positive for presence of IgG if the absorbance was greater than 3 standard deviations above the average absorbance of unmarked *H. armigera* (Hagler & Miller, 2002).

Assessing **Helicoverpa** *spp. predation in the field using gut-content analysis.*

Study plots

The objective of this experiment was to determine whether wolf spiders eat 5th instar *Helicoverpa armigera* and *Helicoverpa punctigera* larvae in Bt cotton fields (both species referred to hereafter as '*Helicoverpa* spp.'). The study plots were located at the Australian Cotton Research Institute (ACRI) near Narrabri, New South Wales, Australia (30ºS, 149ºE). Narrabri has a sub-tropical semi-arid climate (Kottek et al. 2006) and experiences four distinct seasons with a mild winter and a hot summer. The hottest month is January (mean daily maximum of 35ºC and minimum of 19ºC) and July the coldest (mean daily maximum of 18ºC and minimum of 3ºC). Cotton is usually planted in October, and harvested April/May. Mean annual rainfall is 593 mm.

Helicoverpa spp. release and spider collection for gut-content analysis was done in a small plot of unfertilized Bt cotton (Sicot 74 BRF®), 8m wide (=8 rows: 1 cotton row/m) by 160m long (hereafter "focal plot", Figure 1a). This plot was fumigated with 150 g of nitrogen only once pre-season, and no additional fertilizer was added during the season. Cotton was planted on 17 October 2014, sprayed with glyphosate (Round-up®) once in November, and weeds were removed by chipping during the season. Being unfertilized, cotton plants in this plot were shorter than plants in adjacent fertilized cotton fields (mean height ± SD on 18 February 2015 = 56.55 ± 9.0 cm, N=30), and this low canopy facilitated visual searches for wolf spiders between the plant rows.

Helicoverpa spp. *release and spider collection*

Due to species availability, a combination of *H. armigera* (43%) and *H. punctigera* (57%) were used for field predation trials. Larvae of *Helicoverpa* spp. were reared in individual wells in trays with unmarked soy and agar diet (without IgG) and kept in a

controlled environment room (24.4 ± 0.5 ºC, mean ± SD) with a L14:D10 photoperiod, until they reached 5th instar. After reaching 5th instar, each larva was placed in a new well of IgG marked diet (see diet marking protocol), and another 1uL of IgG solution was spread over the body of the larvae using a fine paintbrush. Trays of marked larvae and diet were immediately transferred to a cool room (11.84 ± 0.91 °C, mean ± SD; L24:D0 photoperiod) to prevent larvae from developing into pre-pupae. Under these conditions, larvae still consumed the IgG-marked diet.

Since the focal plot was Bt cotton and so very few larvae would survive to pupation, we simulated a scenario in which *Helicoverpa* spp. larvae survived foraging on Bt-cotton, by releasing IgG-marked larvae on the soil of the focal plot. Approximately 28 hrs after placing *Helicoverpa* spp. larvae on IgG marked diet, 96 larvae were released in the focal plot at sunset (approximately 20:00hr). *Helicoverpa* spp. were placed on the outermost row of the cotton field (edge), and distributed every meter on the soil on top of the ground bed, next to cotton plant stems. *Helicoverpa* spp. were only placed in the middle 96m of the row, excluding the 32 m at each end (Figure 1a). *Helicoverpa* spp. larvae were released in the plot on 5 nights (13 February, 17 February, 19 February, 21 February, and 23 February), and spiders were collected on the following night. After sunset, the three rows on the outer edge of the focal plot and three rows into the fallow field (six transects; Figure 1a) were searched for wolf spiders after sunset (2030hrs) by one investigator walking back and forth along the 160 m rows, for a total of 2 hrs each night. No more than 3 spiders were found during the last 30 minutes of every survey, suggesting that additional surveys would have returned very few additional spiders. All wolf spiders with a cephalothorax width greater than approximately 3.5mm were manually collected in a 70mL clear plastic container. Spiders with distinctive species cephalothorax patterns were classified to species (*Tasmanicosa leuckartii*, *Hogna crispipes*, or *Hogna kuyani*); all other spiders without distinctive patterns were classified as 'Lycosidae'. Immediately after field collecting, spiders were brought to the laboratory and

frozen at -20°C. In an attempt to observe predation events, on 13 February and 17 February, 30 *Helicoverpa* spp. larvae were tethered to fibreglass rods inserted in the soil on the outer row of the focal plot by tying a fine polyester thread to the middle body section using a reef knot and sealing it with a drop of cyanoacrylate glue. Tethered larvae were observed every 30 minutes for 2 hours. Due to the large number of tethered larvae that escaped and were not found within the first hour (53%), this experiment was not repeated on following nights.

To serve as positive/negative controls for gut-content analysis, and to determine predation by different species of wolf spiders, a separate sample of wolf spiders ('predation spiders') was assigned to field container predation trials (N= 79). Wolf spiders with a cephalothorax width greater than approximately 3.5mm were randomly collected in the cotton field surveyed, approximately 100m away from the focal plot row where IgG marked *Helicoverpa* spp. larvae were released. Sample size of each spider species (*T. leuckartii*, *H. crispipes*, *H. kuyani*, or 'Lycosidae') was representative of the species found in the field. Immediately after collection, spiders were placed in clear plastic containers without lids (228 mm height x 238 mm length x 238 mm width, 8.5 L, Décor Tellfresh superstorer®, 'field containers') containing approximately 2 L of moist soil, and placed in between rows of the cotton field. Cotton branches were placed on top of the containers to provide shade and to prevent desiccation. Approximately 5 minutes after the spiders were placed in the containers, one IgG marked *Helicoverpa* spp. larva was placed inside the container. Spiders randomly received either *H. armigera* (N=39) or *H. punctigera* (N= 40). Spiders were left in the containers at ambient conditions for 24 hrs. After 24h, containers were searched for *Helicoverpa* spp. remains as evidence of predation, and the spiders were collected in clear 70mL containers and immediately frozen at -20°C.

Frozen spiders were weighed to the nearest 0.01g using a digital scale (Shimadzu N595). Cephalothorax width was used as a measure of size for all spiders. Smaller spiders

were measured using a calibrated DinoEye camera (ANMO electronics corporation #AM-7023B) fitted in an optical stereoscope (Leica MZ6). Larger spiders were photographed with a digital camera (Panasonic HDC-SD900) set on a tripod, and digital images were measured using ImageJ (National Institutes of Health, Bethesda, Maryland) using the same calibration scale used on the DinoEye camera. After taking measurements, all spiders were tested for *Helicoverpa* spp. predation using the ELISA protocol described above. To assess predictors of *Helicoverpa* spp. predation in control spiders, four variables were chosen for binary logistic regression: (1) spider species, (2) *Helicoverpa* species (*armigera* or *punctigera*), (3) spider size (cephalothorax width), and (4) spider sex (male, female, juvenile). All analyses were carried out using SPSS v. 20 (IBM, 2011).

Field surveys: Mark-recapture and predation rates of wolf spiders.

Visual field surveys were carried out to (1) determine the density and mobility of wolf spiders in the focal plot (mark-recapture), and (2) assess which arthropods are found as wolf spider prey in Bt cotton. Because recapture rate is a factor that potentially influences positive/negative results in gut-content analyses using IgG-marked larvae, a mark-recapture survey was carried out to assess how likely it is to recapture a marked spider. All mark-recapture surveys were carried out in the focal plot (figure 1a) between 29 January and 10 February 2015 during the 'peak flower' stage of cotton growth (Constable 1988), which is the period when adult wolf spiders are most abundant in cotton fields at ACRI (Rendon *et al.*, 2015). Surveys were carried out on dry nights, as wolf spiders remain in their burrows during rain (personal observation).

Mark-recapture surveys were carried out in the focal plot to estimate (1) number of wolf spiders large enough to kill 5th instar *Helicoverpa* spp. larvae in this cotton plot, (2) recapture frequency of wolf spiders, (3) frequency with which wolf spiders crossed the edge between the cotton and fallow fields, and (4) frequency of predation events and prey items of

wolf spiders. The 3 rows on the outer edge of the focal plot ('plant rows') and three rows into the fallow field ('fallow soil') were surveyed after sunset (2030hrs) by one investigator walking along the 160 m rows (transects), for a total of 6 transects. Each transect was surveyed twice, once in each direction. Each wolf spider with a cephalothorax width greater than approximately 3.5 mm was marked with powder dye. Spiders found in the fallow field ('fallow soil') were marked with yellow dye (HCA Colours Australia, VM317), and spiders found in the cotton ('plant row') were marked with blue dye (VM321). These dyes remain clearly visible on spiders for more than 3 weeks even after irrigation and can only be discarded with moulting (personal observation). One limitation of this technique is that marked spiders that moulted could have been recorded as new spiders when recaptured. Every night, for every wolf spider with a cephalothorax width greater than approximately 3.5mm we recorded (1) whether it had a 'fallow soil', 'plant row', or no mark, and (2) if it had been recaptured inside the plant rows or in the fallow soil and (3) whether it was holding prey. Spiders with distinctive species cephalothorax patterns were classified to species (*T. leuckartii*, *H. crispipes*, or *H. kuyani*); all other spiders without distinctive patterns were classified as 'Lycosidae'. To avoid counting the same spider twice in one night, marked spiders were dusted with more dye to show a fresh mark.

To increase sample size of spiders found with prey, a second survey to determine predation frequencies and prey items of wolf spiders was carried out between 26 February and 7 March 2015 in a larger field adjacent to the focal plot (Figure 1b). Visual surveys took place around the edge of a triangular shaped cotton field 240m wide (=240 rows: 1 cotton row/metre) with row lengths ranging from 60 to 160m. The field was fumigated with 150 g of nitrogen pre-season. Cotton was planted on 17 October 2014, sprayed with glyphosate (Round-up®) once in November, and weeds were removed by chipping during the season. The majority of the field (160m wide, 60-140m long) consisted of 4 blocks of Bt cotton (Sicot 74 BRF®), 2 blocks of non-Bt cotton (Sicot 71 RRF®) and 2 blocks of pigeon pea refuge.

This section was flood irrigated every two weeks. The final 80 rows of the field consisted of alternating 8 rows of pigeon pea and Bt cotton, irrigated and unfertilized (See Figure 1b). At this stage of the season, the plant canopy already covered the plot rows, precluding visual search within the crop. Therefore, only the 3m around the edges of the plant rows of the field were surveyed for wolf spiders for 2hrs after sunset. Nocturnal wolf spiders hunt more actively immediately after sunset, and feeding tends to decline over the following hours (Hayes & Lockley, 1990). During surveys, for every spider with a cephalothorax width greater than approximately 3.5mm we recorded whether it was holding a prey in its chelicerae, and the spider species. All spiders holding a prey were captured and taken to the laboratory for identification of prey.

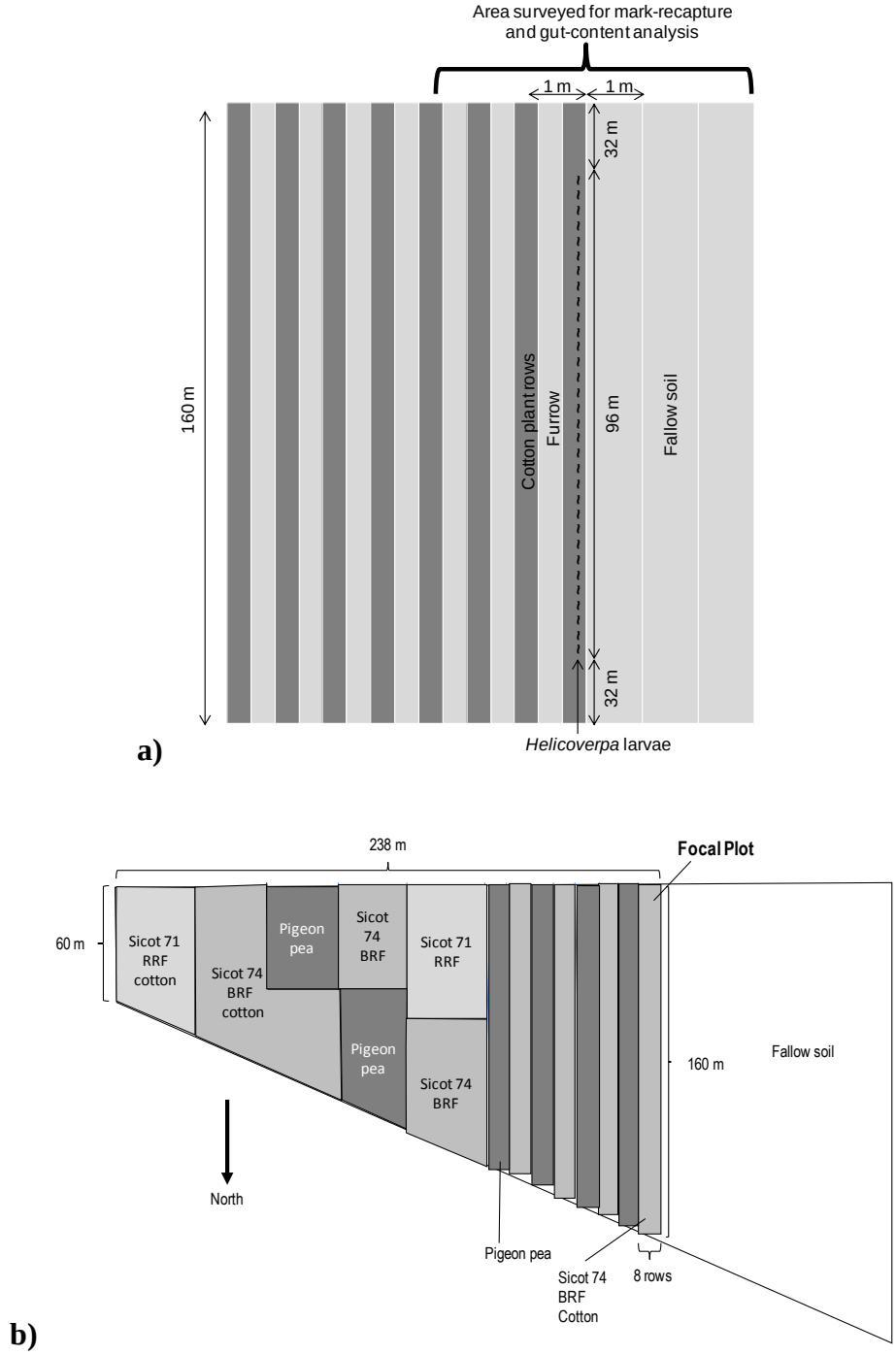

Figure 1. a) Focal plot surveyed for gut-content analysis and mark-recapture. b) Larger cotton field adjacent to focal plot surveyed for wolf spider predation, RRF= roundup ready Flex cotton, which means it contains genetic coding for resistance to glyphosate (Roundup ™), a herbicide; BRF = Bt Roundup Ready Flex cotton, which means the cotton also contains the gene to produce Bt toxins.

Results

IgG mark retention time

All *T. leuckartii* spiders that had been fed marked *H. armigera* tested positive for the presence of IgG, even 72hrs after consumption. However, 36% of the spiders that had occupied the arena but did not kill *H. armigera* tested positive for the presence of IgG (false positives). Spiders that fed on marked *H. armigera* had similar absorbances at all time points, and similar absorbances to false positives (ANOVA, $F = 0.962$, $df = 5,49$, $p = 0.45$; Figure 2).

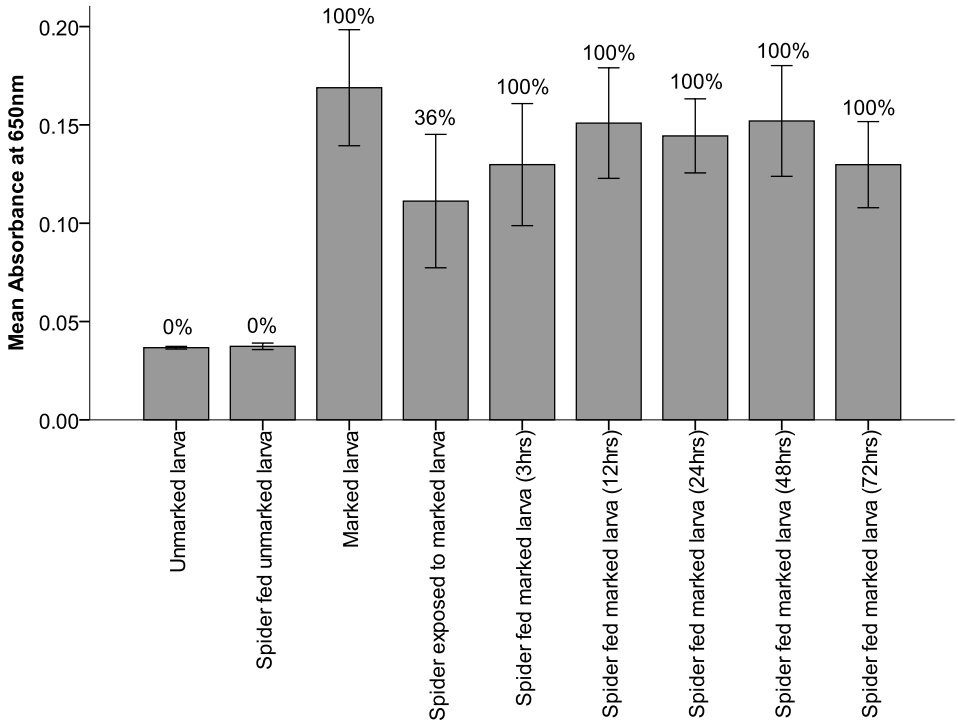

Figure 2. Mean absorbances of gut content of spiders after feeding on *H. armigera* at 25°C. Numbers on top of bars represent percentage of samples scored positive for presence of IgG. *For "spider exposed to marked *Helicoverpa*" treatment, only positive absorbances are graphed. Bars represent 95% confidence intervals.

Helicoverpa *spp. predation in the field*

Ninety four wolf spiders were collected in cotton fields following release of IgG-marked *Helicoverpa* spp. Of these, two spiders (2.1%) tested positive for the presence of IgG (female *T. leuckartii* and female *H. kuyani*, with no dye mark), and both spiders were collected the day after the first *Helicoverpa* spp. release. Of the tethered *Helicoverpa* spp. larvae (30 on each of two nights), we observed 5 larvae being attacked by ants within two hours of being released. Ants were the only *Helicoverpa* spp. predators observed in two hours. Besides the tethered larvae, no other *Helicoverpa* spp. larva was found on the soil.

Forty-nine out of 79 (62%) predation spiders in field containers killed *Helicoverpa.* Spider species, *Helicoverpa* species, spider size, and spider sex were not significant predictors of predation outcome (logistic regression, Nagelkerke $R^2 = 0.18$, $\chi 2 = 11.58$, df = 7, p = 0.11). When spider size and sex were analysed in separate regressions (due to correlation) with the other variables, spider size was a significant predictor of predation outcome, (B = 0.46, Wald = 5.24, df = 1, p = 0.02; Figure 3), with larger spiders more likely to kill *Helicoverpa* spp. There was a non-significant tendency for females to be more likely to kill *Helicoverpa* spp. than males and juveniles were (Wald = 5.848, df = 2, p = 0.054; Figure 4). After analysing gut-contents in field container spiders, all the spiders that ate *Helicoverpa* spp. tested positive for IgG and 7 spiders (23.3%) that had not eaten *Helicoverpa* spp. tested positive for IgG (false positives). Some spiders in field containers ran around and tried to escape when they were placed in the container, while others stayed immobile in the same spot. *Helicoverpa* spp. predation was not observed immediately when the larva was placed in the container.

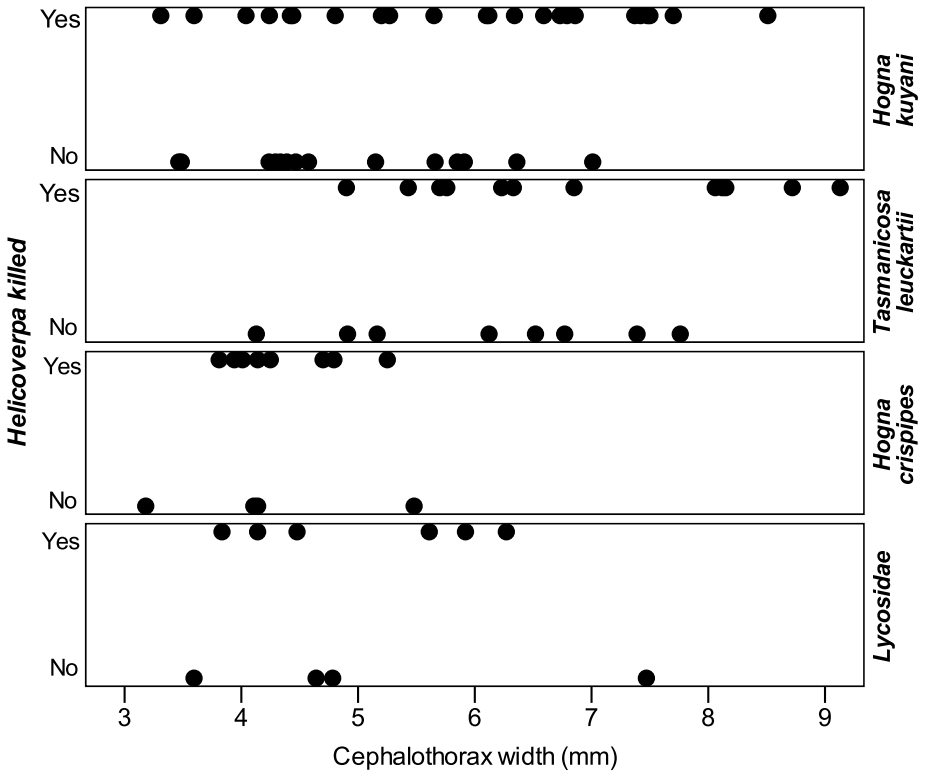

Figure 3. Cephalothorax width of wolf spiders in field containers and *Helicoverpa* spp. predation outcome.

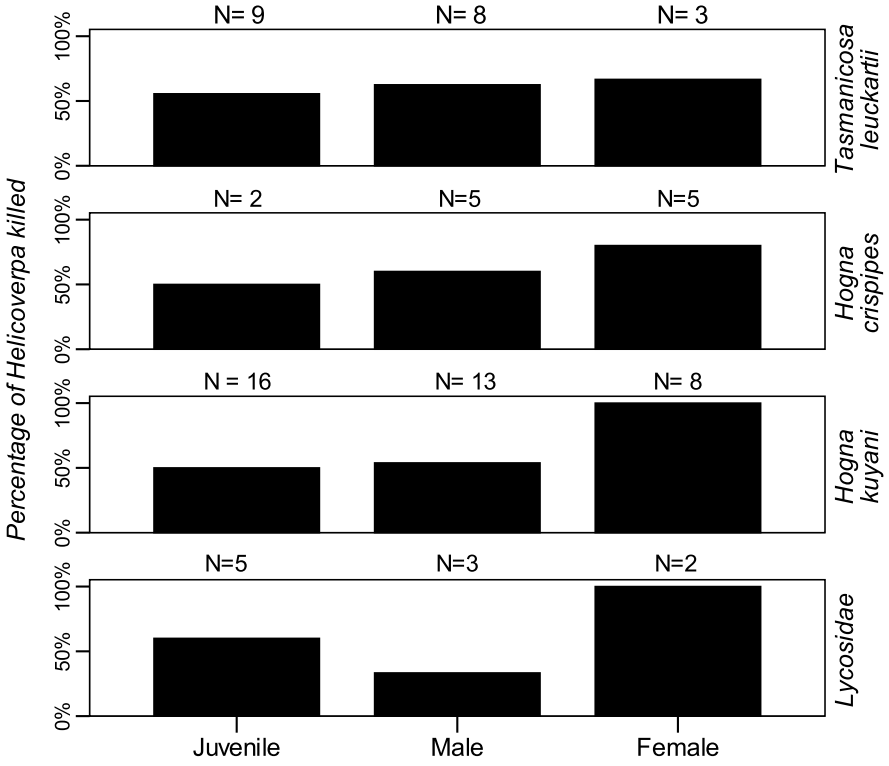

Figure 4. Predation outcomes of wolf spider males, females and juveniles of wolf spiders on *Helicoverpa* spp. in field containers.

Field surveys

A total of 164 spiders were marked on eight nights. The majority of spiders encountered during the mark-recapture surveys in the unmanaged plot were unmarked; a total of 72 unmarked spiders were found on the fallow soil, and 52 unmarked spiders were found inside the plant rows (Figure 5). A total of 25 spiders that were marked on the fallow soil outside the cotton crop were recaptured; 16 were recaptured on the fallow soil and 9 were recaptured inside the plant rows. A total of 14 spiders that were marked within the plant rows were recaptured; 5 were recaptured on the fallow soil and 9 were recaptured inside the plant rows (Figure 6). On the last day of the survey only (excluding all previous nights), 11 spiders were recaptured, representing 6.7% of the total number of spiders marked over eight nights (164). Permanent spider burrows are common in grassy areas outside of cropping fields, but only one permanent burrow was found in the surveyed plot, and spiders commonly hid inside soil cracks.

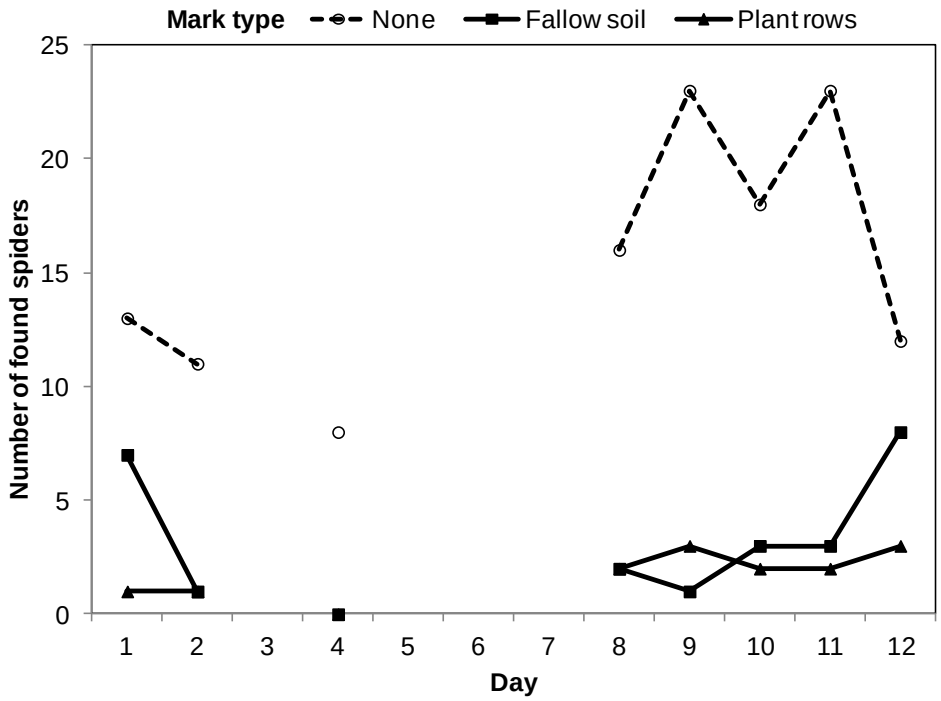

Figure 5. Mark-recapture survey in the focal plot across multiple nights. Days with no spiders represent nights where surveys were not possible due to inclement weather.

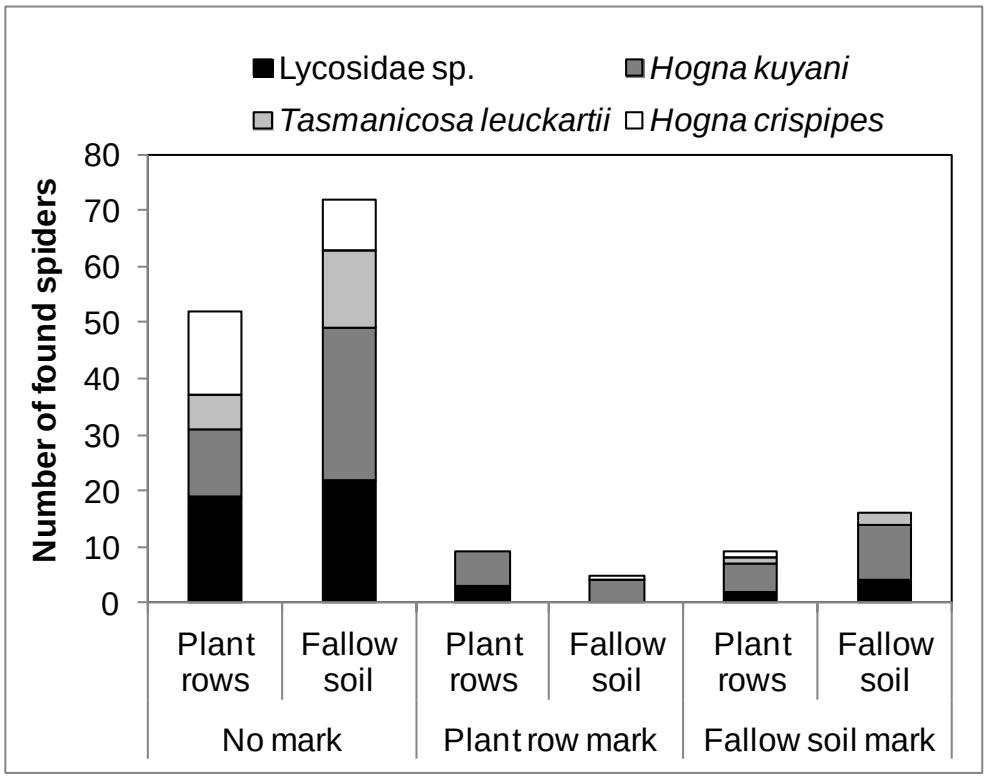

Figure 6. Total mark-recapture data from wolf spider survey in focal plot. Numbers indicate how many spiders of each species were found in total with or without a mark and their location, pooling all nights.

A total of 597 wolf spiders were observed in the fallow 3m beyond the plant rows of the cotton fields, and 18 spiders were recorded holding prey (3.0%; Table 2). Some prey items (including Lepidoptera species) could not be identified to species because they had been masticated by the spider. *Hogna kuyani* was the most abundant wolf spider (n = 254), and also the species most commonly found with prey (4.3%). The most common prey was the ground cricket *Teleogryllus commodus* (33% of all prey observed). Six *H. kuyani* (2.3%), three juvenile lycosids (2.1%), and one *H. crispipes* (1.1%) were found standing on cotton plant leaves, while *T. leuckartii* was always found on the soil.

Table 2. Wolf spider prey observed in visual surveys around edges of cotton fields.

	Hogna kuyani **(N = 254)**	***Hogna crispipes*** **(N = 90)**	***Tasmanicosa leuckartii*** **(N= 114)**	***Lycosidae sp.*** **(N=139)**
Orthoptera				
Gryllidae: *Teleogryllus commodus*	**5**	**1**	**0**	**0**
Dermaptera				
Labiduridae: *Labidura truncate*	**1**	**0**	**1**	**1**
Lepidoptera moth	**1**	**0**	**0**	**1**
Lepidoptera caterpillar	**0**	**1**	**0**	**0**
Lycosidae	**1**	**0**	**0**	**1**
Coleoptera				
Scarabeidae: *Mimadoretus sp.*	**0**	**0**	**0**	**1**
Hymenoptera				
Formicidae: *Iridiomyrmex* sp.	**1**	**0**	**0**	**0**
Unknown	**2**	**0**	**0**	**0**

Discussion

This study provides information about common prey available to sustain wolf spider populations in Bt cotton fields, and examines a molecular gut-content technique to assess wolf spider predation on *Helicoverpa* spp. in a scenario in which Bt-resistant larvae descend from the plant to pupate in the soil. Additionally, we discuss key factors that can influence predation, such as whether spiders move across crop edges and can encounter *Helicoverpa* spp., determining which characteristics of spiders predict likelihood of killing *Helicoverpa* spp., and the frequency with which marked spiders are recaptured.

Predation events were rarely observed in the field, but the abundance of three different species of wolf spiders (*T. leuckartii, H. crispipes,* and *H. kuyani*) suggests that this Bt cotton field harbours sufficient prey for spiders to thrive. From direct observations, only 3% of wolf spiders were found holding prey. Common prey reported in other field studies, such as Diptera and Hemiptera, were not observed as prey in this field. Instead, the most common prey was an abundant ground cricket, *T. commodus.* Interestingly, the second most common prey was the brown earwig, *Labidura truncata,* which is not reported in any of the previous studies listed in Table 1. While prey abundance or availability was not determined in this study, it is a likely factor to influence prey choice in the field. Although we did not find any wolf spiders eating *Helicoverpa* spp., we observed evidence that wolf spiders do kill Lepidoptera in the field, but the moth and caterpillar found as prey had been almost completely consumed by the spider, so it was not possible to determine the species. We found two cases of a spider eating another wolf spider, confirming intraguild predation amongst wolf spiders in cotton fields. Additionally, we found that *H. kuyani* and *H. crispipes* sometimes climb on the plant canopy, and could therefore hunt *Helicoverpa* spp. larvae on both the plant and on the soil.

Since wolf spider predation on *Helicoverpa* spp. was never observed directly in the field, gut-content analysis had the potential to ascertain whether spiders kill *Helicoverpa* spp. in the Bt cotton focal plot. One of the main advantages of ELISA over PCR for gut-content analysis is detectability periods. PCR tends to have a shorter detectability half-life; for example, gut-content analysis of clubionid spiders using PCR revealed that after 24hrs of feeding, only 20% of the assessed spiders tested positive for DNA traces of *H. armigera* (Pearce, 2004). Likewise, *H. armigera* DNA fragments were only detectable in the gut contents of 45% of predator mirid bugs (*Dicyphus tamaninii*) 4hrs after consumption (Agusti *et al.*, 1999b). Owing to short detectability time, after 24 hrs PCR is likely to yield false-negative readings from gut-content analysis. In contrast, antigen-based analyses (both MAb and IgG) can have a longer detectability in the gut of a predator; for example, 18hrs after consumption, 50% of mirid bugs tested positive for *H. armigera* in gut content analysis using indirect ELISA with a MAb (Agusti *et al.*, 1999a). Similarly, 100% of ladybeetles (*Hippodamia convergens*) tested positive 24hrs after consuming IgG-marked *H. armigera* eggs (Mansfield *et al.*, 2008). In this study, spiders were collected at least 24hrs after *Helicoverpa* spp. release; therefore, a longer detection period makes ELISA more suitable for gut-content analysis.

The IgG mark had a very long detectability time; in controlled laboratory trials at 25°C, 100% of the spiders tested positive 72hrs after consuming IgG-marked *H. armigera.* Likewise, 100% of the predation spiders in field containers tested positive 24hrs after consuming IgG marked *Helicoverpa* spp., even when field temperatures exceeded 35°C. This result exceeds the retention periods reported for other arthropods; for example, at 35°C, there were no traces of IgG in the gut-contents of big-eyed bugs (*Geocoris punctipes*) 24hrs after consuming IgG-marked pink bollworms (*Pectinophora gossypiella*). Furthermore, using a Mab ELISA at 25°C, only 25% of the big-eyed bugs tested positive 24hrs after consuming pink bollworms (Hagler & Naranjo, 1997). Spiders can exhibit longer detection periods than

predatory beetles in ELISA, with aphid antigens being detected in the guts of linyphiid spiders for up to 13 days (Sopp & Sunderland, 1989). Because all spiders that consumed IgG-marked *H. armigera* tested positive for IgG after 72hrs in laboratory controlled trials, it is not possible to estimate the half-life of this rabbit IgG mark (Greenstone & Shufran, 2003).

Despite the advantages of using ELISA for gut-content analysis, IgG contamination resulted in high levels of false positives. Other studies using protein for marking have found that cross-contamination of sprayed IgG marks in field-collected arthropods due to mass handling and collection is rare (Hagler *et al.*, 2015; Harwood, 2008). In this study, the source of this contamination is likely from the spiders picking up traces on their legs and abdomen from the soil, or by transferring small amounts of soil inside the containers when hand-collecting spiders. The high prevalence of false positives makes it difficult to interpret positive results from field-collected spiders with complete confidence. However, the likelihood of cross-contamination is greater when the spider and the IgG-marked *Helicoverpa* spp. interact in a smaller container than when spiders wander in an open field, therefore spiders collected from the field that tested positive for IgG are more likely to have come in contact and killed a *Helicoverpa* spp. larva. In future studies, using an internal-only mark might reduce the high incidence of such false positives.

Two out of 96 spiders collected in the focal plot tested positive for the presence of IgG. Assuming that the gut-content analysis is giving a true positive result, this means that a small proportion of spiders (2.1%) found in this plot killed IgG-marked *Helicoverpa* spp. Likelihood of encounter is an important component of predator-prey interactions. *Helicoverpa* spp. are less likely to be found and killed by wolf spiders if spiders are scarce overall, or if spiders are not present in the area where *Helicoverpa* spp. is active. Mark-recapture surveys help to estimate the density and movement of wolf spiders in cotton crops, and assess whether spiders are likely to come into contact with *Helicoverpa* spp. The fact that spiders originally

marked in the plant beds were found on the fallow soil beyond the crop and viceversa confirms that spiders are highly mobile and do routinely cross the field edges. Similarly, Pearce (2004) found that wolf spiders cross field interfaces and bare areas that have little vegetation cover. Spiders crossing the edge between the plant rows and the fallow soil had therefore a chance of encountering and killing IgG-marked *Helicoverpa* spp. where the larvae were released.

Wolf spiders rejecting *Helicoverpa* spp. as suitable prey in the field is an unlikely explanation for the low proportion of spiders in the focal plot testing positive for the presence of IgG. In predation trials in field containers, 62% of spiders attacked *Helicoverpa* spp. While spider size had a significant effect on the likelihood of *Helicoverpa* spp. predation, handling and the un-natural context might also have been important. Spiders were collected, placed into field containers, and offered *Helicoverpa* spp. without any opportunity to adjust to the new environment, so it is possible that spiders spent much of their time trying to escape the container or find burrows, which interferes with hunting behaviour. In a natural setting and without the limitations of artificial enclosures, predation rates of wolf spiders on *Helicoverpa* spp. upon encounter might be higher. Predation trials in field containers showed that *H. kuyani*, *H. crispipes* and *T. leuckartii* were similarly likely to kill *Helicoverpa* spp.; therefore, the proportion of predation in field containers (62%) did not reflect the low proportion of spiders testing positive for IgG in the field (2.1%). Predation is often less frequently detected in field settings than in controlled enclosure settings. For example, despite wolf spiders readily feeding on mayflies in cages, gut-content analysis found no evidence of mayfly predation by wolf spiders in a natural creek setting (Northam *et al.*, 2012). In the present study, the low proportion of spiders captured that tested positive for IgG might not reflect low levels of *Helicoverpa* spp. predation but may instead reflect high levels of emigration and low likelihood of spider recapture after feeding on *Helicoverpa* spp.

After marking 164 spiders on eight nights, only 6.7% of the spiders found on the last day of the survey had a mark. Pearce (2004) also reported low recapture rate of wolf spiders in cropping fields (4.5%). The high number of unmarked spiders found in the focal plot suggests that a low proportion of spiders were 'residents' with permanent burrows in this plot. However, this mark-recapture technique may underestimate the number of 'resident' spiders previously marked. Some of the unmarked spiders could have lost their mark when moulting. Through direct observations in the field it was not possible to distinguish between adult and subadult (penultimate instar) juveniles, so some spiders could have been marked and counted more than once. Nonetheless, assuming that some of the unmarked spiders are immigrants, it can be inferred that wolf spiders routinely walk distances of over 6m (the width of the area surveyed in the focal plot) a night from adjacent fields. Samu *et al.* (2003) found that the wolf spider *Pardosa agrestis* walks on average 4m / day, which is a distance similar to that covered by spiders crossing the cotton plants/ fallow soil interface. Immigrant spiders from adjacent fields may contribute to *Helicoverpa* spp. predation. If such spiders enter the crop to hunt and then leave the plot soon after hunting they would be underrepresented in our mark-recapture study.

In summary, the combination of direct observation, gut-content analysis and predation trials in field containers provide a comprehensive tool to determine which prey wolf spiders hunt in Bt-cotton. Predation trials in field containers showed that *H. kuyani, H. crispipes* and *T. leuckartii* wolf spiders killed *Helicoverpa* spp. immediately after being collected from the field. This suggests that, despite being difficult to detect in field settings, wolf spiders will kill 5th instar *Helicoverpa* spp. larvae that they encounter on the soil. Phenology of these three spider species is staggered across the cotton season (Rendon *et al.*, 2015); *Tasmanicosa leuckartii* is the first colonizer, and adults are present in the field earlier than other wolf spider species (October). *Hogna crispipes* becomes more abundant mid-season (January-February), while *H. kuyani* remains abundant in the field later in the season than the other wolf spider

species (April). In this study site, *H. armigera* moths emerge from winter diapause while *H. punctigera* migrates into the crops from northern areas from mid-October to mid-November (Baker *et al.*, 2011). From December to April, *Helicoverpa* spp. undergoes 4-6 generations; after cotton harvest (between April and May), *H. punctigera* migrates from cropping areas, while *H. armigera* goes into diapause and overwinters underground as pupae in cotton fields (Duffield, 2004; Fitt, 1989; Wilson *et al.*, 1979; Zalucki *et al.*, 1986). As the phenologies of *Helicoverpa* spp. and wolf spiders coincide throughout the cotton growing season, this guild of wolf spider species can target *Helicoverpa* spp. throughout its entire life cycle, from the first moths emerging from diapause, to the last larvae going underground to overwinter. Furthermore, the diverse prey available in these cotton fields can help sustain populations of wolf spiders throughout the season, ensuring that wolf spiders are abundant for controlling rare Bt-resistant *Helicoverpa* spp. larvae.

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CHAPTER 3: Consumptive and non-consumptive effects of wolf spiders on cotton bollworms.

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1

Abstract

Larvae of the cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae), that survive on genetically modified Bt cotton (*Gossypium hirsutum* L., Malvaceae) contribute to the risk of widespread resistance to Bt toxins. Current resistance management techniques include pupae busting, which involves deep tilling of the soil to kill overwintering pupae. Unfortunately pupae busting runs counter to soil and water conserving techniques such as minimum tillage. This problem could be relieved with biological control methods, whereby predators attack either larvae going to ground to pupate or moths emerging from the ground. We found that the wolf spider *Tasmanicosa leuckartii* Thorell (Araneae: Lycosidae), a common inhabitant of Australian cotton agroecosystems, is an effective predator of *H. armigera*, attacking and killing most larvae (66%) and emerging moths (77%) in simple laboratory arenas. *Tasmanicosa leuckartii* also reduced the number of emerging moths by 66% on average in more structurally complex glasshouse arenas. Males, females, and late-instar juveniles of *T. leuckartii* were similarly effective. *Tasmanicosa leuckartii* also imposed non-consumptive effects on *H. armigera*; when a spider was present, larvae in laboratory arenas spent less time on the cotton boll and more time on the soil and, unexpectedly, more mass was lost from the cotton boll. Increased loss of boll mass likely reflects changes in *H. armigera* foraging behaviour induced by the presence of spiders (indirect non-consumptive effects). *Helicoverpa armigera* spent more time as pupae when the spider was present in simple laboratory arenas, but not in more complex glasshouse enclosures. Overall, results indicate that *T. leuckartii* spiders can be effective predators of *H. armigera* late instars and moths but also suggest that, under some conditions, presence of spiders could increase the damage to individual cotton bolls.

Introduction

Larvae of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) are a major pest of Australian cotton crops, costing millions of dollars annually in crop loss and control measures (Fitt et al., 2009). In 1996, the first genetically modified Bt cotton was introduced specifically to control larvae of the genus *Helicoverpa* (Whitehouse et al., 2009b), particularly *H. armigera*, which has developed resistance to most insecticides used in cotton crops (Downes & Mahon, 2012). The current Bt cotton, Bollgard II, *Gossypium hirsutum* L. (Malvaceae), contains two genes of *Bacillus thuringiensis* (Berliner) that code for two different proteins that destroy the gut lining of *Helicoverpa* larvae (Van Rie, 2000).

Helicoverpa armigera larvae feed on cotton plants, and then descend from the plant to pupate underground and later emerge as moths. In temperate regions, *H. armigera* overwinter as pupae in the soil and then emerge as adults in the spring (Fitt, 1989). Survival of overwintering *H. armigera* pupae in Bt cotton crops is an important issue for the cotton industry; overwintering pupae are the end result of 4-6 generations of selection in Bt cotton crops during the previous cotton season (Baker et al., 2011) and potentially convey genes for Bt resistance from one season to the next (Lloyd et al., 2008). Destruction of overwintering Bt-resistant pupae is a key component of the Resistance Management Plan (RMP) deployed by the Australian cotton industry to constrain the proliferation and spread of Bt-resistant *H. armigera* (Fitt et al., 2009). As part of the RMP, growers planting Bt-cotton are required to undertake intensive post-harvest cultivation, referred to as 'pupae busting', to reduce survival of overwintering *H. armigera* pupae (Lloyd et al., 2008). To be effective, pupae busting requires full soil surface disturbance to a depth of 10 cm (Rourke, 2002). However, pupae busting is incompatible with minimum tillage, an agronomic practice commonly used for soil and water conservation before the introduction of Bt cotton (Cooper, 1999).

Minimum tillage involves the maintenance of permanent soil beds, with only occasional furrow delving to build up the ridges (NSW DPI, 1998), so it does not provide

sufficient soil disturbance to substantially reduce survival of overwintering pupae (Duffield, 2004; Lloyd et al., 2008). However, the stable soil beds and complex vegetation present in minimum-tilled grounds may provide an in-crop refuge for predators, thereby enhancing the biodiversity and abundance of natural enemies of *H. armigera*. Conservation of natural enemies is promoted in Bt-cotton crops, which receive 70% fewer insecticide sprays per season compared with conventional crops (Lloyd et al., 2008). Therefore, even though minimum-tillage practices cannot accommodate pupae-busting for control of *H. armigera*, they may promote pest control and management of Bt-resistance through enhanced conservation biological control (Bahar et al., 2013).

Predators in a cotton ecosystem may affect *H. armigera* populations both directly and indirectly. In addition to directly reducing prey densities by killing ('consumptive' or 'density-mediated' effects) predators can also influence trophic webs and contribute to biological control without killing prey ('non-consumptive' or 'trait-mediated' effects; Preisser et al., 2005). For example, the presence of a predator can increase pest prey mortality due to stress, a compromised immune system, or by inducing behavioral changes that compromise fitness, such as reduced energy intake, exposure to harmful environmental conditions, and increased risk from other predators, parasitoids, or pathogens (McCauley et al., 2011; Schmitz et al., 1997). Mortality in prey caused by a non-hunting predator ('lethal non-consumptive effect') has been reported in dragonfly larvae (McCauley et al., 2011) and grasshoppers (Beckerman et al., 1997). Even though the proximate mechanisms for non-consumptive mortality are poorly understood, reduced energy intake and increased vulnerability to pathogens in the presence of predators could be responsible (McCauley et al., 2011). When there is no mortality involved ('non-lethal non-consumptive effect'), risk effects are commonly treated as a trade-off in which any attempt by the prey to reduce predation risk comes at the expense of detrimental alterations to its life history, habitat selection, foraging behavior, physiology (Moya-Larano, 2011), or larval development (McCauley et al., 2011). In

H. armigera, the presence of predators has been associated with changes in feeding location on host plants, increased energy expenditure associated with larval crawling, dropping off host plants, and reduced food intake (Johnson et al., 2007). Non-consumptive effects can have even stronger impacts on prey populations than consumptive effects; this is because a predator can only kill and consume a small number of prey, but the presence of a predator may simultaneously alter the behavior of many potential prey (Beckerman et al., 1997; Preisser et al., 2005; Werner & Peacor, 2003). A more complete understanding of a predator's contribution to an agroecosystem can be obtained when including assessment of both consumptive and non-consumptive effects on community dynamics of pest prey (Schmitz et al., 1997).

Predators can affect plant production through consumptive and non-consumptive effects; this causal chain from predator to herbivore to plant is known as a 'trophic cascade', or 'top-down effect' (Polis, 1994). For example, leaf damage in soybean crops is reduced when spiders are abundant, and this trophic cascade is mediated by spider predation on herbivorous insects (Carter & Rypstra, 1995). Plant growth can be increased in the presence of chemical cues left by wolf spiders (Hlivko & Rypstra, 2003) or predatory mites (Bowler et al., 2013) that induce non-consumptive effects on herbivorous insects. Beckerman et al. (1997) found that damage from grasshoppers on herbs in enclosures was significantly lower in the presence of wolf spiders that could not kill than in enclosures without spiders. In cotton systems, cotton bolls suffer less damage from mirids in the presence of lynx spiders (Whitehouse et al., 2011). If the presence of a predator causes herbivores to alter activity and foraging, then the predator could alter the impact of herbivores on plants to an extent as great as that from density changes (Werner & Peacor, 2003).

Ground-hunting spiders can play an important role as stabilizing agents of insect pest populations in agroecosystems, and can contribute to maintaining pest populations below an economic threshold (Nyffeler & Benz, 1987). Wolf spiders (Araneae: Lycosidae) are active

ground hunters that do not build webs. They are the most abundant family and have the largest body size of ground spiders in many agroecosystems (Oberg & Ekbom, 2006; Pearce et al., 2004) including Australian cotton (Whitehouse et al., 2009a). Despite wolf spider abundance, they are often overlooked as biological control agents because they are not sampled on plants during the day, and are difficult to identify (Pearce et al., 2004). In cotton fields, wolf spiders feed on *H. armigera* larvae as well as other pests (Johnson et al., 2000). Since hunting spiders usually do not attack prey larger than 80% of their own body size (Nentwig & Wissel, 1986) wolf spiders in Australian cotton agroecosystems are more likely than smaller spiders to attack and subdue final instar *H. armigera* as they descend from the plant to pupate in the soil or as they emerge as moths.

The wolf spider *Tasmanicosa leuckartii* (formerly *Lycosa*) (Thorell) is the largest species of ground spider inhabiting cotton agroecosystems in Narrabri, New South Wales (NSW), Australia. *Tasmanicosa leuckartii* is found in the cotton fields as soon as cotton is sowed in late October – earlier in the season than adults of any other species of wolf spider (Rendon et al., 2015). Because they are abundant early in the season when other predators are rare, *T. leuckartii* may be a particularly important predator of emerging *H. armigera* moths. *Tasmanicosa leuckartii* is highly abundant throughout the cotton-growing season, and is found in the fields until early April (Rendon et al., 2015) when most cotton bolls are open and *H. armigera* larvae descend to the ground to overwinter as pupae. The predator-prey interactions, consumptive effects, and non-consumptive effects between *T. leuckartii* and late instar *H. armigera* and recently emerged moths have not been investigated before.

The aim of the present study was to assess the efficacy of *T. leuckartii* as a predator of late instars and recently emerged moths (consumptive effects) of *H. armigera*, and to also assess effects of spider presence on the behavior of *H. armigera* larvae (non-consumptive effects) and on cotton plants (trophic cascades). In these experiments we ask (1) whether late juvenile and adult life stages of *T. leuckartii* are effective hunters of late instars or emerging

moths of *H. armigera*, (2) whether the presence of a non-hunting spider influences microhabitat choice, behavior, mortality, or development of *H. armigera* in simple laboratory arenas and more complex glasshouse arenas, and (3) whether presence of a spider triggers a trophic cascade affecting cotton boll mass in laboratory arenas.

Materials and methods

Study site and specimen collection

This study was carried out at the Australian Cotton Research Institute (33ºS, 149ºE), near Narrabri, NSW, Australia. Experiments started in November 2012 (1 month after cotton sowing), and ended in April 2013 (before cotton harvest). *Tasmanicosa leuckartii* wolf spiders were collected in and around cotton fields after sunset. Spiders were found by visual search using a headlamp (Petzl Tikka, 140 lumens) and were collected manually in clear 70-ml cylindrical plastic containers. On the day of the experiments, cephalothorax width of each spider was measured to the nearest 0.1 mm using a manual caliper (resolution 0.1 mm), and the spider's sex and life stage (adult or juvenile) was recorded. Since abdomen size and body weight can vary with nutrition or reproductive state of females, cephalothorax width has been used as a measure of overall spider size in previous studies (Hagstrum, 1971; Buddle et al., 2003). Adult males, females, and juveniles that already bore the distinctive cephalothorax pattern characteristic of this species were used in the experiments. Each spider was used in laboratory or glasshouse experiments only once within 72 h of collection, and was then released back into the field.

Larvae of *H. armigera* were supplied by the Commonwealth Scientific and Industrial Research Organization (CSIRO) Agricultural flagship Bt Resistance Monitoring Group (Australian Cotton Research Institute, Narrabri, NSW, Australia), which has well established colonies of *H. armigera*. Larvae were reared in individual wells on a soy and agar diet

(Teakle & Jensen, 1985; Downes et al., 2009) and maintained in a controlled temperature room (mean ± SD = 24.4 ± 0.5 °C) with a L14:D10 photoperiod. *Helicoverpa armigera* larvae were randomly assigned to treatments for use in assays as larvae or pupae. Those to be tested as larvae were reared until they reached 5th instar and were then weighed to the nearest 0.01 g using a Sartorius A200S digital scale (mean larval weight ± SD = 0.42 ± 0.06 g). Those to be tested as pupae were reared to pupation and were then held to develop as pupae for a further 12 days (pupal weight = 0.39 ± 0.08 g). On the day that larvae pupated, pupae were rinsed with a 3.3% (wt/vol) sodium hypochlorite solution diluted in 10 parts of water to prevent fungal growth, and then kept in a dry plastic container until they were used in trials.

Laboratory experiments

To assess predation capacity, we first established how many *H. armigera* larvae *T. leuckartii* ate before rejecting prey. A *T. leuckartii* male (n = 11) was paired with two fifth instar *H. armigera*. After 2 h, the number of *H. armigera* eaten was recorded. Eaten *H. armigera* were replaced with new larvae every 2 h. After 8 h, we recorded how many *H. armigera* had been eaten in total.

To investigate effects of spider presence on mortality and behavior of *H. armigera* larvae and emerging moths, four treatment groups were established: (1) a larva paired with an intact spider (referred to hereafter as 'predation spider'; n = 36); (2) a larva paired with a spider unable to kill [referred to hereafter as 'risk spider', terminology following Beckerman et al. (1997) and Schmitz et al. (1997); n = 53]; (3) a larva without a spider ('no spider'; n = 41); and (4) a 12-day pupa paired with a predation spider (n = 31). The chelicerae of risk spiders were glued together with a drop of cyanoacrylate 'Super glue' (Bostik SuperBond; Bostik, Victoria, Australia) on the day of the experiment. This method has been used in other studies (Beckerman et al., 1997), and our previous observations showed that spiders with glued chelicerae still attempt to attack *H. armigera*. Laboratory experiments were carried out

under the same controlled temperature room conditions (see above). In these conditions, *H. armigera* reliably start emerging as adults after 12 days of pupation; thus, we were able to video-record the moment when the moth left the pupal case and emerged to the soil surface. To further investigate whether the presence of a spider influences pupal development, 23 trials from the no spider treatment and 16 trials from the risk spider treatment were maintained until *H. armigera* completed development and emerged as moths.

Collected spiders were housed in clear plastic containers (228 × 238 × 238 mm, 8.5 l; Décor Tellfresh Superstorer, NSW, Australia) containing 2 l of moist soil, and were kept in a controlled temperature room (see above). Each container had a retreat in one corner, consisting of a hole in the soil approximately 2 cm deep and 1 cm diameter. The retreat was partially covered by a 3 × 3 cm sheet of bark from a paperbark tree (*Melaleuca* spec.). Each spider was kept in the laboratory for 48-72 h before being used in experiments, and no prey items or additional water were supplied.

After weighing, each spider and larva was dusted with fluorescent dye to improve contrast for recording (VM311 Pink for *H. armigera* larvae and VM317 Yellow for spiders; HCA Colours, Kingsgrove, NSW, Australia). In previous observations, the dye did not seem to affect spider attack behaviour, or larvae foraging behaviour. Approximately 30-60 min after the dark phase commenced in the laboratory, one *H. armigera* was introduced in each spider's container. In treatments using larvae, one larva was placed on top of a previously weighed green cotton boll (mean boll weight ± SD = 17.22 ± 0.07 g) held approximately 3 cm from the soil on a wooden skewer. In the treatment with pupae, one pupa was placed inside a 1.5-ml plastic microcentrifuge vial without a cap and buried in the soil, covered by a 5-mm layer of soil to simulate the space conditions of a pupal chamber.

Arenas were video recorded continuously for 24 h (day 0), commencing immediately after *H. armigera* was released. Our recording system comprised a 1/3" CCD monochromatic infra-red camera (CCS-Sony Go Video; Sony, Cairo, Egypt) with a 4-mm lens positioned

above each arena, which recorded to a 2TB DVR 4-100 hard drive recorder. One infrared illuminator (IR-covert, 940 nm) was placed approximately 10 cm to the side of each arena. For each predation spider experiment, we recorded whether the spider killed and ate *H. armigera*, and the time it spent feeding (defined as the time during which the spider held the prey with its chelicerae and was not performing any other behavior). Trials were terminated on day 5, and the spiders and the green cotton boll were weighed again to determine any changes in mass. To estimate relative profit, we measured spider percent weight gain. Additionally, to estimate prey conversion efficiency we divided the spider's percent weight gain by the time spent feeding (a higher score indicates higher prey conversion efficiency in terms of rate of relative weight gain against prey handling costs). Attacks that occurred after 24 h, and therefore fell outside of the period of video recording, were excluded from analysis of prey conversion efficiency. A Student's t-test was used to test for differences in prey conversion efficiency of larvae and emerging moths. For larvae in the risk spider treatment, we recorded the time that each larva spent on the plant, on the soil, and underground in the first 24 h of the experiment. Because any larva body movement poses risk of detection or can trigger an attack, an activity score was created by recording the position of each larva and spider every 30 min and counting the times in a 24-h period each larva and spider moved. Larvae and spiders were scored as having moved if any part of their body changed position between observations, or if the whole larva or spider had moved to a different part of the arena. In trials that were maintained for longer to assess the effect of spider presence on pupal development, the spider remained in the enclosure throughout the experiment. We recorded the number of days from when the larva burrowed into the soil to pupate until it emerged as a moth.

A contingency table was analyzed using a Pearson χ^2 test to test whether there was a difference in the proportion of spiders that attacked either larvae or emerging moths, and to test whether spider stages (adult male, adult female, or juvenile) differed in frequency of

attacks on larvae and moths. A binary logistic regression was used to test whether probability of attack was predicted by spider cephalothorax width or *H. armigera* weight.

A permutational analysis of variance (maximum permutations = 999; PERMANOVA v.1.0.5, Anderson, 2005) was used to test for differences in the amount of time spent by *H. armigera* on the plant, soil, and underground among treatments (no spider, n = 37; risk spider, n = 35). This non-parametric test is a robust alternative for data with bimodal distributions, and does not assume independence between variables (Anderson, 2001). An analysis of covariance (ANCOVA) was used to test whether *H. armigera* activity scores and duration of pupation differed in the presence and absence of a risk spider, using treatment as a fixed factor and initial larval weight as a covariate. To determine whether *H. armigera* and *T. leuckartii* have similar activity scores during the 24-h period in risk spider treatments, an ANOVA was carried out using time, species (*H. armigera* or *T.leuckartii*), and time*species interaction as fixed effects. To account for activity scores being measured over time during the same trial, trial number was included in the model as a random effect. An ANCOVA with post-hoc Least Significant Difference (LSD) was used to test whether change in cotton boll mass differed in the presence of a risk spider, a predation spider, and no spider, using treatment as a fixed factor and initial larval weight as a covariate. All data were screened for normality using a Shapiro-Wilk test, and were log-transformed when necessary to meet requirements of parametric analysis. All analyses besides non-PERMANOVA were run using SPSS v.20 (IBM, Armonk, NY, USA).

Glasshouse experiments

Glasshouse experiments more closely emulated field conditions in which Bt-resistant *H. armigera* are found, in order to ascertain how the presence of a wolf spider affects density, development, and behavior of *H. armigera* in a more natural setting. These experiments were carried out in glasshouse enclosures that comprised 12- to 20-week-old cotton plants (variety

Sicot 71 RRF conventional, non-Bt) planted in plastic crates 60 (l) × 40 (w) × 40 (h) cm and covered by a clear plastic and mesh insect cage adapted to fit the crate, 60 (l) × 40 (w) × 60 (h) cm (MegaView Bug Dorm, model 2120). Plants were watered every day, and fertilizer (Thrive® soluble all-purpose plant food; Yates products, Padstow, NSW, Australia) was applied to each crate 1 day before each trial following manufacturer's instructions. No pesticides were applied to the plants during the 5-month duration of all the trials, but the leaves were manually washed with a 1:10 (vol:vol) household detergent water solution (Palmolive unscented dishwashing liquid) 1 day before each trial to control mite infestations, and rinsed with water to eliminate detergent residues. To evaluate both consumptive and non-consumptive predator effects, each enclosure was randomly assigned to one of three treatments: (1) no spider, (2) predation spider, or (3) risk spider (i.e., chelicerae glued). The experiments were repeated 3× in the same enclosures (November, n = 4 for each treatment, mean ± SD maximum temperature = 39.8 ± 4.7 ºC, minimum temperature = 21.2 ± 1.7 ºC; January, n = 4 for each treatment, maximum temperature = 43.8 ± 5.9 ºC, minimum temperature = 22.8 ± 1.2 ºC; March, n = 6 for each treatment, maximum temperature = 41.4 ± 3.6 ºC, minimum temperature = 20.9 ± 0.7 ºC). Treatments were rotated so that all enclosures were used for every treatment, thereby avoiding bias associated with a specific enclosure.

Spiders were collected from the fields in the evening and placed individually in clear plastic containers (18 × 10 × 5 cm) containing a 1-cm layer of dry soil and stubble and a clear plastic 30-ml vial containing water and a cotton wick to provide moisture. Each container was placed inside a randomly assigned glasshouse enclosure for acclimation. On the day after collecting, we sexed and measured the cephalothorax width of each spider, and glued together the chelicerae of risk spiders. Spiders were released into the glasshouse enclosures immediately after being measured. Before sunset (between 19:00 and 20:00 hours), six fifth instar *H. armigera* were placed inside each enclosure on the upper surface of a cotton plant leaf. Each *H. armigera* larva was marked with fluorescent dye (HCA Colours Australia) of a

different color for individual identification, and to facilitate observation and tracking. Following the release of larvae (day 0, hour 0), the behavior and location of the spider and each larva was recorded once every hour for 5 h. The behaviors assigned to *H. armigera* larvae were (1) feeding (mandibles touching a plant part), (2) digging (using head to displace soil), (3) still (not positioned to feed or dig and not moving any part of the body), and (4) moving (displacing any body part and not digging or feeding). The behaviors assigned to spiders were (1) still (not moving any body part), (2) grooming (rubbing any body part against chelicerae), (3) feeding (holding a *H. armigera* between the chelicerae), and (4) moving (displacing any body part and not grooming or feeding). Locations assigned for both *H. armigera* and spiders were (1) on plant, (2) on soil, or (3) underground.

Every day at sunset, the numbers of live and dead visible larvae and emerged moths were recorded. For larval behaviors, we recorded the number of larvae performing each behavior from the total number of visible larvae in the enclosure. If no larval remains were found on the soil surface, missing larvae in each treatment were assumed to have burrowed underground to pupate. Three separate developmental stages were defined for each enclosure: larval period, pupation period, and emergence period. Larval period lasted from day 0 until the day the last larva was observed. Pupation period lasted from the day when no more larvae were observed, until the day the first moth emerged. Emergence period lasted from the day of first moth emergence, until the day after the last moth emerged. Trials were terminated on the day after no moth emergence was recorded for any enclosure (2nd day with no emerged moths). On the last day of the trial, the soil was sieved and live pupae (if any) were counted. The two juvenile spiders that died during trials (one ‘risk spider’ and one ‘predation spider’) both died during the pupation period and were replaced by juvenile spiders of a similar size on the same day they were found dead.

A priori tests revealed no interaction between dates of trials and the three treatments (no spider, predation spider, risk spider) for all the variables examined in glasshouse

experiments; therefore, trials from all three dates (November, January, March) were pooled for analysis. An ANOVA was used to test for differences among the three treatments (no spider, predation spider, risk spider) in the total number of moths emerged in each enclosure. A repeated measures multivariate ANOVA (RM-MANOVA) was used to test for differences among treatments in the proportion of larvae per enclosure in each location (plant, soil, underground), and performing each behavior (feeding, moving, still, digging) at every hour during 5 h. An RM-MANOVA was used to test for differences between no spider and risk spider treatments in the duration (days) of each developmental stage (larval, pupation, emergence) for trials in January and March (n = 10).

All data were tested for normality using a Shapiro-Wilk test, and log-transformed when necessary to meet requirements of parametric analysis. Data that violated the sphericity assumption in RM-MANOVA were analyzed using a Greenhouse-Geisser transformation; The sphericity assumption states that the variances of the different values in a repeated-measures test are equal across the groups, and it is equivalent to the homogeneity of variance assumption in independent-samples ANOVA; the Greenhouse-Geisser transformation adjusts the degrees of freedom to decrease the chance of a type-I error (Greenhouse & Geisser, 1959). All analyses were conducted using SPSS v.20 (IBM, Armonk, NY, USA).

Results

Laboratory experiments

Wolf spider attack behavior. In laboratory arenas, spiders oriented towards *H. armigera* larvae when the larvae moved within a field of approximately 30º to either side the spider's front sagittal plane, or after being touched by a wandering larva. Spiders approached moving larvae even if the larva was located in the most distant part of the container. Attacking spiders tapped the larva with legs I, grabbed it with legs I and II, and bit it. Larvae were attacked on

the soil after they descended from the boll, and in one instance a spider was observed using legs I to knock down a larva that was on the bottom of the boll. *Helicoverpa armigera* larvae were safe from attack after going underground, as there were no observations of spiders excavating larvae or pupae. Spiders were sometimes observed to tap *H. armigera* larvae with legs I and then walk away without attacking. After delivering an initial bite, no spider rejected a larva and all attacks resulted in consumption of *H. armigera*. Immediately after emergence, moths walked away from the pupal chamber, and climbed up the first vertical surface they encountered. Some crawled up and down the walls of the container and then back onto the soil. Attacks occurred when the moths were low on the cage walls within the spider's reach, when they were walking on the soil after emergence, or when they descended to the soil from the walls. Spiders only attacked moths that had fully emerged from underground. In all cases, spiders oriented towards the moths after moths made movements. Spiders then rapidly approached the moth and grasped it with legs I and II before biting. Spiders spent all of the time on the soil or inside their retreat and were never observed climbing on the walls of the container or on the cotton bolls.

Larva and moth mortality. In predation capacity trials *T. leuckartii* males killed (mean ± SD) 2.54 ± 1.8 *H. armigera* larvae in 8 h, with a maximum of six larvae in 8 h. In some occasions, *T. leuckartii* attacked a new larva offered while it was still feeding on another larva. For trials investigating predation on larvae, we tested 15 adult *T. leuckartii* males (mean cephalothorax width ± SD = 8.26 ± 0.77 mm), 10 adult females (8.5 ± 0.31 mm), and 11 juveniles (6.9 ± 1.00 mm). For trials investigating predation on moths, we tested 14 adult males (7.96 ± 1.00 mm), 8 adult females (9.1 ± 0.73 mm), and 9 juveniles (6.97 ± 1.22 mm). In both the treatment with predation spiders and larvae, and the treatment with predation spiders and moths, 24 *H. armigera* were killed (66.6% larvae, 77.4% emerging moths). There was no significant difference in the proportion of larvae and moths killed by spiders (Pearson χ^2 =

0.948, d.f.= 1, P = 0.24).

The likelihood that larvae would be killed was not predicted by spider cephalothorax width, *H. armigera* weight, nor their interaction (binary logistic regression: $\chi^2 = 1.395$, d.f.= 3, P = 0.71; Cox-Snell $R^2 = 0.038$). The same variables also did not predict probability that emerging moths would be killed ($\chi^2 = 4.541$, d.f. = 3, P = 0.21; Cox-Snell $R^2 = 0.136$). Moreover, there was no difference in the tendency of *T. leuckartii* adult males, females, or juveniles to kill larvae ($\chi^2 = 1.070$, P = 0.59) or moths ($\chi^2 = 0.036$, P = 0.98, both d.f. = 2). There was no evidence of difference between larvae and moths in prey conversion efficiency (spider % weight gain / prey handling time) (t-test: t = -0.09, P = 0.93), or in relative prey profit (spider % weight gain; t = 0.52 P = 0.75, both d.f. = 41).

Larva and moth behavior. Larvae spent significantly less time on the cotton boll and more time on the soil when a risk spider was present (PERMANOVA, plant: t = 2.411, P = 0.034; soil: t = 2.445, P = 0.009, both d.f. = 70; Figure 1). In risk spider and no spider treatments, respectively, larvae spent (mean ± SD =) 9.60 ± 10.52 and 15.28 ± 9.47 h on the cotton boll, but 4.01 ± 5.74 and 1.57 ± 1.88 h on the soil. There was no evidence that presence of a risk spider influenced the time *H. armigera* larvae spent underground (t = 1.105, d.f. = 70, P = 0.27). The time that a moth spent on the soil in the presence of a risk spider was highly variable (median = 0.80 h, range: 0.1-21.29 h; n = 8).

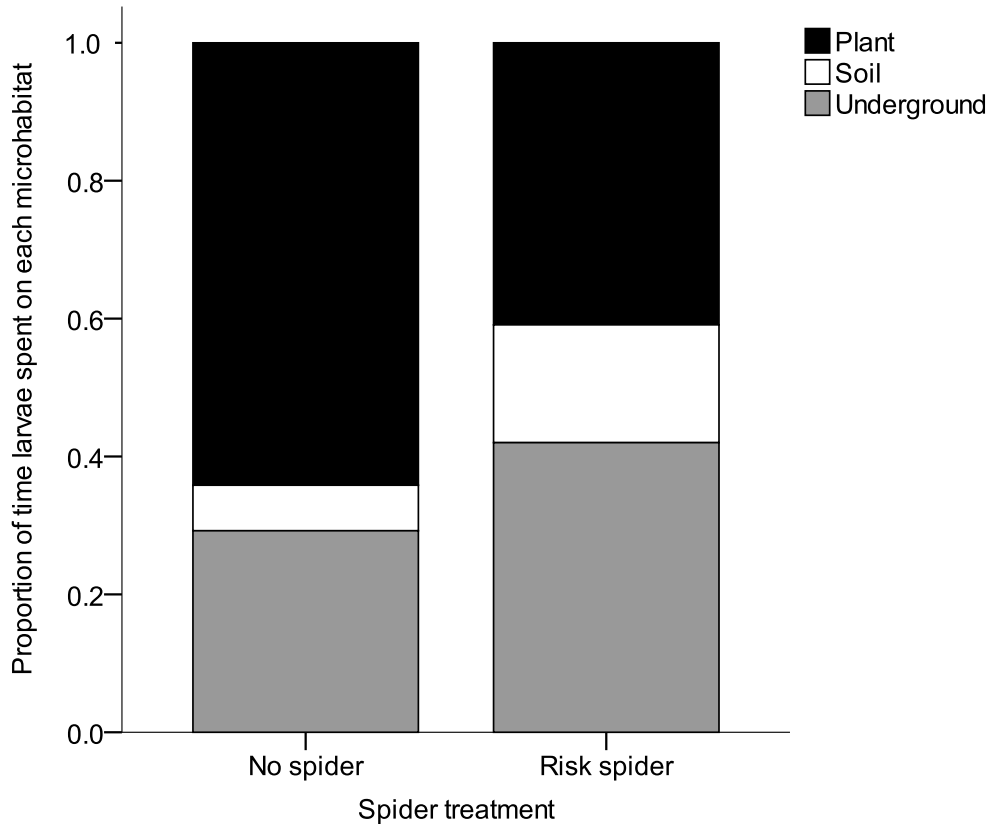

Figure 1. Average proportion of time *Helicoverpa armigera* larvae spent on plant (cotton boll), on soil, or underground in laboratory trials with no spider or a risk spider.

Analysis of activity revealed that both *H. armigera* and risk *T. leuckartii* are more active during the dark phase, and both species show similar activity patterns over the 24-h observation period (ANOVA, time: $F_{23,358} = 3.874$, $P<0.01$; time*species: $F_{23,358} = 0.705$, $P = 0.84$). There was no evidence that presence of a risk spider influenced larval activity scores compared to no spider (ANCOVA: $F_{1,69} = 0.365$, $P = 0.55$).

Helicoverpa armigera spent longer in the pupal stage in the presence of a risk spider (mean ± SD = 18.44 ± 1.26 days) compared to when no spider was present (14.96 ± 1.33 days; ANCOVA: $F_{1,36} = 64.41$, $P<0.01$). The initial boll weight did not influence the time *Helicoverpa* spent on the boll (Pearson correlation, no spider: $r = 0.51$, $P = 0.90$; risk spider: $r = -0.40$, $P = 0.82$), or boll weight loss (no spider: $r = 0.043$, $P = 0.80$; risk spider: $r = 0.263$, $P = 0.17$). In all trials, there was physical evidence of *H. armigera* larvae feeding on the cotton boll.

There was a significant effect of treatment on cotton ball mass loss (ANCOVA: $F_{2,122}$ = 6.714, P = 0.002; Figure 2). Although there was no significant difference between predation and risk spider treatments (LSD = 0.0211, P = 0.16), cotton bolls lost more mass when a spider was present than when no spider was present regardless of whether it was a risk spider (LSD = 0.054, P<0.01) or a predation spider (LSD = 0.033, P = 0.037).

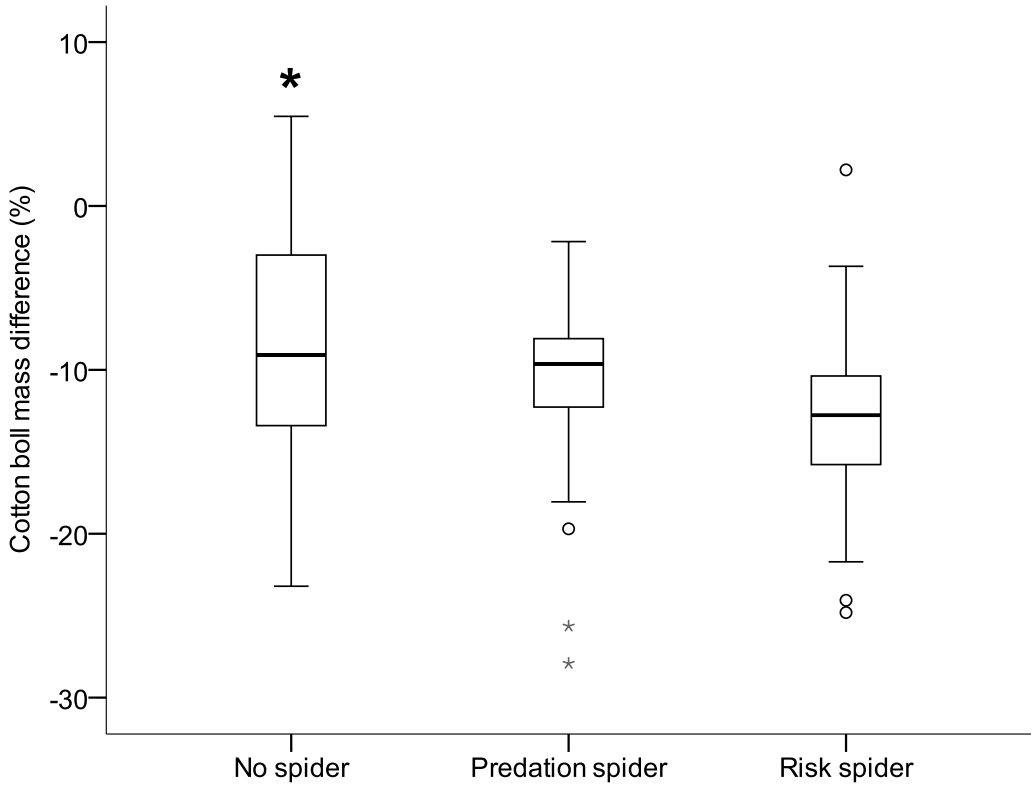

Figure 2. Mass loss (%) by cotton boll in laboratory trials with no spider and risk spider. Asterisk on top of bars represents significantly different treatments (ANCOVA, $p < 0.05$). Horizontal line represents median, box length represents interquartile range, whiskers length are upper and lower quartiles, dots represent suspect outliers in untransformed data (values greater than 1.5 box lengths), light grey stars represent extreme outliers in untransformed data (values greater than 3 box lengths).

Glasshouse experiments

In glasshouse enclosures, spiders spent most of the time on the soil, but three spiders were observed on the leaves of the cotton plant, and 17 spiders climbed onto the sides of the cages. Even though in these trials we could not observe the exact moment of attack, all predation spiders were observed eating a larva.

The number of moths emerging from each enclosure differed among the three treatments (no spider, risk spider, predation spider; ANOVA: $F_{2,39} = 21.72$, $P<0.01$; Figure 3). Post-hoc Tukey tests revealed that significantly fewer moths emerged when a predation spider was present (mean ± SD = 2.00 ± 1.66) than when no spider (4.71 ± 1.13) or a risk spider (4.71 ± 0.82) was present (predation spider – no spider, mean difference = -2.71, SE = 0.44, $P<0.01$; predation spider - risk spider, mean difference = -2.71, SE = 0.84, $P<0.01$). There was no evidence of difference in the number of moths that emerged when no spider or a risk spider was present. In two trials, no moths emerged when predation spiders were present.

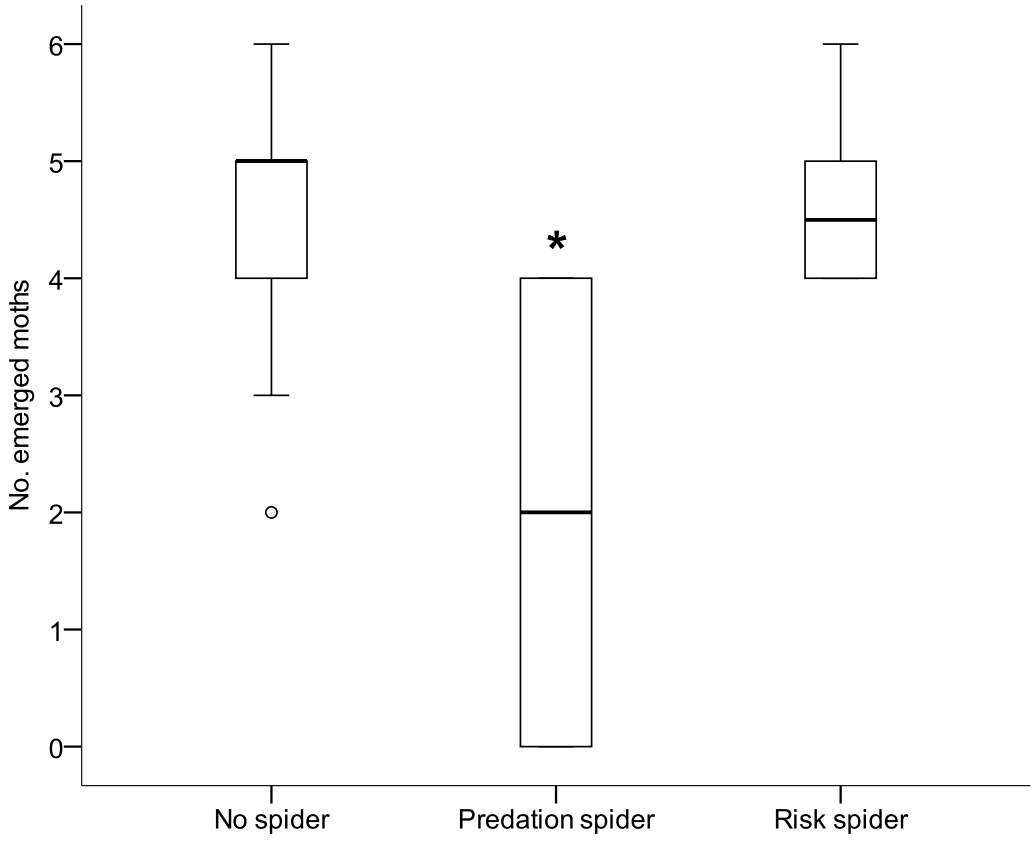

Figure 3. Number of moths emerged from enclosures with no spider, predation spider, and risk spider. Asterisk represents significantly different treatments. (ANOVA, $p < 0.05$). Horizontal line represents median, box length represents interquartile range, whiskers length are upper and lower quartiles, and dots represent suspect outliers in untransformed data (values greater than 1.5 box lengths).

The proportion of larvae on the plant, on the soil, or underground in each enclosure differed among treatments (no spider, risk spider, predation spider) over time (RM-

MANOVA: Wilk's $\lambda = 0.022$, $F_{8.78,257.07} = 1.960$, $P = 0.046$; Figure 4). To further understand what variables drive this difference, we looked at the effect of treatment, location, and time separately. There was no effect of treatment on the overall proportion of larvae on the plant, on the soil, or underground (between-subjects effect: $F_{2,114} = 0.527$, $P = 0.72$). We then examined the effect of each timepoint on proportion of larvae on the plant, on the soil, or underground among treatments. Only when time 1 (1 h after start) was excluded from the analysis, the proportion of larvae on the plant, on the soil, or underground was similar among treatments over time (RM-MANOVA excluding time 1: Wilk's $\lambda = 0.271$, $F_{6.40,187.33} = 1.391$, $P = 0.22$; excluding all other times, $P<0.05$). When examining each location individually, proportion of larvae on the plant (RM-MANOVA: Wilk's $\lambda = 0.843$, $F_{3.79,73.91} = 1.235$, $P = 0.30$), on the soil (Wilk's $\lambda = 0.737$, $F_{4.38,85.41} = 2.172$, $P = 0.073$), or underground (Wilk's $\lambda = 0.785$, $F_{4.49,87.58} = 0.893$, $P = 0.48$) was not influenced by treatment over time. There was a trend for proportionally more larvae to be on the soil when no spiders were present at time 1, but this was not significant (ANOVA: $F_{2,39} = 2.76$, $P = 0.076$; Figure 4).

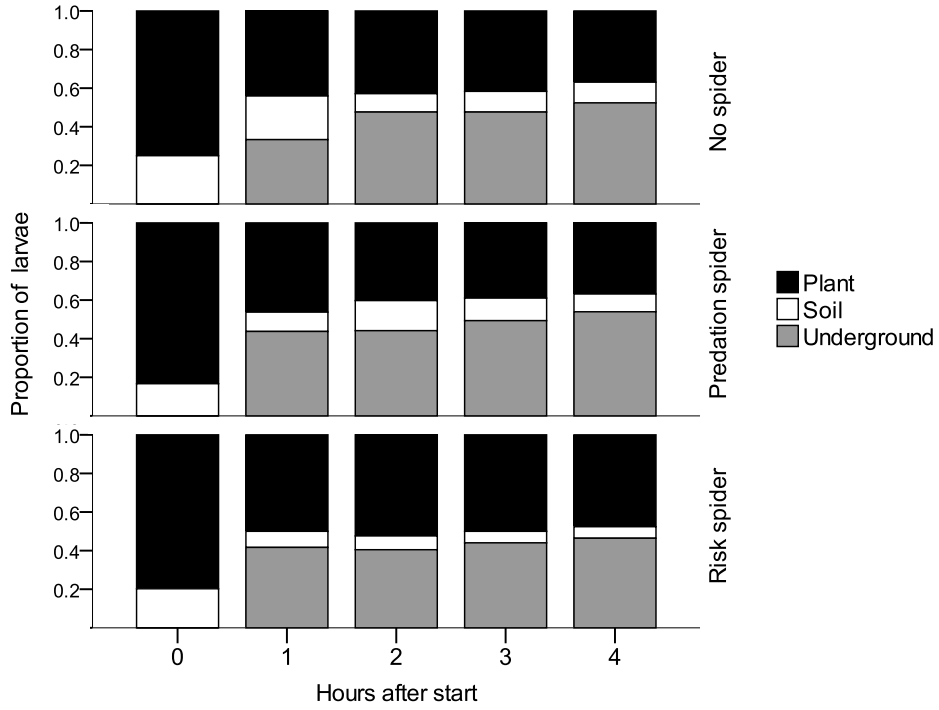

Figure 4. Average proportion of live *Helicoverpa armigera* larvae in each of three locations in cotton plant enclosures with no spider, predation spider, and risk spider. Bar size represents average proportion of larvae observed in each location per enclosure.

The proportion of larvae feeding, moving, still, or digging in each enclosure was not influenced by spider treatment over time (RM-MANOVA: Wilk's $\lambda = 0.343$, $F_{22.30,579.38} = 1.167$, $P = 0.27$; Figure 5). Duration of the larval, pupal, and emergence periods in each enclosure did not vary between no spider and risk spider treatments (Wilk's $\lambda = 0.683$, $F_{2,36} = 0.523$, $P = 0.60$; Figure 6).

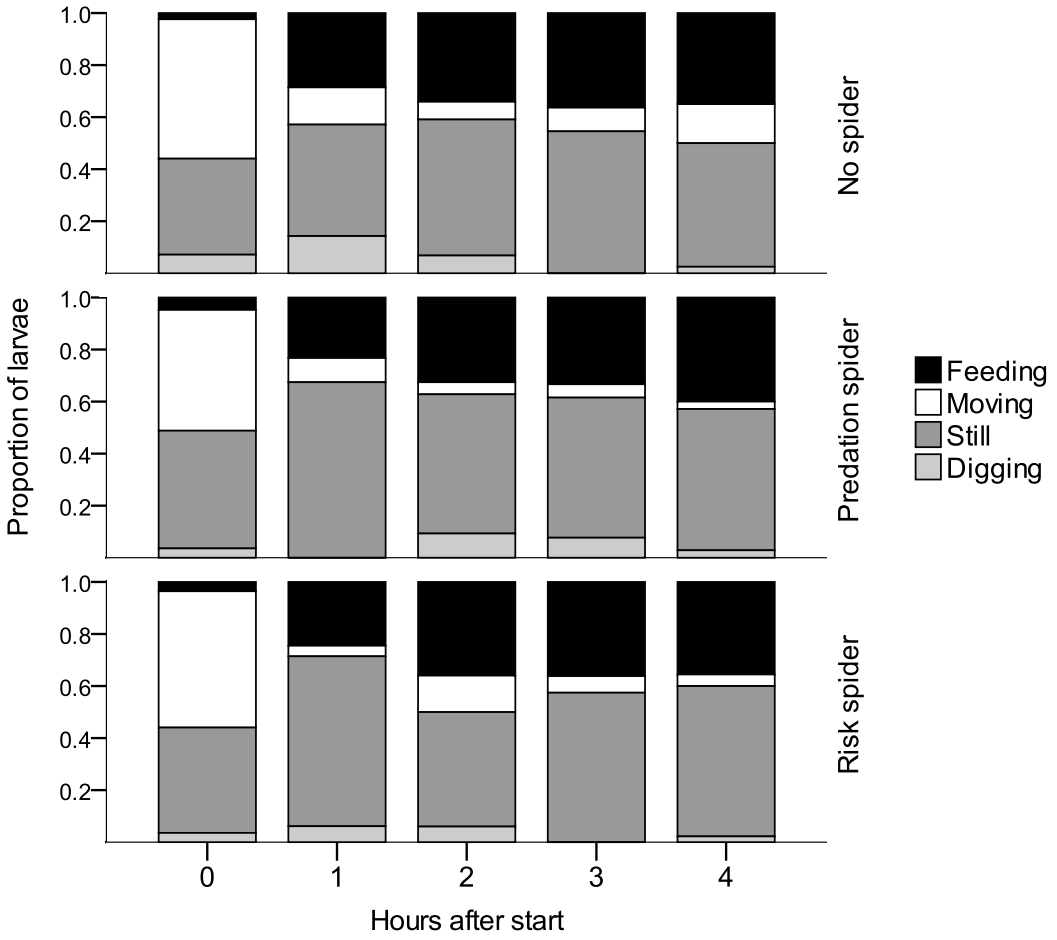

Figure 5. Average proportion of live *Helicoverpa armigera* larvae performing each of four behaviors inside cotton plant glasshouse enclosures with no spider, predation spider, and risk spider. Bar size represents average proportion of larvae observed performing each behavior per enclosure.

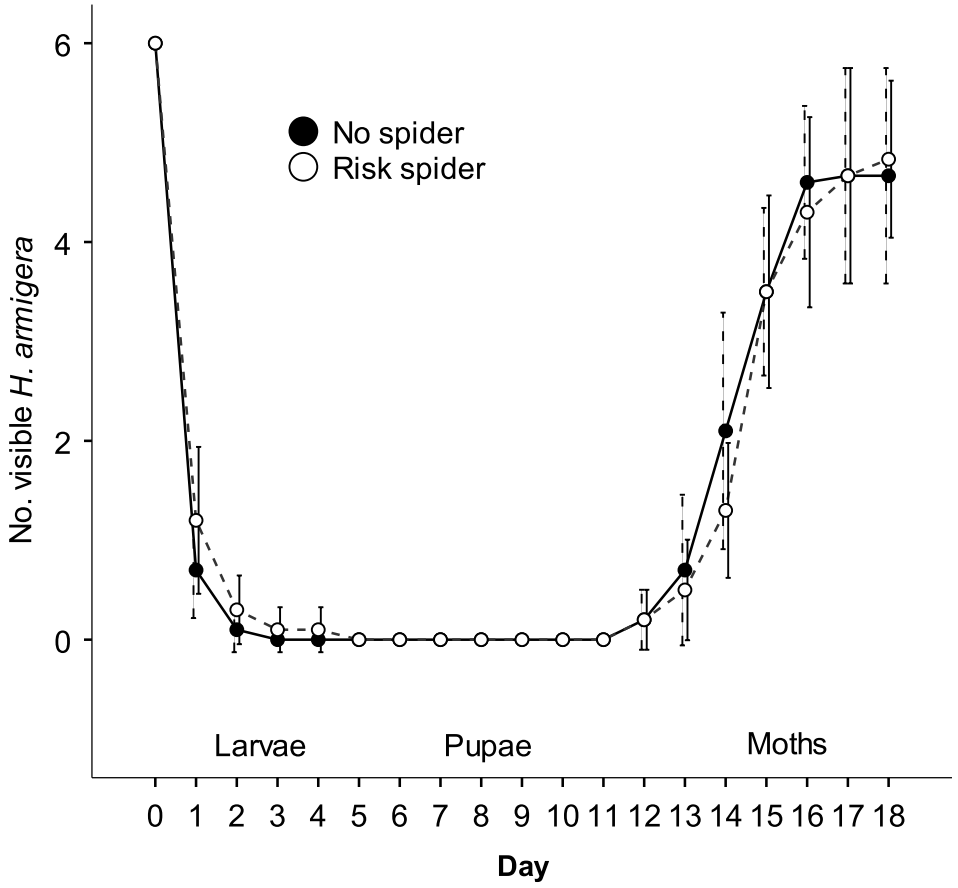

Figure 6. Number of visible *Helicoverpa armigera* through different life stages in glasshouse enclosures by day, with no spider and risk spider. Bars represent 1 SE.

Discussion

Helicoverpa armigera larvae that have succeeded in developing on Bt cotton and have the potential to convey genes for resistance to the next generation, are exposed to attack by ground-dwelling predators as they descend from the plant to pupate in the soil and again as they emerge from the soil as moths. The present study indicates that the wolf spider *T. leuckartii* cannot only affect *H. armigera* abundance directly through predation, but their presence is also associated with changes in *H. armigera* development and can influence trophic cascades that affect cotton production.

Consumptive effects

Tasmanicosa leuckartii is an effective predator of *H. armigera*, both when larvae descend from the plant to pupate and when the moths emerge. In this study, the density of *H. armigera* larvae offered in glasshouse enclosures (25 larvae / m^2) is much higher than economic threshold larval densities in cotton fields (2 larvae / m^2; Williams et al., 2011). This suggests that even in scenarios of very high pest densities, *T. leuckartii* can reduce the number of emerging *H. armigera* by 66% on average. This result is supported by laboratory observations, where spiders are not satiated after a single consuming a single larvae, and will continue attacking *H. armigera* even while it is still feeding. In some wolf spiders, females are more voracious predators than males (Nyffeler & Benz, 1988), but in the present study we did not find a difference in the likelihood of adult males, females, or late-instar juveniles attacking larvae and moths of *H. armigera*. Because a range of sex and age classes of *T. leuckartii* attack *H. armigera* at both the larval and moth stage, this spider has the potential to reduce proportions of Bt-resistant *H. armigera* throughout the cotton season.

Some studies of other predator/prey interactions in agro-ecosystems have failed to detect consumptive effects. For example, Van den Berg & Cock (1995) found no evidence that exclusion of natural predators in the field affected survival of late instars of *Helicoverpa* spp., or that consumptive effects of predators added significantly to natural mortality. Likewise, Beckerman et al. (1997) found no evidence that spider density affects the densities of grasshoppers in plant enclosures. In contrast, we observed that *T. leuckartii* has a consumptive effect on *H. armigera* that is significantly beyond the mortality exerted by other natural causes, as evidenced by the significant reduction in number of emerging moths in glasshouse enclosures compared to risk spiders, and recorded observations of predation.

Susceptibility to attack may differ between larvae and moths through differences in the amount of time that each remains near the ground where they potentially encounter a wolf spider. Recently emerged *H. armigera* moths have been reported to spend only a few seconds

on the soil before climbing up to the first plant they encounter (Riley et al., 1992), but in our experiments moths spent longer on the soil (between 0.1 and 21 h). Riley et al (1992) found that moths remain on stems between 2 and 25 cm from the ground, and the time from when their wings harden until they start crawling or fluttering is highly variable, ranging from 10 min to 8 h. Moths that rest on the lower stems are also vulnerable to spider predation, and many attacks may also occur after moths have climbed onto the plant from the soil. In our laboratory experiments, larvae exposed to a risk spider stayed on the soil for an average of 4 h before burrowing in the soil. Our study did not find a difference between larvae and moths in susceptibility to attack, suggesting that spiders may exert similar biological control pressure during moth emergence periods and pupation periods.

Non-consumptive effects: Mortality

In addition to assessing mortality due to consumptive effects, we also analyzed *H. armigera* mortality caused by the presence of *T. leuckartii* (lethal non-consumptive effects). Even in the absence of predation, natural mortality of first instar *H. armigera* in conventional cotton plant enclosures is ca. 40%, although it is expected that later instars are less vulnerable to natural mortality factors (Kyi et al., 1991; Van den Berg & Cock, 1995). We did not observe lethal non-consumptive effects in our study, since the level of mortality of *H. armigera* exposed to risk spiders did not differ from mortality in the absence of spiders, and was significantly lower than mortality due to consumptive effects. In contrast, Schmitz et al. (1997) reported that the mortality of grasshoppers exposed to predation spiders did not differ from those exposed to risk spiders and that both treatments had significantly higher prey mortality than in the absence of a spider, but the causes for non-consumptive mortality are not well understood (McCauley et al., 2011). In our study, the presence of a spider does not seem to increase the vulnerability of *H. armigera* to non-consumptive mortality factors.

Non-consumptive effects: Development

Helicoverpa armigera spent longer in the pupal stage in the presence of risk spiders than in the absence of spiders in the laboratory arenas. Studies of how predators influence development of holometabolous arthropods have tended to focus on motile larval stages (Van Buskirk & Saxer, 2001; Orizaola & Brana, 2005), overlooking how predator pressure affects the pupal stage. In laboratory experiments, emergence was delayed but not stopped in the presence of a risk spider. It is possible that a longer pupation period allows moths to develop stronger structures such as wings that promote faster escape and dispersal, or allows the pupa to remain safe underground until the predators move away. Insects will be advantaged if they can emerge from a pupal stage when predation risk is low (Koenig & Liebhold, 2013) or if they can minimize predation by synchronizing mass emergence in which each individual moth has decreased risk of being attacked due to the simultaneous abundance of conspecifics (Ito, 1998). In cotton fields, *H. armigera* tend to emerge in bouts of distinct cohorts through the cotton growing season (Baker et al., 2011). If the presence of a spider triggered synchronized emergence as an antipredatory strategy in *H. armigera*, then moths would have a reduced emergence period in the presence of *T. leuckartii*. However, in our glasshouse setting, the emergence period did not differ between treatments, therefore we detected no evidence of increased synchrony of emergence as an antipredator tactic.

The extended period of pupation in laboratory trials in the presence of a risk spider was not observed in glasshouse enclosures. Experimental settings could influence developmental times; for example, emergence of moths of the tobacco budworm has been found to occur later in field conditions than in enclosures (Lopez & Hartstack, 1983). It is possible that the larger arena and presence of a whole plant structure in glasshouse enclosures offered more protection from a ground predator than the smaller, structurally simpler, laboratory arenas, or that predators are more easily detected and can influence development in smaller settings. There is little information about the interactions between predation risk and

abiotic factors such as humidity and temperature, or biotic factors such as densities of conspecifics and predators on development in invertebrates (Stoks, 2001; Hawlena & Schmitz, 2010), but the results of our study suggest that developmental responses of *H. armigera* to predation may depend on the setting and environment.

Non-consumptive effects: Behavior

In glasshouse enclosures, we did not find evidence that the presence of a wolf spider affected *H. armigera* behaviour. Our results contrast those of Johnson et al. (2007), who found that early instar *H. armigera* change their feeding and resting behavior in the presence of a big-eyed bug predator. There are several possible explanations for the lack of behavioral responses of *H. armigera* to predators in glasshouse enclosures, including that (1) predator density in the glasshouse enclosures (1 per 0.5 m^2) was not high enough to induce behavioral effects, (2) the structural complexity of the enclosures did not allow larvae to detect the spider, or (3) *H. armigera* larvae do not respond to the killing of nearby conspecifics. Because *T. leuckartii* spent most time on the soil rather than on the plant, perhaps they are not detected or do not present sufficient risk to induce behavioral shifts in activity and feeding of larvae in glasshouse enclosures. Other studies have shown density-dependent responses to predators. For example, Whitehouse et al. (2011) found that mirids (another common cotton pest) modified their microhabitat use only in the presence of two spiders or more. Similarly, Bowler et al. (2013) found that differences in antipredatory behavior and trophic cascades were only detected at higher densities of predatory mites in a patch of herbivorous spider mites. Therefore, it is possible that *H. armigera* larvae would show changes in their behavior if exposed to a higher density of spiders in enclosures.

Habitat shifts are a common response of herbivores to the presence of predators (Werner & Peacor, 2003). An unexpected habitat shift was detected in laboratory arenas: in the presence of a risk spider, larvae spent more time on the soil (where they are more likely to

be attacked) and less time on the cotton boll (where the larvae forage), than when no spider was present. Plant drop-off is a common source of mortality for *H. armigera* neonates (Perovic et al., 2008), but the tendency to drop off (regardless of predation risk) declines in older instars. Because the plant foliage is the first microhabitat where larvae are exposed to predators in nature, *H. armigera* might leave the plant and move to the soil if any predator is detected (Johnson et al., 2007). In smaller, simpler laboratory arenas it is possible that *H. armigera* were able to detect volatile chemicals (Dicke & Grostal, 2001) or vibrational cues from a predator, even when the larva was on the cotton boll, a few centimeters from where the spider was on the soil. Wolf spiders have been reported to release volatile kairomones that enable prey to detect their presence from a distance (Schonewolf et al., 2006). It is possible that larvae cannot discriminate between foliage or ground predator cues, and therefore leaving the plant is a general response of a late instar when detecting any predator cues. The proportion of larvae on the plant over time was not influenced by treatment in glasshouse enclosures, illustrating again how different environmental settings may affect how prey detect and respond to predators, and influence non-consumptive effects.

Even though wolf spiders are mainly ground rather than foliage predators, there were instances in which the spider climbed onto the plant in glasshouse enclosures, or knocked the larva from the cotton boll in laboratory arenas. Even though wolf spiders can detect prey vibrations through soil (Wrinn & Uetz, 2008), we never observed spiders digging larvae or pupae out from the ground. Therefore, underground is the safest microhabitat for *H. armigera* to avoid this predator. However, we did not observe (in either laboratory or glasshouse experiments) larvae digging and burrowing underground sooner in the presence of a risk spider, which suggests *H. armigera* do not dig under the soil as an immediate escape strategy to evade *T. leuckartii*.

Trophic cascades

There was evidence in the present study of a trophic cascade that connects spiders as predators to the cotton plant. Indirect effects from predators through consumers can lead to negative as well as positive effects on primary producers (Werner & Peacor, 2003). In the present study, the presence of a spider was associated with increased loss of mass from the cotton boll in the laboratory experiment. This finding differs from other studies, in which the presence of a predator more often triggers reduced foraging due to increased vigilance or escape behavior (Joern et al., 2006; Johnson et al., 2007). However, some other studies have shown trophic cascades that reduced plant productivity; for example, the presence of wolf spiders has been associated with diminished production in cucurbit crops (Snyder & Wise, 2001). Spiders have been reported to trigger increased loss of biomass in herbs by inducing habitat shifts in grasshoppers (Beckerman et al., 1997). Nevertheless habitat shifts do not explain results of the present study, as *H. armigera* larvae spent less time on cotton bolls when a spider was present. Furthermore, presence of a risk spider did not induce changes in the proportion of feeding larvae in glasshouse enclosures.

There are several observations that suggest that cotton boll mass loss is mediated through *H. armigera* behavior. First, the mass lost in the cotton bolls was not different between predation and risk spider treatments. If trophic cascades in our experiments were influenced by consumptive effects, then cotton bolls in the predation spider treatment would have lost less mass than cotton bolls in the risk spider treatment. Second, the initial mass of the boll did not correlate with the percentage of mass lost, or whether larvae spent more or less time foraging on it. This suggests that boll weight loss was influenced by the larva and the spider, and not the boll's initial mass. A possible explanation for this result is that larvae forage more intensively when they are initially placed on the plant, and before they descend to the soil (and are possibly killed). The presence of a spider may trigger a 'dine and dash' effect in *H. armigera* larvae: given that a larva spends less time on a cotton boll in the presence of a spider, it is possible that the larvae increase the amount of plant mass that they consume in a

shorter period of time – i.e., that they eat faster. Another possibility is that *H. armigera* modified the way it forages, rather than the mass it consumes. Mass lost from the cotton boll is also caused by water loss, therefore it is possible that bolls with more extensive superficial damage lost more water than bolls with a single deep cavity. Altogether, these results support the idea that trophic cascades triggered by wolf spiders are mediated by behavior (non-consumptive effect) rather than by predation (consumptive effect).

Overall, this study shows that consumptive and non-consumptive effects may affect cotton production in a positive or negative way. However, the positive effects of consumptive effects are more likely to overshadow any negative non-consumptive effects. For example, although there was a negative trophic cascade which affected cotton boll mass in the presence of a spider in a small-scale container, we did not find evidence of wolf spiders exerting non-consumptive effects in larger glasshouse enclosures, a scenario more similar to a large-scale cotton field. Additionally, from an industry perspective, once a boll is damaged it cannot be harvested, so increasing the damage inflicted on one boll has less effect than many larvae damaging many bolls less intensively. Therefore, an increase in the amount of damage caused to individual bolls would not detract from the presence of wolf spiders reducing *H. armigera* numbers.

Second, *T. leuckartii* was an effective predator of *H. armigera* larvae as they descend to pupate in soil and later when they emerge as moths, both in small laboratory containers and larger glasshouse enclosures, emphasizing the importance of the wolf spiders consumptive effect on *H. armigera*. Third, predatory capacity trials showed that spiders kill multiple larvae, even when they are still feeding. Consumptive effects can have a positive effect on the efficacy of Bt cotton by reducing Bt-resistant *H. armigera*. In a cotton field where *H. armigera* cannot be destroyed by 'pupae busting', the presence of *T. leuckartii* offers an alternative method to control this pest, and should be taken into consideration when designing and implementing integrated pest management programs.

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CHAPTER 4: Intraguild interactions between two wolf spider species lead to non-additive effects on the biological control of the cotton bollworm

Chapter formatted for publication in *Agricultural and Forest Entomology*

Abstract

1. Biological control of pests exerted by a guild of predators might be hindered by intraguild interactions. This study examined the predator-prey interactions between two species of wolf spiders (*Tasmanicosa leuckartii* [Thorell] and *Hogna crispipes* [Koch]), and the effect of such interactions on the control of the cotton bollworm, *Helicoverpa armigera* (Hübner).
2. Experiments in glasshouse enclosures investigated whether the number of *Helicoverpa* larvae surviving to pupation changed in the presence of two wolf spiders (either conspecifics or heterospecifics) compared to one spider. These trials revealed no difference in *Helicoverpa* survival when one or two conspecific spiders were present. However, significantly more *Helicoverpa* were killed when both *Tasmanicosa* and *Hogna* were in the same enclosure compared to *Tasmanicosa* alone, indicating that a community with a mix of *Tasmanicosa* and *Hogna* can have an improved (but not additive) effect on controlling *Helicoverpa* than one with only *Tasmanicosa*. The lack of additive effects in biological control of *Helicoverpa* was attributed to prey choice and satiation, reduced hunting time, and behavioral modifications.
3. Video recorded laboratory experiments described the predator-prey interactions among *Tasmanicosa, Hogna* and *Helicoverpa. Tasmanicosa* killed fewer *Helicoverpa* in the presence of *Hogna* compared to when *Hogna* was not present in the arena. In contrast, *Hogna* killed *Helicoverpa* in similar proportions regardless of whether *Tasmanicosa* was present in the same arena or not. We also observed that spiders did not kill *Helicoverpa* once they had fed on another spider.
4. Spider size and weight were strong predictors of intraguild predation and, as a consequence, the dynamics of intraguild interactions are likely to vary throughout the season according to the phenology of each spider species.

Introduction

Predators are an important element of biological control in many agricultural systems, and pest management practices are often developed to maximize predator abundance. But in addition to hunting target pests, predators commonly interact with one another, influencing biological control (Letourneau *et al.*, 2009; Snyder & Tylianakis, 2012). In some cases, the presence of multiple predators in agricultural systems has been found to have additive or synergistic effects on the efficacy of either or both predators on reducing pest densities (Dinter, 2002; Lang, 2003; Sigsgaard, 2007). However, other studies have found no net effect of predator diversity on biological control (Denno *et al.*, 2004; Venzon *et al.*, 2001). Further, owing to interference amongst predators, the presence of multiple predators may even diminish biological control (Finke & Denno, 2003; Finke & Denno, 2004; Snyder & Wise, 2001). Given these diverse biological control outcomes, it is important to understand the interactions between predators, and their consequences for pest control.

There are several mechanisms through which biological control may be hindered by interactions among predators. Most research has focused on direct predation among members of the same guild (intraguild predation – IGP). Intraguild predators benefit not only by directly obtaining nutrients, but also by reducing potential competition (Hodge, 1999). If a top predator species hunts an intermediate predator (or 'mesopredator'; Mueller & Brodeur, 2002) that is more specialized and effective at attacking a target pest species, then intraguild predation reduces the density of more effective biological control agents (Prasad & Snyder, 2004). Additionally, a top predator that usually hunts a pest may switch prey preferences in the presence of another predator, choosing to hunt the other predator instead (Bjorkman *et al.*, 2011; Finke & Denno, 2003). Not all intraguild interactions involve a predator killing and consuming another predator (consumptive effects). The presence of other predators can also affect each predator's hunting behavior and microhabitat selection, thereby constraining each predator's capacity to hunt a targeted pest (non-consumptive effects; Sato *et al.*, 2005;

Whitehouse *et al.*, 2011). By understanding how biological control might be disrupted by interactions amongst predators, it may be possible to design farming practices that maintain an optimal predator community to maximize control (Mueller & Brodeur, 2002).

The present study focuses on predator-prey interactions between the cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae; referred to hereafter as '*Helicoverpa*'), and two species of wolf spider, *Tasmanicosa leuckartii* (Thorell) and *Hogna crispipes* (Koch) (Araneae: Lycosidae; referred to hereafter as '*Tasmanicosa*' and '*Hogna*', respectively) that are native to cotton fields (*Gossypium hirsutum* L., Malvaceae) in the Namoi valley, New South Wales, Australia. Larvae of *Helicoverpa* are a major pest of Australian cotton crops, historically costing millions of dollars annually in crop loss and chemical control measures (Fitt *et al.*, 2009). *Helicoverpa* larvae feed on cotton plants, and then descend to pupate in soil (Fitt, 1989). To control *Helicoverpa*, genetically modified 'Bt cotton' was introduced in 1996 (Whitehouse et al., 2009b). Bt cotton is highly effective in controlling *Helicoverpa*, but emerging resistance to Bt toxins is a constant concern to the cotton industry (Downes & Mahon, 2012). Having completed four to six generations during the cotton season (Baker *et al.*, 2011), surviving Bt resistant *Helicoverpa* pupae overwinter in the soil and emerge in spring, conveying genes for Bt insecticide resistance from one season to the next (Lloyd *et al.*, 2008). Persistence and proliferation of Bt resistance could be countered by identifying and exploiting non-insecticidal control measures, such as predators that attack Bt-resistant *Helicoverpa* during their descent as late instar larvae or emergence as moths.

Wolf spiders are abundant generalist predators that attack a wide range of pests in agroecosystems (Kuusk & Ekbom, 2012; Nyffeler & Benz, 1988; Oberg & Ekbom, 2006; Pearce *et al.*, 2004), and are usually the largest ground dwelling spiders in Australian cotton agroecosystems (Whitehouse *et al.*, 2009). Wolf spiders have been reported to feed on *Helicoverpa* spp. larvae as well as other pests in cotton fields (Johnson *et al.*, 2000).

However, wolf spiders are also intraguild predators that attack other spiders (Sitvarin & Rypstra, 2014), often including their own species (Balfour *et al.*, 2003; Wise, 2006). Intraguild predation among spiders is especially prevalent in non-web builders such as wolf spiders, as the active hunting behavior of these spiders increases the frequency with which they encounter other spiders (Hodge, 1999).

Tasmanicosa is commonly found in cotton fields in the Namoi valley, New South Wales, Australia, and has the largest body size of all ground spiders recorded in fields surrounding the Australian Cotton Research Institute. *Tasmanicosa* is more abundant in minimum-tilled fields than in conventionally tilled fields (Rendon *et al.*, 2015). Because of its large body size (see methods) *Tasmanicosa* can attack late-instar *Helicoverpa* larvae, but can also engage in intraguild predation by killing and eating smaller species of wolf spiders present in cotton fields. *Hogna*, another ground-hunting nocturnal predator present in cotton fields, is a potential prey and competitor of *Tasmanicosa*. *Hogna* is more abundant than *Tasmanicosa* in minimum-tilled cotton fields at the Australian Cotton Research Institute (Rendon *et al.*, 2015), but has a smaller body size (see methods). *Hogna* is an effective colonizer of tilled cotton fields (Rendon *et al.*, 2015), and is usually found on the edges of springs and in the ephemeral wet zone beyond the permanent vegetated wetland (Framenau *et al.*, 2006). Both *Tasmanicosa* and *Hogna* are abundant in cotton fields when the first generation of *Helicoverpa* larvae of each season descend from the plant to pupate (Baker *et al.*, 2011), through until the last generation of *Helicoverpa* larvae descends to overwinter. As *Tasmanicosa* and *Hogna* overlap in their seasonal abundance and are both generalist predators, these two spider species are likely to compete for resources in cotton fields and their combined efficacy as biological control agents may be impeded by intraguild predation or behavioral interference.

The aim of the present study is to determine how interactions between *Tasmanicosa* and *Hogna* might affect each species' efficacy in controlling *Helicoverpa*. Specifically we

ask: 1) is predation on *Helicoverpa* larvae by *Tasmanicosa* or *Hogna* decreased by presence of the other spider species; 2) what is the tendency of each spider species to attack the other spider species and *Helicoverpa* larvae, and 3) does the presence of two spider species have an additive effect in reducing *Helicoverpa* numbers?

Materials and methods

Spider collection and Helicoverpa rearing

This study was carried out at the Australian Cotton Research Institute (33ºS, 149ºE), near Narrabri, New South Wales, Australia, during the 2013 - 2014 cotton-growing season. Specimens of *Tasmanicosa* and *Hogna* wolf spiders were collected in and around cotton fields after sunset (approximately 2030 hrs) from November 2013 (one month after cotton sowing) until April 2014 (before cotton harvest). Spiders were found by visual search using a headlamp (Petzl Tikka, 140 lumens), and were collected manually in clear 70 mL cylindrical plastic containers. During the time spent collecting spiders, it was also noted where spiders were located (soil, grass, plants), and whether spiders were holding any prey. After collecting, spiders were brought to the laboratory where cephalothorax width was measured to the nearest 0.1 mm using a manual caliper (resolution 0.1 mm), and the spider's sex and life stage (adult or juvenile) was recorded. Since abdomen size and body weight can vary with nutrition or reproductive state of females, cephalothorax width has been used as a fixed measure of spider size in previous studies (Buddle *et al.*, 2003; Hagstrum, 1971; Humphreys, 1976), and will be used hereafter to refer to 'size'. A measure of body condition was determined by extracting the residuals of a linear regression between $\log_{10}$(cephalothorax width) and $\log_{10}$(body weight) separately for males, females and juveniles. This residuals method has the advantage of controlling for variation in body sizes (Jakob *et al.*, 1996), while a logarithmic transformation accounts for differences in unit residuals for different body sizes (Kotiaho, 1999). Adult males, females, and juveniles that already bore the distinctive cephalothorax

pattern characteristic of each species were used in the experiments. All spiders were used in experiments only once, and were then released back in the fields.

Larvae of *Helicoverpa* were supplied by the Commonwealth Scientific and Industrial Research Organization (CSIRO) Agricultural Flagship Bt Resistance Monitoring Group. Larvae were reared in individual wells in trays with soy and agar diet (for protocol, see (Downes *et al.*, 2009; Teakle & Jensen, 1985) and were maintained in a controlled temperature room (24.4 ± 0.5 ^{o}C, mean ± SD) with a L14:D10 photoperiod. Larvae were reared until they reached 5th instar and were then weighed to the nearest 0.01 g using a digital scale (Sartorius model A200S).

Wolf spider effect on* Helicoverpa *survival

Glasshouse experiments were designed to test whether the presence of *Tasmanicosa* and *Hogna* individually and together affects the number of *Helicoverpa* larvae surviving to pupation. This experiment was carried out in 18 glasshouse enclosures that comprised 24-week old cotton plants (variety Sicot 71 RRF conventional, non-Bt) planted in plastic crates 60 (l) × 40 (w) × 40 (h) cm and covered by a clear plastic and mesh insect cage adapted to fit the crate, 60 (l) × 40 (w) × 60 (h) cm (MegaView Bug Dorm, model 2120). Plants were watered every two days, and fertilizer (Thrive™ soluble all purpose plant food, Yates products) was applied to each crate one day before each trial following manufacturer's instructions. No pesticides were applied to the plants during the trials, but the leaves were manually washed with a 1:10 (vol.) household detergent water solution (Palmolive unscented dishwashing liquid) one day before each trial to control mite infestations, and any detergent residues were rinsed with water. Each enclosure was randomly assigned to one of six treatments: (1) No spiders, (2) one *Tasmanicosa* (cephalothorax width = 7.6 ± 1.1 mm; mean ± SD), (3) one *Hogna* (cephalothorax width = 5.2 ± 0.2 mm, mean ± SD), (4) two *Tasmanicosa* (cephalothorax width larger spider = 8.2 ± 1.1 mm, cephalothorax width smaller spider = 6.4 ± 0.9 mm; mean ± SD), (5) two *Hogna* (cephalothorax width larger spider = 5.7 ±

0.9 cm, cephalothorax width smaller spider = 5.0 ± 0.9 mm; mean ± SD), and (6) one *Tasmanicosa* (cephalothorax width= 8.5 ± 1.2 mm; mean ± SD) with one *Hogna* (cephalothorax width= 5.0 ± 0.6 mm; mean ± SD). All spiders were assigned to a treatment according to sex and size on the night they were collected; paired spiders were of the same sex or were juvenile (to prevent mating behavior), and of different sizes (expressed as cephalothorax width of larger spider / cephalothorax width of smaller spider; *Tasmanicosa* was always larger than *Hogna*). Experiments were carried out in February and March 2014 (late summer/early autumn, glasshouse daily maximum temperature = 33.98 ± 3.21 ºC, daily minimum temperature = 22.72 ± 2.06 ºC; mean ± SD). Experiments were repeated four times, with a two-day interval between the end of one trial and the start of the next. Each assigned treatment was rotated across enclosures so that no enclosure received any treatment twice (N= 3 for each treatment on four dates, for a total of N= 12 for each treatment).

Collected spiders were sexed, measured, and released inside the glasshouse containers on the evening that they were collected. The released spiders were isolated from each other inside the glasshouse enclosures by placing an opaque PVC pipe (100 mm diameter x 150 mm height) over them. Trials were started the following evening (day 0). At sunset, between 1930 h and 2030 h, six 5th instar *Helicoverpa* larvae were placed on the leaves of the cotton plants in each enclosure, and the PVC pipes were removed so that spiders could explore and interact. Trials were terminated after three days, time by which all 5th instar larvae have already descended from the plant and gone underground to pupate (Rendon et al, in press). In each enclosure the top 10 cm of soil was removed and sieved to count number of pupae. Number and species of surviving spiders was recorded.

A-priori tests were carried out to test for interactions between dates and treatment on number of *Helicoverpa* pupae. Since no effects were found, a one-way Kruskal-Wallis ANOVA was carried out on untransformed data, with post-hoc pair-wise Mann-Whitney tests

used to test for differences in number of pupae among the six treatments. All tests were carried out using SPSS version 20 (IBM, 2011).

Intraguild competition and predation

Laboratory experiments were carried out to investigate how interactions between *Tasmanicosa* and *Hogna* influence survival of *Helicoverpa*. One 5th instar *Helicoverpa* larva (weight = 0.41 ± 0.06 g, mean ± SD) was paired with (1) one *Tasmanicosa* (cephalothorax width = 7.4 ± 1.1 mm, mean ±SD; N=27), (2) one *Hogna* (cephalothorax width = 4.7 ± 0.9 mm, mean ±SD; N=19), or (3) one *Tasmanicosa* (cephalothorax width = 7.2 ± 1.4 mm) with one *Hogna* together (cephalothorax width = 5.9 ± 0.9 mm; N=29). Each spider (male, female or juvenile) was randomly assigned to treatment on the night it was collected. Experiments commenced in November 2013 (one month after cotton sowing), and ended in March 2014.

Collected spiders were housed in clear plastic containers (228 mm height x 238 mm length x 238 mm width, 8.5 L, Décor Tellfresh superstorer®) containing 2 L of moist soil, and were kept in a controlled temperature room (24.4 ± 0.5 ^{o}C, mean ± SD) with a L14:D10 photoperiod. Each container had a retreat in each of two opposite corners (one for each spider), comprising a hole in the soil approximately 2 cm deep and 1 cm diameter. The retreat was partially covered by a 3 x 3 cm sheet of bark from a 'Paper Bark tree' (*Melaleuca sp.*). Spiders in treatment 3 (two spiders, one *Helicoverpa*) were housed in the same container, but were initially isolated from each other by placing an opaque PVC pipe (100 mm diameter x 150 mm height) over the retreats. Single spiders were housed individually, but were isolated from the rest of the container by a PVC pipe as well, to ensure uniform methods across treatments. Each spider was kept in the laboratory for 24 hours before being used in experiments. No food or water was supplied.

Trials in treatment 3 (*Tasmanicosa* and *Hogna* with *Helicoverpa*) were video recorded for 24 hours; the recording system comprised a 1/3" CCD monochromatic infra-red camera (CCS- Sony Go Video) with a 4 mm C mount lens positioned above each container, which

recorded to a 2TB DVR4-100 hard drive recorder. One infrared (940nm) illuminator (IR-covert) was placed 10 cm to the side of each container. To improve video contrast, each animal was dusted with fluorescent dye (HCA Colours Australia, VM311 Pink for *Helicoverpa* larvae, VM317 Yellow for *Tasmanicosa*, VM315 Orange for *Hogna*). Trials started 30-60 minutes after the dark phase commenced in the laboratory; one 5th instar *Helicoverpa* larva was placed inside the container, and the PVC pipes removed to allow spiders to explore and hunt. Continuous video recording began immediately after *Helicoverpa* was released, and ended 24 hrs later. At the end of each trial, we recorded (1) if *Tasmanicosa* or *Hogna* killed *Helicoverpa*, (2) whether one spider had killed the other, and (3) which spider had killed *Helicoverpa* when paired together.

To determine time budgets and activity scores of each spider, we observed the videos of treatment 3 (*Tasmanicosa* and *Hogna* paired together), once each hour for 24 hrs. The activity of the spiders was evaluated and scored as follows: a score of 2 was given if after one hour the spider had displaced its whole body from one part of the enclosure to a different part. A score of 1 was given if after one hour the spider had remained in the same location, but had moved its limbs or oriented to face a different direction. Finally, a score of 0 was given if after one hour the spider had remained completely in the same location and facing the same direction. We recorded 24 activity scores in total for each trial (one for each hour assessed). To test for differences in activity between spider species, or for species differences in spider activity at a particular time, a univariate analysis of variance (ANOVA) was carried out on activity scores, using spider species, time, and species*time as fixed effects and trial number as a random effect.

A 2x3 contingency test (Pearson chi-square) was used to test for differences in the proportion of *Helicoverpa* that survived in each treatment. An additional contingency test was used for treatment 3 to test for differences in the proportions in which *Tasmanicosa* and *Hogna* each killed *Helicoverpa* or the other spider. It should be noted that predation outcomes

for each spider species in treatment 3 were not intended to be independent from each other; to elucidate potential interdependent effects, Chi-square tests were performed on each spider species as a focal spider separately, but in the same trial. To determine whether natural variation in spiders affects predation outcomes, six predictors were chosen: 1) focal spider size, 2) focal spider weight, 3) focal spider body condition 4) focal spider sex, 5) *Helicoverpa* weight, and 6) spider size ratio (*Tasmanicosa* cephalothorax width / *Hogna* cephalothorax width). A binary logistic regression was used to determine whether each of the six predictors independently predicted the odds of: 1) *Tasmanicosa* killing *Helicoverpa*, 2) *Tasmanicosa* killing *Hogna*, 3) *Hogna* killing *Helicoverpa*, and 4) *Hogna* killing *Tasmanicosa*. Due to sample size restrictions and multicollinearity violations, each predictor was individually evaluated in one-way regressions. All tests were carried out using SPSS version 20 (IBM, 2011).

Results

Wolf spider behavior in the field

Both *Tasmanicosa* and *Hogna* were abundant at the time of collecting, which spanned between 30 minutes to 2 hours after sunset. Spiders were mostly found immobile, or went into burrows as soon as the light shone on them. *Tasmanicosa* was always found on the soil or grass at ground level, while *Hogna* was mostly found on the soil or grass, but sometimes sitting on the blades of long grasses or weeds, raised 5-10 centimeters above ground. Intraguild predation between *Tasmanicosa* and *Hogna* was not observed in the field. Predation events in general were very rare: in over 50 hours of observation while collecting spiders for experiments, *Hogna* was found holding prey (the ground cricket *Teleogryllus commodus*) on only three occasions, while *Tasmanicosa* was never observed holding prey.

Wolf spider effect on Helicoverpa *survival*

In the glasshouse experiments, the number of larvae surviving to pupation in the no-spider treatment was significantly higher than that of all the other treatments (Kruskal-Wallis ANOVA, χ^2= 29.05, df= 5, p< 0.01; post-hoc Mann-Whitney, all pairwise comparisons p< 0.01). There were no significant differences in the number of larvae surviving to pupation in cages containing one or two *Tasmanicosa*, and likewise, there was a similar number of surviving pupae in cages containing one or two *Hogna.* Cages containing one *Tasmanicosa* had significantly more larvae surviving to pupation than cages with one *Tasmanicosa* together with one *Hogna* (Mann-Whitney U= 35.50, p= 0.033; Figure 1). There was no significant relationship between spider size ratio and number of *Helicoverpa* larvae surviving to pupation in any spider treatment (Spearman's Rho, p>0.05 for all treatments). Number of spiders alive at the end of the trial did not affect the number of larvae surviving to pupation (ANOVA, F= 0.066, df= 1, 30, p= 0.80).

When two *Tasmanicosa* were paired, one spider killed the other in 41.6% of the trials. When two *Hogna* were paired, one spider killed the other in 58.3% of the trials. When one *Tasmanicosa* and one *Hogna* were paired, *Tasmanicosa* killed *Hogna* in 58.3% of the trials. In these trials *Tasmanicosa* were always larger than *Hogna*, and there were no instances of *Hogna* killing *Tasmanicosa* in the glasshouse trials. Number of spiders alive at the end of the trial was similar between treatments with two *Hogna*, two *Tasmanicosa*, and one *Hogna* with one *Tasmanicosa* (χ^2= 0.892, df = 2 p= 0.640).

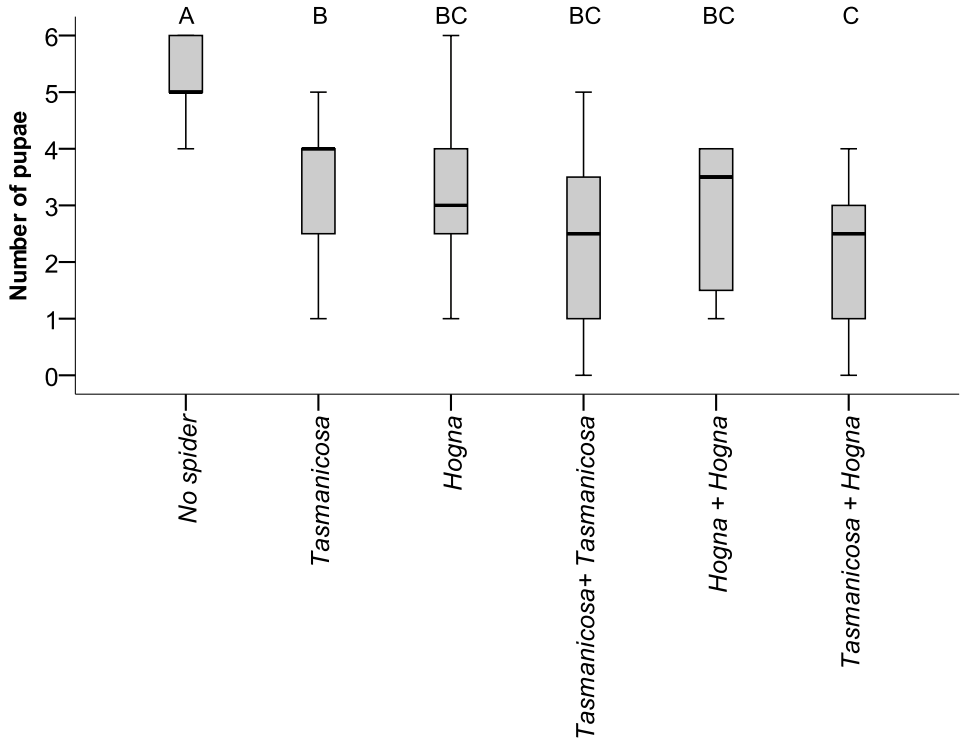

Figure 1. Number of surviving *Helicoverpa armigera* pupae in glasshouse enclosures in each spider treatment. Different letters represent significantly different treatments. Horizontal line represents median, box length represents interquartile range, whiskers length are upper and lower quartiles.

Intraguild competition and predation

In laboratory trials there was no significant difference in the proportion of *Helicoverpa* killed with (1) one *Tasmanicosa,* (2) one *Hogna,* and (3) both spider species together (χ^2= 1.059, df= 2, p= 0.589). *Helicoverpa* mortality was high in all treatments (*Tasmanicosa* = 96.5%, *Hogna* = 89.6%, *Tasmanicosa* + *Hogna* = 89.6%). *Tasmanicosa* killed *Helicoverpa* more often when alone than when paired with *Hogna* (χ^2= 32.63, df= 1, p< 0.01). In contrast, the frequency with which *Hogna* killed *Helicoverpa* was not significantly influenced by the presence of *Tasmanicosa* (χ^2= 2.733, df= 1, p= 0.094; Figure 2). When both spiders were present together, *Hogna* was more likely than *Tasmanicosa* to kill *Helicoverpa* (χ^2=13.663, df=1, p< 0.01). This result also reflects encounter rate: in 20 out of 29 trials, *Hogna* came in contact with *Helicoverpa* before *Tasmanicosa* did. When both spiders were present, *Tasmanicosa* was similarly likely to kill *Helicoverpa* or *Hogna,* but *Hogna* was more likely to

kill *Helicoverpa* than to kill *Tasmanicosa* (χ^2=4.786, df = 1, p = 0.029). On three occasions *Hogna* killed *Tasmanicosa* after killing *Helicoverpa.* In contrast, *Tasmanicosa* only killed either *Helicoverpa* or *Hogna*, but never both. *Helicoverpa* was never killed in trials in which one spider had eaten the other. *Tasmanicosa* stole *Helicoverpa* from *Hogna* on three occasions, whereas *Hogna* stole *Helicoverpa* from *Tasmanicosa* once.

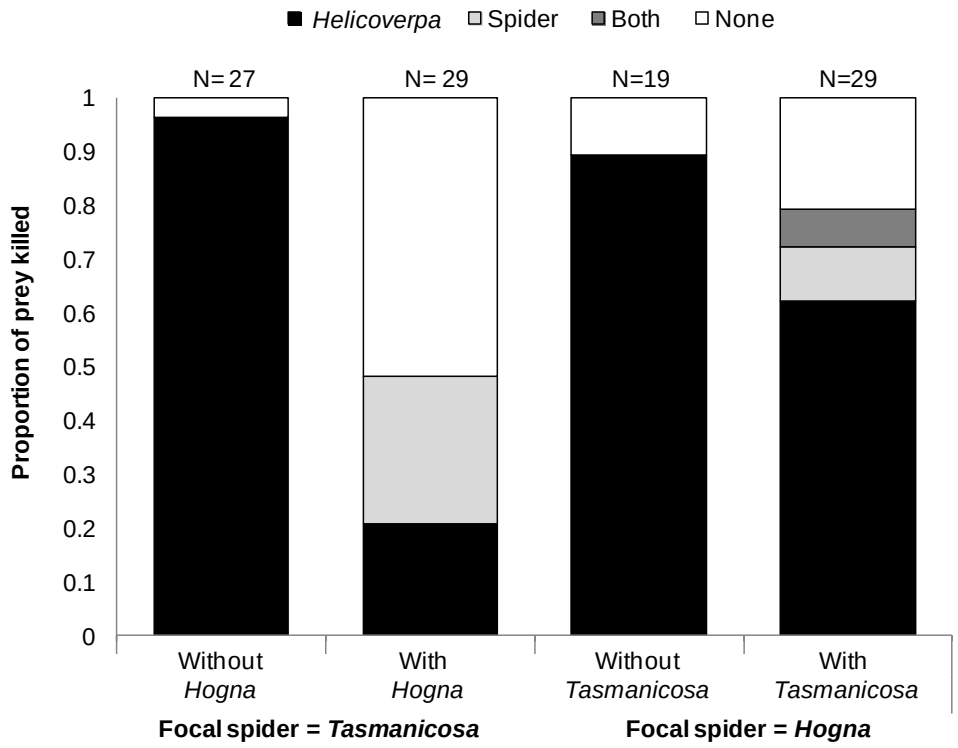

Figure 2. Proportion of attacks by either *Tasmanicosa leuckartii* or *Hogna crispipes* on *Helicoverpa armigera* or each other when tested in isolation or paired together with the other spider species in laboratory arenas. Males, females and juveniles were pooled together as the analysis failed to find a relationship between spider sex and intraguild predation. The second bar for each spider represents the same trials in treatment 3, but analyzed separately for each spider species.

For *Tasmanicosa*, spiders with a lower body condition were more likely to kill *Helicoverpa* (binary logistic regression, χ^2 = 4.96, p = 0.02, R^2 = 0.24; Table 1) but none of the other independent variables (spider size, spider weight, spider sex, *Helicoverpa* weight, spider size difference) influenced likelihood of *Tasmanicosa* killing *Helicoverpa*. For *Hogna*

none of the independent variables significantly predicted likelihood of killing *Helicoverpa* (Table 1). For *Tasmanicosa*, spider size, spider weight, body condition, *Hogna* weight and spider size ratio significantly predicted the likelihood of killing *Hogna*. *Tasmanicosa* that were larger or heavier, had a higher body condition, were proportionally larger than *Hogna*, or were exposed to heavier *Helicoverpa* were more likely to kill *Hogna.* For *Hogna*, spider size, spider weight, and spider size ratio significantly predicted the likelihood of killing *Tasmanicosa*. *Hogna* that were larger, heavier, or proportionally larger than *Tasmanicosa* were more likely to kill *Tasmanicosa* (Table 1, Figure 3).

Tasmanicosa and *Hogna* did not differ significantly in activity rank when both species were paired in the same arena (ANOVA, $F= 2.160$, $df= 1, 475$, $p= 0.142$). Also, there was no spider species*time interaction on activity rankings, meaning that both spiders show similar patterns of activity through the day (ANOVA, $F= 0.735$, $df= 23, 475$, $p= 0.811$). Both spider species were more active throughout the 10 hours of the night (ANOVA, $F= 20.252$, $df= 23, 475$, $p< 0.01$).

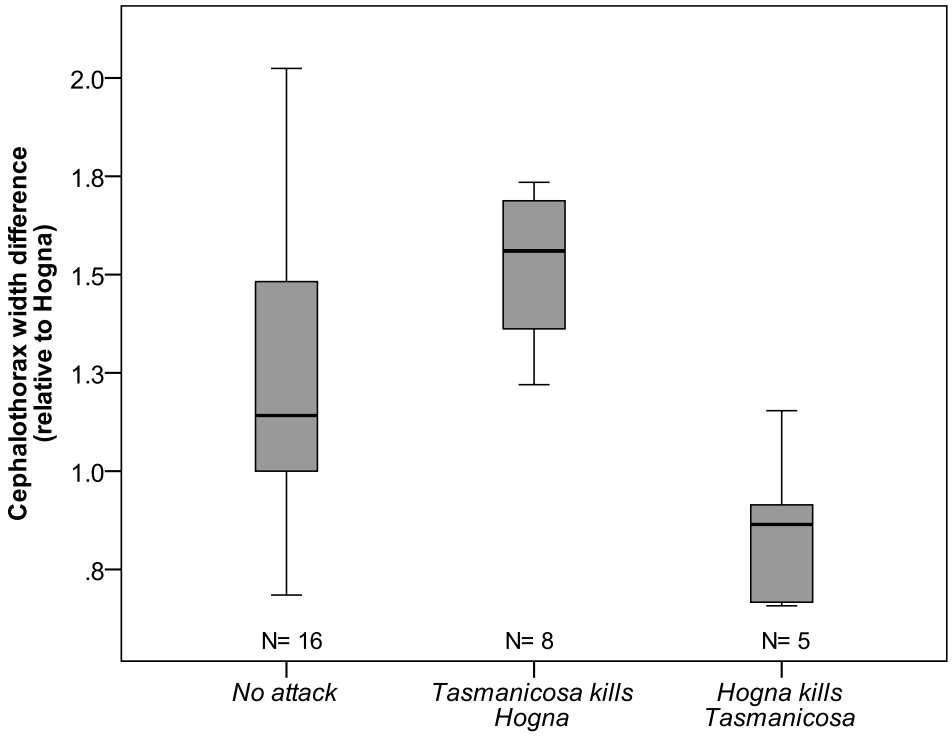

Figure 3. Spider size difference (percent relative to *Hogna crispipes* cephalothorax weight) and the occurrence of attack between *Tasmanicosa leuckartii* and *Hogna crispipes* paired together in laboratory arenas. Horizontal line represents median, box length represents interquartile range, whiskers length are upper and lower quartiles.

Table 1. One-way logistic regression relationships between six predictors and predation outcomes in laboratory trials where *Tasmanicosa*, *Hogna* and *Helicoverpa* are paired together.

	Tasmanicosa* kills *Helicoverpa						***Tasmanicosa* kills *Hogna***					
	Constant	**B**	**Wald χ^2**	**p**	**Odds ratio**	**Nagelkerke's R^2**	**Constant**	**B**	**Wald χ^2**	**p**	**Odds ratio**	**Nagelkerke's R^2**
Focal spider size	-2.29	1.31	0.16	0.68	3.73	0.01	-7.69	8.93	4.36	0.03*	7611.46	0.28
Focal spider weight	-0.68	-1.03	0.68	0.40	0.35	0.04	-4.33	4.19	7.28	<0.01**	66.64	0.48
Body condition	-2.03	-10.62	3.75	0.02*	0.00	0.24	-0.85	10.24	3.94	0.04*	28140.4	0.25
Focal spider sex (male)	-2.07	1.06	0.77	0.37	2.90	0.04	-2.07	1.06	0.77	0.37	2.90	0.17
Focal spider sex (female)	-2.07	0.69	0.20	0.65	2.00	0.04	-2.07	2.48	3.15	0.07	12.00	0.17
***Helicoverpa* weight**	-5.01	8.60	1.22	0.26	5460.22	0.07	4.29	-12.04	3.61	0.04*	0.09	0.19
Spider size ratio	-1.20	-0.11	0.00	0.93	0.89	0.00	-5.13	3.11	4.75	0.02*	22.62	0.27

	Hogna* kills *Helicoverpa						***Hogna* kills *Tasmanicosa***					
	Constant	B	Wald χ^2	p	Odds ratio	Nagelkerke's R^2	Constant	B	Wald χ^2	p	Odds ratio	Nagelkerke's R^2
Focal spider size	1.54	-1.25	0.09	0.76	0.28	0.00	-9.95	13.38	3.45	0.02*	647083	0.25
Focal spider weight	1.35	-1.20	0.59	0.44	0.30	0.02	-4.70	5.65	5.14	0.02*	286.41	0.37
Body condition	0.80	0.30	0.06	0.93	0.73	0.00	-2.03	-10.62	3.75	0.03*	0.00	0.24
Focal spider sex (male)	1.01	20.19	0.00	0.99	5874	0.15	-2.63	-12.56	0.00	0.99	0.00	0.25
Focal spider sex (female)	1.01	-0.82	0.84	0.32	0.43	0.15	-2.63	2.07	2.95	0.08	8.00	0.25
***Helicoverpa* weight**	2.67	-4.48	0.53	0.46	0.01	0.02	-8.10	15.01	2.29	0.13	33246	0.16
Spider size ratio	1.68	-1.09	0.49	0.48	0.36	0.02	-7.56	6.06	5.90	0.01*	431.77	0.46

Discussion

Predation tendency of each spider species

In a setting where *Tasmanicosa* and *Hogna* wolf spiders encountered *Helicoverpa* individually, these species were similarly likely to kill *Helicoverpa* larvae. However, when these spiders were housed together, the presence of *Hogna* diminished the likelihood of *Tasmanicosa* killing *Helicoverpa.* Interestingly, the presence of *Tasmanicosa* did not affect frequency of predation on *Helicoverpa* by *Hogna.* Given that *Tasmanicosa* was usually the larger spider, *Hogna* might be expected to reduce its foraging activity to reduce risk (Martinou *et al.*, 2010). However, even though *Hogna* tended to encounter *Helicoverpa* before *Tasmanicosa*, we found no evidence of difference in the overall activity levels of *Hogna* and *Tasmanicosa*. The explanation for the different tendencies to hunt *Helicoverpa* may relate to body condition. *Tasmanicosa*, spiders with a lower body condition were more likely to kill *Helicoverpa*, but this was not the case for *Hogna*. Body condition is commonly used as a measure of nutritional state or to estimate previous foraging events (Moya-Larano *et al.*, 2008). Perhaps *Hogna* collected from the field were in a more stable nutritional state, such that variation in body condition of this species was not large enough to detect effects on predation of *Helicoverpa*. However, the relationship between body size and mass can also indicate water retention or eggloads in spiders (Anderson, 1974) therefore caution must be taken when interpreting this measurement as an index of nutritional state of the spider. *Tasmanicosa* with lower body condition might be hungrier spiders that are more motivated to kill *Helicoverpa*, but spiders with a lower body condition (i.e., lighter) might also be more agile and effective at finding and killing prey.

In the laboratory experiments, *Hogna* killed *Helicoverpa* more frequently than they killed *Tasmanicosa,* but *Tasmanicosa* had a similar tendency to kill *Hogna* or *Helicoverpa.* For both spider species, spider weight was the strongest predictor of its likelihood to kill the

other spider. This result is in accord to other studies that report effects of size difference on direction of intraguild predation, with larger heavier spiders tending to kill smaller spiders, but not spiders of a similar size (Balfour *et al.*, 2003; Hogg & Daane, 2014). Predation frequencies reflect the influence of spider size; *Hogna* was more often smaller than *Tasmanicosa*, so more often selected *Helicoverpa* as a less dangerous prey than a bigger spider (Rypstra & Samu, 2005). When *Tasmanicosa* encountered a smaller *Hogna*, the risks of attacking the predator may have been compensated by the higher nutritional quality of the spider, compared to the easier, but protein-poor prey of *Helicoverpa* (Denno & Fagan, 2003). That *Tasmanicosa* either killed only *Helicoverpa* or only *Hogna*, but not both in the same trial, indicates that the presence of smaller spiders in the same space and time as *Tasmanicosa* can interfere with the biological control of *Helicoverpa* by *Tasmanicosa*.

Effect of wolf spider abundance and diversity on* Helicoverpa *control

In glasshouse experiments, one spider (either *Tasmanicosa* or *Hogna*) was just as effective as two conspecific spiders in reducing *Helicoverpa* survival. Increasing spider density did not have a significant effect on biological control. Controlling for density (two spiders in each enclosure), increasing diversity (two species vs. single species) also did not have an effect on biological control. *Helicoverpa* survival was higher in enclosures with one *Tasmanicosa* compared to enclosures with *Tasmanicosa* + *Hogna*. However, *Helicoverpa* survival was similar comparing enclosures with one *Tasmanicosa*, two *Tasmanicosa*, and two *Hogna*. This result suggests that while there is not an effect on increasing abundance, there is a positive (yet not additive) effect on biological control when increasing diversity along with abundance.

It is argued that the strength of biological control can been improved by enhancing the diversity of prey-specific predators, rather than the abundance of generalist predators (Denno & Finke, 2006; Finke & Denno, 2005). Several studies have shown how the addition

of predator arthropods does not improve biological control. For example, planthopper suppression was diminished in a system when two wolf spider species (*Hogna* and *Pardosa*) where added, compared to a system with a mirid predator with no spiders (Finke & Denno, 2004). Similarly, Hogg & Daane (2014) found that presence of two hunting spider species (Miturgidae: *Cheiracanthium mildei* and Anyphaenidae: *Anyphaena pacifica*) did not suppress densities of leafhoppers in vineyards beyond the suppression exerted by one individual of each spider species only. The same pattern has also been observed in ants, where the addition of two and three ant species does not improve biological control on the coffee borer beetle beyond that exerted by a single ant species (Philpott *et al.*, 2012). In contrast, another study found that enhancing spider diversity improves biological control; the combination of two hunting spider species (Lycosidae: *Pardosa pseudoannulata* and Linyphiidae: *Atypena formosana*) were more effective at controlling brown planthoppers than a group of single spider species (Sigsgaard, 2007). The main limitation of interpreting these studies is that while they make their conclusions based on increased diversity, they actually do not address the confounding effects of increasing predator abundance while increasing diversity (Jolliffe, 2000). It is also possible that in a field scenario, where spider densities are lower, the increase in abundance or diversity has a different effect on *Helicoverpa* mortality that the effect observed in high density glasshouse enclosures. In the present study, we determined that while increasing spider abundance (one individual compared to two individuals of the same species) did not improve biological control, there seemed to be an interaction between abundance and diversity that lead to increased *Helicoverpa* mortality (*Tasmanicosa* compared to *Tasmanicosa* + *Hogna*).

Mechanisms for disruption of biological control

Given the different effects reported by studies of multiple spiders on biological control, it is important to not simply describe the effects of multiple predators on prey

densities, but to understand the mechanisms that drive the disruption of biological control. Intraguild interactions between *Tasmanicosa* and *Hogna* may lead to a non-additive effect on *Helicoverpa* control through three mechanisms: prey choice and satiation, reduced time for hunting *Helicoverpa* (consumptive effects), and behavioral inhibition (nonconsumptive effects).

Prey choice and satiation. Predation on *Helicoverpa* can be hindered if one spider rejects *Helicoverpa* because of satiation after eating another spider. Previous studies have found that spiders might reject target pest prey after killing intermediate predators. For example, Finke & Denno (2003) found reduced efficacy of biological control of planthoppers (pests) due to wolf spiders (primary predators) killing mirids (mesopredators) instead of planthoppers. Wolf spiders are voracious predators, and can hunt and handle more than one prey item at a time (Framenau *et al.*, 1999); predatory capacity trials have shown that *Tasmanicosa* can kill as many as six fifth instar *Helicoverpa* larvae in eight hours (chapter 3). However, in the laboratory the only cases in which *Helicoverpa* survived were when one spider had killed the other. These results indicate that even though wolf spiders can kill multiple *Helicoverpa* larvae, spiders may still become sated after killing another spider and then reject *Helicoverpa.*

Reduced hunting time. Even if a spider is not sated after eating another spider, biological control might still be disrupted by spiders having reduced time to hunt *Helicoverpa*. In laboratory trials, *Helicoverpa* was never killed while one spider was eating another. Consequently, *Helicoverpa* larvae can descend from plants while spiders are feeding and pupate underground where they are safe from wolf spiders. These results suggest that the likelihood of either spider species killing *Helicoverpa* depends on two factors: whether a spider encounters another spider before it encounters *Helicoverpa*, and whether the size difference between these two spiders is large enough to trigger intraguild predation.

Numerous scenarios can influence biological control. For instance, if one spider kills another spider before it kills *Helicoverpa,* then biological control will be hindered because 1) *Helicoverpa* has opportunity to descend and pupate while one spider is eating another spider, and 2) the second spider is now dead and cannot kill remaining *Helicoverpa*, and 3) the first spider might be sated and consequently reject remaining *Helicoverpa.* In contrast, if one spider encounters and kills another spider after both have eaten *Helicoverpa,* then biological control would not be disrupted by intraguild predation. Since the number of surviving pupae was similar in glasshouse trials where one spider had killed the other spider compared to trials where both spiders were alive, our results suggest that intraguild predation likely occurred after both spiders had already killed *Helicoverpa.*

Inhibited predatory behavior. Biological control on *Helicoverpa* can be mediated by behavioral intraguild interactions between spiders (nonconsumptive effects), or by intraguild predation (consumptive effects) after one spider kills another spider. If intraguild predation (consumptive effects) was the only mechanism mediating biological control, then we would have observed that glasshouse enclosures in which one spider killed the other had more surviving *Helicoverpa* than glasshouse enclosures in which two spiders were paired but neither was killed. As the number of surviving pupae was similar between glasshouse trials with or without intraguild predation, intraguild predation was not the only mechanism mediating biological control. Even though laboratory trials did not show activity differences between spider species, we did observe that *Hogna* was more likely to encounter and kill *Helicoverpa* before *Tasmanicosa* did. These results suggest that the presence of one spider does exert some behavioral effect on the other spider (beyond what we could assess from recordings). Behavioral changes of mesopredators in the presence of dominant predators have been documented in ladybugs (Sato *et al.*, 2005), predatory mites (Walzer & Schausberger, 2013) and jumping spiders (Okuyama, 2002). Overall, our study suggests a lack of additive

effects on biological control when number of spiders increased from one to two, and this is likely due to a combination of modified hunting behavior caused by the presence of another spider (non-consumptive effect), satiation, and a reduced window of time for spiders to kill *Helicoverpa* if they are hunting other spiders (consumptive effects).

Spider natural history and implications for crop management

Given the strong effect of spider size and weight on predatory interactions between *Tasmanicosa* and *Hogna*, it might be possible to predict the direction of intraguild interference and predation based on the life history of each spider species, and the relative abundance of adults and juveniles through the season (Lensing & Wise, 2004). Both spider species emerge from their burrows at the same time in the evening and are inactive during the day, but the direction and intensity of interference and intraguild predation is likely to vary through the cotton season. Adult males of *Tasmanicosa* appear in cotton fields in late October/early November, earlier than adults of *Hogna* (Rendon et al., 2015), and when *Helicoverpa* moths emerge from diapause. The lack of potential spider competitors early in the growing season could mean that emerging *Helicoverpa* moths are a more common prey item for *Tasmanicosa*. Unidirectional intraguild predation might be more intense when the first juveniles of *Hogna* colonize the field and can be killed by *Tasmanicosa*. As the season progresses and *Hogna* adults become more abundant, intraguild predation may decrease (Balfour *et al*., 2003) as the size difference decreases between *Tasmanicosa* and *Hogna.* At this stage, generations of *Helicoverpa* larvae and moths overlap with a peak in abundance of wolf spiders in the cotton fields. Towards the end of the season (March/April), cotton fields contain mostly large adult females and dispersed spiderlings of *Tasmanicosa*, and a mix of juveniles and adults of both sexes of *Hogna* (chapter 2, personal observation). At this stage intraguild predation may be bidirectional, with adult *Tasmanicosa* females preying on smaller juveniles of *Hogna*, and *Hogna* hunting *Tasmanicosa* spiderlings – this intraguild predation

could reduce biological control of the last seasonal generation of *Helicoverpa* larvae that descend to overwinter in the soil. Even though *Tasmanicosa* is the largest ground spider in these fields, the abundance of smaller spider species is not suppressed by intraguild predation (Rendon et al., 2015).

While there was no difference in the number of *Helicoverpa* larvae surviving to pupation when two spiders of the same or different species were paired together, subtle interactions between abundance and diversity suggest that *Tasmanicosa* and *Hogna* might not always behave as a single functional group (Sokol-Hessner & Schmitz, 2002). To optimize biological control, there is value in maintaining diverse guilds of wolf spiders that can serve in different niches, such ground and stubble predators. It may be possible to make spatial modifications to the cropping fields that might maximize availability of microhabitats for multiple spider species and at the same time minimize interactions and predatory inhibition between spiders. Both *Tasmanicosa* and *Hogna* are active ground hunters, but do not overlap completely in their microhabitat use. *Hogna* is sometimes observed on tall grasses, small shrubs and plant stubble, a few centimeters from the ground. In contrast, *Tasmanicosa* was always observed at soil level, and never climbed onto plants. Sitvarin & Rypstra (2014) argue that to predict multiple predator effects in wolf spiders the combination of microhabitat (soil or vegetation), hunting mode, and the direction of IGP should be considered in conjunction. A spatial separation may reduce encounter rates of these spiders in the field, thereby reducing the predatory and behavioral effects of intraguild interference and predation (Denno *et al.*, 2004). In the present study a lack of structural complexity forced spiders to share the same soil space; similar studies have suggested that additional vegetation relaxes antagonistic interactions between intraguild predators and has a flow-on effect on pest control (Finke & Denno, 2006; Janssen *et al.*, 2007; Noppe *et al.*, 2012). The effect of multiple microhabitats also has implications for farm management; minimum-tillage practices have been found to

increase wolf spider diversity (Rendon *et al.*, 2015) and this may in part be a consequence of retained plant stubble creating multiple spatial niches that allow several species of spiders to co-exist with reduced frequency of antagonistic interactions (Finke & Denno, 2006). By reducing interference and predation among spiders through provision of complex habitat architecture, minimum-tillage may maximize biological control of *Helicoverpa* from multiple spider species.

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CHAPTER 5: Prey encounter, prey vulnerability and nutritional content in a ground arthropod trophic web in cotton crops.

1

Abstract

Wolf spiders are abundant and voracious predators that inhabit the soil-plant interface in cotton crops. Bt-cotton, which contains insecticidal properties from *Bacillus thuringiensis* (Bt) bacteria, is widely used as a pest control strategy against the cotton bollworm, *Helicoverpa armigera* (Hübner). Bt-cotton imposes selection pressure on *Helicoverpa* that promotes evolution of resistance, but ground predators could inhibit the emergence of Bt-resistance by attacking resistant larvae as they descend from the plants to pupate in the soil. However, the predator-prey interactions between wolf spiders and *Helicoverpa* could be constrained by the presence of alternative prey and intraguild predators. This study describes predation by the wolf spider *Tasmanicosa leuckartii* (Thorrell) on another wolf spider *Hogna crispipes* (Koch) (intraguild predator), *Teleogryllus commodus* (Walker) crickets (minor pest) and *Helicoverpa armigera* (Hübner) larvae (major pest) in laboratory enclosures. First encounter rate (defined as physical contact between individuals) and prey vulnerability were evaluated as mechanisms that may mediate frequency of predation. Although *Tasmanicosa* first encountered *Teleogryllus, Hogna* and *Helicoverpa* in similar proportions when paired together, the proportion of first encounters did not match the proportions of first attacks. In both 3-way (without *Hogna*) and 4-way (with *Hogna*) trophic webs, *Tasmanicosa* had a tendency to attack *Helicoverpa* before attacking *Teleogryllus*. As *Teleogryllus* escapes quicker than *Helicoverpa* and *Hogna*, it might be less vulnerable to predation by *Tasmanicosa*. *Helicoverpa* (protein-poor) and *Hogna* (protein-rich) were consumed by *Tasmanicosa* in similar proportions, suggesting that *Tasmanicosa* might benefit from nutrient balance as an outcome of multiple predation in this trophic web. That *Tasmanicosa* readily attacked *Helicoverpa* larvae in the presence of alternative prey is an encouraging result that supports the potential of *Tasmanicosa* predation as a means of controlling *Helicoverpa* larvae that survive on Bt cotton.

Introduction

Interactions between a predator and its prey rarely occur in isolation. Instead, predatory interactions usually occur within trophic webs that also involve primary producers, alternative prey, and other predators. When multiple prey is present, predation outcomes may be driven by prey availability (passive selection – independent of predator behaviour) or by the predator-prey interactions upon prey encounter (Sih & Christensen, 2001). Variables influencing passive selection of prey include prey dispersal (Pastorok, 1981) and camouflage (Endler, 1978). After encounter, predation outcomes may be determined by the prey's ability to escape (Lang & Gsodl, 2001; Provost *et al.*, 2006), prey defences (Provost *et al.*, 2006), prey size (Bence & Murdoch, 1986; Downes, 2002; Turesson *et al.*, 2002), predator hunger state (Lang & Gsodl, 2001; Molles & Pietruszka, 1987), or prey nutritional content (Schmidt *et al.*, 2012; Simpson *et al.*, 2004). Predators may not attack prey that is difficult to catch and subdue, or impose risk of injury or toxicity. Optimal foraging theory combines these influences on predation outcomes, and states that after predators encounter prey they should aim to maximize their energy intake (Krebs, 1978) while minimizing the risks and energy spent in prey capture (Sih, 1980). Understanding the mechanisms that mediate predation tendencies is crucial to predict the structure and function of trophic webs.

In agricultural trophic webs, generalist predators do not target pest prey exclusively, yet these predators are still an important component of integrated pest management (Symondson *et al.*, 2002). Prey diversity can be essential to sustain generalist predator populations; when pest densities are low, predators may rely on alternative prey to improve growth and survival, allowing the predator population to be maintained (Harwood & Obrycki, 2005). In some cases, a diet consisting of only single pest prey may be nutritionally deficient and can drastically reduce the survival of generalist predators (Harwood *et al.*, 2009). In

addition, foraging on diverse prey may enable predators to balance nutrient intake (Greenstone, 1979; Matsumura *et al.*, 2004; Mayntz *et al.*, 2005; Toft *et al.*, 2010). Alternative prey therefore may be essential for predators to thrive in agricultural systems. Since the presence of alternative prey may influence predator-prey interactions and trophic web dynamics, it is important to understand how the presence of alternative prey may affect the biological control of pest species.

Abundance of alternative prey may influence whether a generalist predator feeds on a particular pest predominantly or switches prey preference (Murdoch, 1969). Interference of the biological control of pests due to the presence of alternative prey has been reported in several studies; for example, in the presence of Collembola (alternative prey), spiders kill fewer aphids (pest) (Gavish-Regev *et al.*, 2009; Harwood *et al.*, 2004; Kuusk & Ekbom, 2010). In other cases, generalist predators can still effectively suppress pest populations despite the availability of diverse prey. For example, the wolf spider *Hogna* sp. continued feeding on cucurbit pests, even when alternative prey were available (Wise *et al.*, 2006). Predators may respond differently to different types of alternative prey. For instance, the wolf spider *Pardosa prativaga* consumes fewer aphids when fruit flies (*Drosophila* spp.) are available, but does not change its predation rate on aphids when collembola are available (Madsen *et al.*, 2004). The interactions between generalist predators, pest prey, and alternative prey are difficult to predict and vary among species and agricultural systems.

In this study, we examine the predator-prey interactions of four arthropods commonly found on the soil surface of cotton fields in New South Wales, Australia: two species of wolf spider (Araneae: Lycosidae: *Tasmanicosa leuckartii* (Thorrell) and *Hogna crispipes* (Koch); referred to hereafter as *Tasmanicosa* and *Hogna*, respectively), a ground cricket *Teleogryllus commodus* (Walker) (Orthoptera: Gryllidae; referred to hereafter as *Teleogryllus*), and a

cotton pest, the bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae; referred to hereafter as *Helicoverpa*).

Genetically modified Bt-cotton was introduced to control the larvae of *Helicoverpa* spp. (Whitehouse *et al.*, 2009), an important cotton pest (Fitt *et al.*, 2009). However, the long term viability of Bt cotton is constantly threatened by the potential of *Helicoverpa* to develop resistance to Bt-toxins (Downes & Mahon, 2012). Bt-resistant larvae descend from the cotton plants to pupate underground, and later emerge as moths. After foraging on cotton, these larvae are exposed to predators on the plant-soil interface. By killing larvae of *Helicoverpa* as they descend to pupate, or later as they emerge as moths, ground predators present in cotton crops can delay the proliferation of Bt-resistant genes in *Helicoverpa* populations (Liu *et al.*, 2014).

Wolf spiders are commonly classified as generalist predators, and will readily accept crickets, Lepidoptera larvae, and other wolf spiders as prey (Nentwig, 1986). The wolf spiders *Hogna* and *Tasmanicosa* are the second and third most abundant species of wolf spiders in our study site, with *Hogna* representing approximately 35% and *Tasmanicosa* representing approximately 12% of the wolf spider community (Rendon *et al.*, 2015). Moreover, these two spider species are large enough to be capable of subduing 5th instar *Helicoverpa* larvae as they descend to pupate in the soil. The ground cricket *Teleogryllus* is commonly found in cotton fields, and has been reported to be an occasional pest in cotton when they are abundant. Adults and late-instar nymphs can be early-season pests, as they feed on the leaves and stems of cotton seedlings (Williams *et al.*, 2011). Additionally, *Teleogryllus* is the most commonly observed prey item of wolf spiders in the field (Rendon *et al.*, unpublished data, Chapter 2). This ground trophic web includes two abundant predators in Bt cotton fields (*Tasmanicosa* and *Hogna*), an abundant but economically minor cotton pest (*Teleogryllus*), and a less abundant, yet economically far more important cotton pest (*Helicoverpa*).

The aim of this study is to describe the predator-prey interactions trophic web between *Tasmanicosa, Hogna, Teleogryllus* and *Helicoverpa*, and determine how these interactions influence predation outcomes. Specifically, we aim to answer: 1) whether predation outcomes are linked to prey encounter and prey vulnerability, 2) whether predation on *Helicoverpa* by *Tasmanicosa* is influenced by the presence of *Teleogryllus* as alternative prey and *Hogna* as competitor and intraguild prey, and 3) whether predation outcomes are related to lipid and protein content of available prey.

Materials and methods

Collection of spiders and Teleogryllus

This study was carried out at the Australian Cotton Research Institute (ACRI; 33ºS, 149ºE), near Narrabri, New South Wales, Australia. Adult males, females and late-instar juveniles of the wolf spiders *Tasmanicosa* (cephalothorax width = 7.1 ± 1.2 mm; mean ± SD); and *Hogna* (cephalothorax width 5.7 ± 1.1 mm; mean ± SD) were collected in and around Bt-cotton fields after sunset from December until March 2014, and in March 2015. Since *Teleogryllus* nymphs shelter in soil cracks and are difficult to collect from cotton fields, *Teleogryllus* nymphs (body length = 12.8 ± 2.4 mm, mean ± SD) were collected around the buildings at ACRI. Spiders and crickets were found by visual search using a headlamp (Petzl Tikka, 140 lumens), and were collected manually in clear 70 ml cylindrical plastic containers. After collecting, all animals were brought to the laboratory, and the spider's cephalothorax width and cricket body length was measured to the nearest 0.01cm using a manual caliper (resolution 0.01cm. Each animal was weighed to the nearest 0.01 g using a digital scale (Sartorius model A200S).

Collected spiders were housed in clear plastic containers (220 mm height x 228 mm length x 228 mm width, 8.5 L, Décor Tellfresh superstorer®; referred to hereafter as 'spider container') containing 2 L of moist soil, and were kept in a controlled environment room (24.4 ± 0.5ºC, mean ± SD) with a L14:D10 photoperiod. Each container had a retreat in each of two opposite corners comprising a hole in the soil approximately 2 cm deep and 1 cm diameter. The retreat was partially covered by a 3 x 3 cm sheet cut from the bark of a 'Paper Bark tree' (*Melaleuca sp.).* All spiders were isolated by placing an opaque PVC pipe (100 mm diameter x 150 mm height) over each spider's retreat until experiments started. Each spider was kept in the laboratory for 24 hours before being used in experiments, during which no prey or water were supplied.

Experiment 1: **Helicoverpa** ***predation***

This experiment was done to assess whether *Tasmanicosa* kills both *Helicoverpa* larvae reared in artificial diet (used in food web experiments 2 and 3) and larvae reared in cotton plants (field scenario). Larvae of *Helicoverpa armigera* were supplied by the Commonwealth Scientific and Industrial Research Organization (CSIRO) Agricultural Flagship Bt Resistance Monitoring Group. Larvae were reared in individual wells in trays with soy and agar diet (Downes *et al.*, 2009; Teakle & Jensen, 1985; referred to as 'diet-reared' larvae), and kept in a controlled environment room (24.4 ± 0.5ºC, mean ± SD) with a L14:D10 photoperiod. A separate group of larvae were reared from neonates on plant material (referred to as 'plant-reared' larvae). Larvae were placed in individual wells in trays containing a mixture of cotton leaves, flowers, and squares (Sicot 71® conventional, non-Bt, RRF). Larvae were transferred into new trays with fresh plant material every two days. Both diet-reared and plant-reared larvae were maintained until they reached 5th instar and were then weighed to the nearest 0.01 g (body weight = 0.40 ± 0.07 g, mean ± SD) using a digital scale (Sartorius model A200S).

To assess whether spiders kill both plant-reared and diet-reared larvae, one *Tasmanicosa* was paired simultaneously with one diet-reared 5th instar larva and one plant-reared 5th instar larva (N= 10). To distinguish larvae, both were marked with different colour dyes (VM311 Pink and VM315 Orange), alternating colours in different trials (so that 5 plant-reared larvae and 5 diet-reared larvae had pink dye, and 5 of each had orange dye). Larval mortality from predation was recorded after 24hrs.

Experiment 2: First encounter and attack in 3-way and 4-way trophic webs

To determine the effect of alternative prey and intraguild predators on the first prey attacked by *Tasmanicosa*, 3-way (*Helicoverpa – Teleogryllus – Tasmanicosa*; N=27) and 4-way (*Helicoverpa – Teleogryllus – Hogna – Tasmanicosa*; N=23) trophic webs were set up in which animals were present together in the same spider container. This experiment was done to examine how the frequency of first prey encounter is related to the frequency of first attack for *Tasmanicosa* and *Hogna.* All trophic web experiments were carried out in the same controlled environment room described above, with diet-reared *Helicoverpa* larvae. Approximately 30 minutes after the dark phase commenced in the controlled environment room, one 5th instar *Helicoverpa* larva and one *Teleogryllus* nymph were placed inside a container housing either (1) one *Tasmanicosa;* or (2) one *Tasmanicosa* and one *Hogna.* PVC pipes were immediately removed to allow spiders to explore and hunt. Continuous video recording began immediately after *Helicoverpa* and *Teleogryllus* were released, and ended 24 hrs later; the recording system comprised a 1/3" CCD monochromatic infra-red camera (CCS-Sony Go Video) with a 4 mm C mount lens positioned above each container, which recorded to a 2TB DVR4-100 hard drive recorder. One infrared (940nm) illuminator (IR-covert) was placed 10 cm to the side of each spider container. To improve video contrast, each animal was dusted with fluorescent dye (HCA Colours Australia, VM311 Pink for *Helicoverpa* larvae and *Teleogryllus*, VM317 Yellow for *Tasmanicosa*). For each trial, we recorded which prey item

was first encountered (physical contact between predator and prey) and first attacked by *Tasmanicosa* and *Hogna* (the spider lunging towards the prey).

Experiment 3: Multiple predation in 4-way trophic webs

To further examine whether *Tasmanicosa* consumes multiple prey after a first attack, a second 4-way trophic web experiment was carried out in March 2015 (N = 31). *Tasmanicosa, Hogna* and *Teleogryllus* were collected in the same area as the experiments in 2014, and all trophic web experiments were carried out in the same controlled environment room described above, with diet-reared *Helicoverpa* larvae. *Teleogryllus* nymphs were housed together in clear 8.5L plastic containers with a wet cotton wick to provide moisture, and were fed *ad libitum* soy and wheat agar diet cubes for 3 days before being used in experiments. Approximately 30 minutes after the dark phase commenced in the laboratory, one 5^{th} instar *Helicoverpa* larva and one *Teleogryllus* nymph were placed inside the spider container housing one *Tasmanicosa* and one *Hogna;* the PVC pipes were immediately removed to allow spiders to explore and hunt. Each spider container was observed 1, 5, 15, 30, and 60 minutes later during the first hour of the trial, and then every hour for 4 hours after the first 60 minutes. During each focal point, we recorded whether *Tasmanicosa* and *Hogna* were feeding and the identity of each prey item. After 24 hrs, we recorded whether *Tasmanicosa, Hogna, Teleogryllus* and *Helicoverpa* were alive. Trials in which prey attacks fell outside the observation period were excluded from predation frequency analysis. Although spiders sometimes steal dead prey from each other, only instances in which the spider killed the prey were included in multiple predation analysis.

Experiment 4: Lipid and protein content

To determine the relationship between prey protein and lipid content, and predation outcomes in food webs, a protein and lipid analysis was done in *Tasmanicosa, Hogna,*

Teleogryllus and *Helicoverpa.* Because spiders were caught from the field 24 hrs before experiments and were not offered prey before trials, we made the assumption that spiders were responding to the *Helicoverpa* and *Teleogryllus* offered to them as if the prey were sourced from the field. Therefore, we assumed that any link between predation outcomes and nutrient content would be based on the typical protein and lipid contents of prey in the field. Juvenile *Tasmanicosa* (N = 8), juvenile *Hogna* (N = 12), field-collected *Teleogryllus* (N = 12), diet-reared *Teleogryllus* (N = 13) and diet-reared *Helicoverpa* (N = 21), and plant-reared *Helicoverpa* (N = 12) were collected for nutritional analysis. *Tasmanicosa, Hogna* and *Teleogryllus* were collected from the same fields described above, and immediately frozen at -20°C for nutritional analysis. Diet-reared *Helicoverpa* larvae were raised in soy and agar diet as described above, and frozen at -20°C once they reached 5th instar. Plant-reared larvae were raised from neonates in a mix of cotton leaves, flowers, and squares (Sicot 71® conventional, non-Bt, RRF) as described above, and frozen at -20°C once they reached 5th instar. Diet-reared *Teleogryllus* nymphs were collected in the field as early nymphs (body length 1.8 ± 0.2 mm, mean ± SD), and then housed together in a single clear 8.5L plastic container with a wet cotton wick to provide moisture, and fed *ad libitum* soy and agar diet cubes for 25 days until the nymphs reached a body length of 12.2 ± 1.6 mm (mean ± SD) before being frozen at -20°C for nutritional analysis.

After freezing, each arthropod was dried in an oven at 60ºC for 48 hrs before lipid and protein analysis. Lipid content was measured gravimetrically by submerging each dried arthropod in chloroform for 24 hrs, discarding the chloroform, and repeating for another 24 hrs. The lipid content was estimated by taking the difference in the dry weight of samples before and after soaking them in chloroform (Wilder *et al.*, 2013). Protein was extracted from ground sub-samples (3-5mg) using 0.1 M NaOH and heat (90°C for 30 minutes) after which samples were centrifuged and the supernatant was collected for analysis. Protein content was then measured using the Bradford Assay modified for use in 96 well microplates following

manufacturer's instructions (Bio-Rad protein assay kit, product #500-001). We analysed each sample in triplicate and all samples were run together on the same plate reader (Biotek EL808) with a calibration curve created using a protein standard (Bio-Rad bovine globulin gamma, Bio-Rad #500-001).

Statistical analysis

A test of independence on a contingency table with post-hoc z values was used to determine if there were differences in the proportions of first encounter, first attack, and overall predation frequencies for *Tasmanicosa* and *Hogna* towards *Teleogryllus* and *Helicoverpa.* Percent protein and percent lipid were analysed for normality using a Shapiro-Wilk test, log-transformed where necessary and three extreme outlier values were removed (based on biologically unrealistic protein values that could only reflect errors) to meet the assumptions of parametric testing. A multivariate analysis of variance (MANOVA) was used to test for differences in percent lipid and protein between diet-reared *Helicoverpa,* plant-reared *Helicoverpa,* field-collected *Teleogryllus,* diet-reared *Teleogryllus,* and field- collected *Hogna* and *Tasmanicosa*, using dry body mass as a covariate. Post-hoc least significant differences (LSD) were carried out to determine differences in percent lipid and percent protein separately between each arthropod. All statistical analyses were carried out using SPSS V.20 (IBM, 2011).

Results

Experiment 1:* Helicoverpa *predation

At the end of *Helicoverpa* predation trials, all spiders had killed both the plant-reared and the diet-reared larvae. In 6 out of 10 trials, *Tasmanicosa* killed plant-reared *Helicoverpa* first, but no larvae were rejected, and both *Helicoverpa* larvae were completely consumed.

Experiment 2: First encounter and attack in 3-way and 4-way trophic webs

Videorecorded 3-way trophic web trials revealed that *Tasmanicosa* encountered *Teleogryllus* and *Helicoverpa* first in similar proportions (Pearson $\chi^2 = 0.30$, df = 1, p = 0.58, cases in contingency table = 27; Figure 1). There was a non-significant tendency for *Tasmanicosa* to attack *Helicoverpa* before attacking *Teleogryllus* (Pearson $\chi^2 = 3.65$, df = 1, p = 0.056, cases in contingency table = 27). After being encountered first, 90% of *Helicoverpa* (n= 10) and 75% of *Teleogryllus* (n= 12) were immediately attacked by *Tasmanicosa*. Videorecorded 4-way trophic web revealed that there were no differences in the proportions of first encounters between *Tasmanicosa* and *Hogna* with *Teleogryllus, Helicoverpa,* or the other spider (Pearson $\chi^2 = 2.40$, df = 2, p = 0.30, cases in contingency table = 40; Figure 2). There was a tendency for a non-random proportion of first attacks between *Tasmanicosa, Hogna, Teleogryllus* and *Helicoverpa* (Pearson $\chi^2 = 5.53$, df = 2, p = 0.06, cases in contingency table = 36); post-hoc tests revealed that *Tasmanicosa* had a tendency to attack *Helicoverpa* before attacking *Teleogryllus* or *Hogna* (z= 2.2). In contrast, there was no difference in the likelihood of *Hogna* attacking *Helicoverpa* or *Teleogryllus* first. After being encountered first, 84% of *Helicoverpa* (n = 6), 50% of *Teleogryllus* (n = 10) and 50% of *Hogna* (n = 4) were immediately attacked by *Tasmanicosa*. The same tendency of *Tasmanicosa* to attack *Helicoverpa* before attacking *Teleogryllus* first was observed in both 3-way and 4-way trophic web trials.

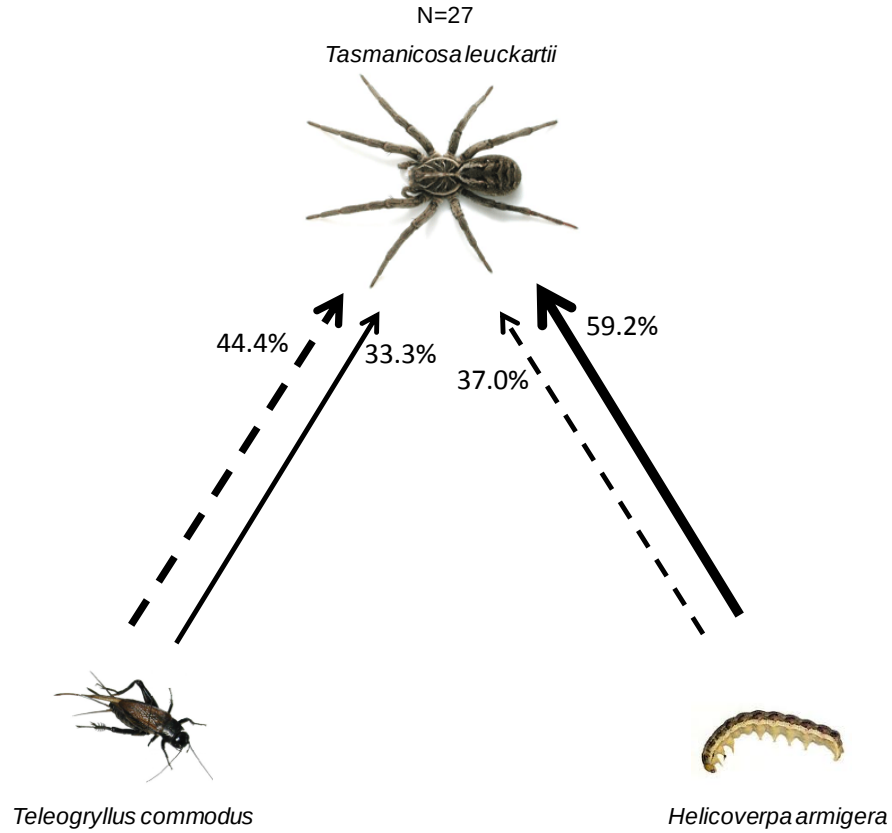

Figure 1. Percentages of first encounter (dashed arrows) and first attack (solid arrows) of *Tasmanicosa* in a 3-way videorecorded trophic web arena (experiment 2). Direction of arrows point to the predator that made the encounter/attack. Percentages indicate cases in which prey was encountered first (out of n = 27), or attacked first (out of n = 27).

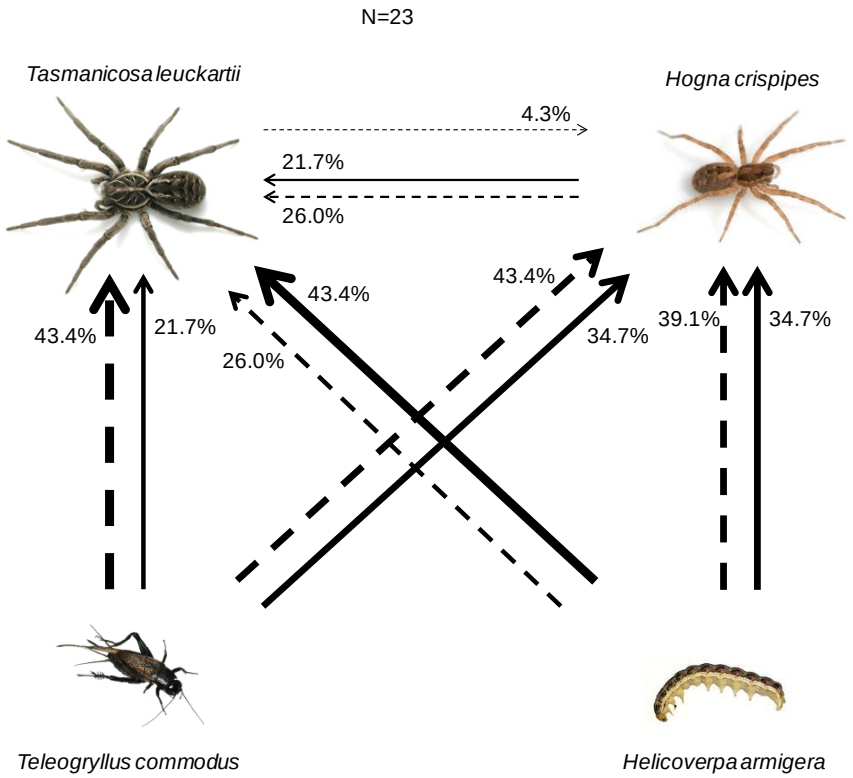

Figure 2. Percentages of first encounter (dashed arrows) and first attack (solid arrows) of Tasmanicosa and Hogna in a 4-way videorecorded trophic web arena (experiment 2). Direction of arrows point to the predator that made the encounter/attack. Percentages indicate cases in which prey was encountered first (out of n = 23), or attacked first (out of n = 23).

In all trials, no prey escaped a spider attack, and all attacks resulted in the spider eating the prey. When attacked by a spider, *Helicoverpa* displayed behaviours such as biting, bobbing its body from side to side, ejecting faeces and regurgitating. Very large *Helicoverpa* larvae sometimes lifted a smaller spider off the ground. None of these *Helicoverpa* behaviours were life threatening for a spider, and no spider died from attacks on *Helicoverpa,* or retreated after initiating attack. When being in contact or being seized by a spider, *Teleogryllus* exhibited behaviours such as kicking and head-butting spiders. No spiders died from mechanical injuries inflicted by *Teleogryllus*. *Teleogryllus* quickly jumped away when coming into contact with a spider, or even when a spider moved within a few centimetres. *Hogna* usually ran away after detecting an approaching *Tasmanicosa,* but never counter-attacked or bit, and were quickly seized by larger spiders. No *Tasmanicosa* died from attacking *Hogna.*

Experiment 3: Multiple predation in 4-way trophic webs

Four-way trophic web trials showed that over 24hrs, in 50% of the instances in which *Tasmanicosa* killed prey (n=26), more than one prey was killed. Prey kill in a 4-way trophic web between *Tasmanicosa*, *Hogna*, *Teleogryllus* and *Helicoverpa* was not random (Pearson $\chi^2 = 13.05$, df = 2, p = 0.01, valid cases in contingency table = 67; Figure 3). Post-hoc z tests revealed that *Tasmanicosa* was more likely to attack *Helicoverpa* and *Hogna* than *Teleogryllus* (z = 6.3). In contrast, there was no difference in the likelihood of *Hogna* attacking *Helicoverpa* or *Teleogryllus*.

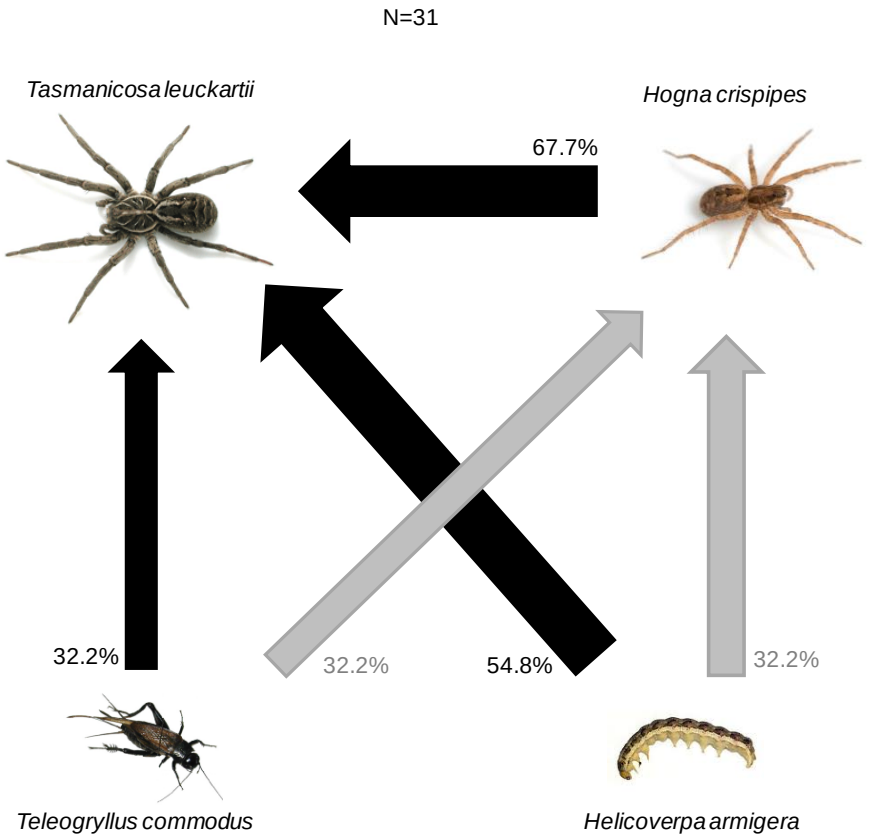

Figure 3. 4-way trophic web between *Tasmanicosa, Hogna, Teleogryllus* and *Helicoverpa* (multiple predation trials; experiment 3). Direction of arrows point to the predator that made the attack (energy flow). Percentages indicate cases in which the prey was attacked by the predator (out of n= 31). Black arrows indicate *Tasmanicosa* predation, grey arrows indicate *Hogna* predation. Arrow width represents proportion of predation from total trials.

Experiment 4: Lipid and protein content

Comparing protein and lipid contents of all animals as if they had been encountered in the field, plant-reared *Helicoverpa,* field-collected *Teleogryllus, Hogna* and *Tasmanicosa* had different protein contents (Wilks' Lambda = 0.07, F = 32.69, df = 3, 39, p<0.01; Figure 4), but similar lipid contents (Wilks' Lambda = 0.07, F = 1.02, df = 3, 39, p = 0.39). Post hoc LSD tests revealed that plant-reared *Helicoverpa* had lower protein content than field-collected *Teleogryllus, Hogna* and *Tasmanicosa.*

Prey diet changed lipid but not protein contents in *Helicoverpa*; diet-reared *Helicoverpa* had higher lipid content than plant-reared *Helicoverpa* (Wilks' Lambda = 0.19, F = 91.13, df = 1, 31, p < 0.01), whereas diet-reared *Helicoverpa* had similar protein content as plant-reared *Helicoverpa* (Wilks' Lambda = 0.19, F = 0.23, df = 1, 31, p = 0.63), Prey diet changed both lipid and protein content in *Teleogryllus;* diet-reared *Teleogryllus* had higher lipid content than field-collected *Teleogryllus* (Wilks' Lambda <0.01, F = 81.84, df = 1, 22, p <0.01), and diet-reared *Teleogryllus* had lower protein content than field-collected *Teleogryllus* (Wilks' Lambda <0.01, F = 20.19, df = 1, 22, p <0.01).

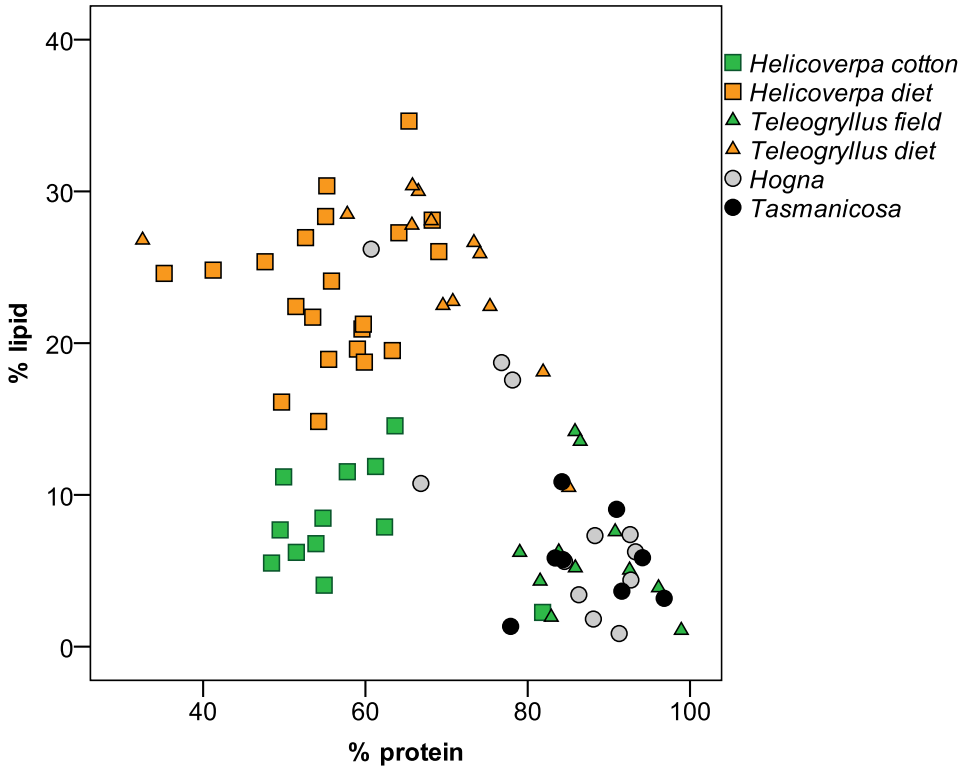

Figure 4. Percent body content of protein and lipid in four arthropods, caught near cotton fields, reared on cotton plant material, or reared on soy and agar diet.

Discussion

In the present study, we observed that in the presence of alternative prey, *Helicoverpa* was still targeted as prey by *Tasmanicosa* and *Hogna.* Predation outcomes can result from a combination of prey driven or predator driven variables; we here discuss how prey encounter, vulnerability and likelihood to escape can mediate predation by *Tasmanicosa,* the top predator in this ground arthropod food web. Additionally, we discuss how protein and lipid intake correlates with the structure of this food web.

Prey encounter and attack

Wolf spiders are considered as generalist predators that exhibit little prey selectivity, tending to feed according to availability. Wolf spiders respond principally to prey movement

(Persons & Uetz, 1997), and have been assumed to attack whenever a moving suitable prey is detected and comes within reach (Edgar, 1969). Prey encounter rate is the first mechanism that determines predation outcomes. From 3-way and 4-way trophic web trials, we observed that *Tasmanicosa* encountered all prey at a similar rate. However, prey encounter proportions did not match first attack proportions. The mismatch between first encounter and first attack by *Tasmanicosa* to *Teleogryllus* and *Helicoverpa* may be influenced by both the predator's decisions and the prey's defence mechanisms. Prey encounters (Brechbuehl *et al.*, 2011) or prey abundance (Kuusk & Ekbom, 2010) are not always the decisive factor for predation tendencies in hunting spiders. Other intrinsic characteristics of available prey should be taken into account to understand the factors that drive frequency of predation, such as prey's defences or ability to escape.

Potential risks and costs associated with attacking after prey encounter can be an important predictor of predatory decisions. Risks could involve physical injury or death due to counter attack, while costs involve energetic expenditure for subduing prey such as chasing and restraining. In the present trials, *Helicoverpa* and *Teleogryllus* exhibited defence behaviours against *Tasmanicosa.* Since *Tasmanicosa* was never physically injured while attempting to subdue *Teleogryllus* and *Helicoverpa,* it could be assumed that under the circumstances and prey sizes used for this study, *Teleogryllus* and *Helicoverpa* pose a similarly low risk to *Tasmanicosa.* Additionally, the body-bobbing behaviour of *Helicoverpa* might intensify the spider's attack behaviour as a visual stimulus (Bardwell & Averill, 1996). Because spiders are venomous predators themselves, spiders can be considered a dangerous prey to attack, due to the risk of counter-attack or inflicted bites. However, in 4-way trophic webs *Hogna* did not counter-attack as a defence mechanism, and when accounting for multiple predation, *Hogna* and *Helioverpa* were similarly attacked by *Tasmanicosa.*

A more likely mechanism underlying predation outcomes is the ability of the prey to escape and how a predator responds to escaping prey. Compared to *Helicoverpa* and *Hogna,*

Teleogryllus is a very mobile prey. After encounter, *Tasmanicosa* might not even have the chance to attempt attack if *Teleogryllus* quickly jumps away. (Dangles *et al.*, 2006) found that wood crickets (*Nemobius*) could detect a wolf spider (*Pardosa*) 5mm away and still escape; additionally, attack success was correlated to prey distance and attack velocity, but wolf spiders did not modify their attack velocity depending on prey distance. Some crickets can also detect chemical cues from wolf spiders and modify their behaviour to avoid predation (Storm & Lima, 2008), therefore crickets might not need to see or touch the spider to escape. From a predator's perspective, *Tasmanicosa* had a 100% success rate at killing *Teleogryllus* after attacking in an enclosed container, but *Teleogryllus* might be better at escaping attacks in field conditions. If *Tasmanicosa* has failed attacks towards *Teleogryllus* in the field, it is possible that spiders chose not to attack *Teleogryllus* immediately after encounter to avoid wasting energy in failed predation attempts, and instead choose to wait until they are positioned in a direction and distance that maximizes attack success. Thus, both *Teleogryllus'* ability to escape and *Tasmanicosa* hesitance to attack a quick prey can mediate predation outcomes in this food web. In the presence of slower prey such as *Helicoverpa* and *Hogna, Teleogryllus* is relatively costly and difficult to pursue, and may therefore represent a less vulnerable prey to *Tasmanicosa* than *Helicoverpa* and *Hogna*.

Since antipredatory behaviour and predator choice are not mutually exclusive in determining predation outcomes, disentangling their confounding effects is not straightforward. In some cases, passive selection mechanisms underlie what might seem a predator's active choice. For example, predatory midge larvae (*Chaoborus*) appear to select prey that are medium-sized, but this size selection is in fact confounded by the rate of prey encounter and capture success, thereby indicating a combination of passive prey selection and active predator choice (Pastorok, 1981). Other studies support the hypothesis of active prey choice regardless of passive selection variables. For example, predatory mirids selected two-spotted mites over phytoseiid mites regardless of how easily they are found and captured,

suggesting that mirids actively choose prey based on nutritional benefits (Provost *et al.*, 2006). In the present study, *Tasmanicosa* had a tendency to attack *Helicoverpa* before *Teleogryllus* despite similar encounter rates, and this trend was consistent in both in 3-way and 4-way trophic webs. This suggests that predation outcomes are not always determined by rate of encounter, and that prey vulnerability and the predator's response to such vulnerability can mediate the trophic web connections between *Tasmanicosa, Hogna, Teleogryllus* and *Helicoverpa.*

Prey nutritional content

Spider nutrition has the potential to influence prey choice, yet few studies have explored the links between spider nutrition and trophic webs (Wilder, 2011). Nutrient intake by predators is important to regulate development, health, and reproduction (Jensen *et al.*, 2011), therefore prey selectivity in hunting spiders may be mediated by nutrient optimization and toxin aversion (Toft, 1999). Some studies have argued that predatory arthropods are limited by nitrogen intake (necessary for building proteins; Fagan & Denno, 2004; Matsumura *et al.*, 2004). From this perspective, spiders would benefit more by preying on other predators or omnivores (Denno & Fagan, 2003), as nitrogen content enhances growth rates and survival in spiders (Okuyama, 2008), and herbivores have lower protein content (Wilder *et al.*, 2013). However, this nitrogen-limitation view has been challenged (Wilder & Eubanks, 2010), arguing that the predator's life stage, the way the predator differentially extracts nutrients, and the value of other macronutrients such as lipids and carbohydrates have a stronger effect on nutrient-mediated arthropod trophic webs.

Considering the nutritional contents of *Teleogryllus, Hogna* and *Helicoverpa* as if they had been encountered in the field, we observed that plant-reared *Helicoverpa* had lower protein content compared to field-collected *Teleogryllus* and *Hogna,* which is expected from a plant diet. Field-collected *Teleogryllus* had higher protein content than would be expected for a herbivore, similar to that of *Hogna.* A protein-enriched diet has been shown to increase

lifespan in *Teleogryllus* (Zajitschek *et al.*, 2012). Such a benefit could lead *Teleogryllus* to be omnivorous in the field by complementing their plant diet with scavenged animal remains (Fagan *et al.*, 2002). Diet-reared *Teleogryllus* had lower protein content than field-collected *Teleogryllus*, which shows that the high protein content of *Teleogryllus* is not only indigestible protein from its exoskeleton (chitin), but that there is also protein content in the gut or tissue of field-collected *Teleogryllus* that the spider can ingest.

Tasmanicosa did not reject protein-poor prey (*Helicoverpa)* in the presence of protein-rich prey (*Teleogryllus* and *Hogna),* as they killed *Hogna* and *Helicoverpa* in similar proportions in 4-way trophic webs. By having lower protein and similar lipid contents as *Hogna, Helicoverpa* might not seem to provide a nutritional advantage to *Tasmanicosa,* especially considering that the gut of *Helicoverpa* in the field is likely to have a high content of plant cellulose, a carbohydrate indigestible to the spider. However, there is evidence that wolf spiders (*Lycosa helluo*) develop quicker and survive longer on a diet of mixed arthropods (Uetz *et al.*, 1992). Spiders might benefit from varied proportions of different amino acids and essential micronutrients rather than just bulk protein (Mayntz & Toft, 2001). Greenstone (1979) found that the wolf spider *Pardosa ramulosa* preys on three different species of flies (Diptera: *Ephydra*, *Trichocorixa* and *Aedes*) in quantities such that the proportions of essential amino-acid are balanced and reflect the proportion of each amino acid present in the spider's haemolypmph. *Helicoverpa* contains essential amino acids and digestible carbohydrates (Lawo *et al.*, 2010) which can contribute to a balanced nutrient intake. Studies have shown that other spiders can represent as much as 38% of a wolf spider's mixed diet (for a review, see Hodge, 1999), yet a diet consisting solely of conspecifics can be detrimental to spider development. For example, lycosid spiderlings fed only spiders died sooner than spiderlings fed fruit flies or aphids (Oelbermann & Scheu, 2002), suggesting that conspecifics, despite their high protein content, still lack essential nutrients for development and survival.

Pest management implications

From a pest control perspective, the results of this study show that *Tasmanicosa* still kills *Helicoverpa* even when other common prey are available. In an agricultural landscape dominated by Bt-cotton, *Helicoverpa* larvae are less commonly encountered than are other wolf spiders or *Teleogryllus*. Predators often become more adept at killing and handling common prey, which may lead to development of a preference over unfamiliar prey (Murdoch, 1969). However, results of this study indicate that if *Tasmanicosa* encounters a *Helicoverpa* larva that has descended from foraging in a cotton plant in the same field as many other more common prey, it is likely that it will still kill *Helicoverpa,* thereby supporting the value of *Tasmanicosa* as an effective predator that can contribute to the control of Bt-resistance in *Helicoverpa.*

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CHAPTER 6: A killer killed? Wolf spider mortality after feeding on ground crickets

Manuscript formatted as a short communication for *Journal of Applied Entomology*

Abstract

Wolf spiders are abundant ground predators in a cotton agroecosystem where they can contribute to the biological control of insecticide-resistant cotton bollworms, *Helicoverpa armigera* (Hübner). However, their ability to serve as biological control agents may be impeded in the presence of toxic prey that affect their survivorship or behaviour. In this study, we describe the first record of the ground cricket *Teleogryllus commodus* (Walker) being toxic to the wolf spiders *Tasmanicosa leuckartii* (Thorell) and *Hogna crispipes* (Koch). Additionally, we describe the trophic web that includes *Tasmanicosa* and *Hogna* wolf spiders, *Teleogryllus* crickets, and *Helicoverpa* larvae, and consider the effects of *Teleogryllus* toxicity to wolf spiders on *Helicoverpa* survival. After eating one seemingly healthy *Teleogryllus* nymph collected from grassy areas around buildings, 30-40% of wolf spiders displayed symptoms of poisoning approximately 3 hours later and then died. No spiders died after feeding on commercially purchased house crickets, *Acheta domesticus* (Linnaeus). *Helicoverpa* larvae were more likely to survive in enclosed settings with wolf spiders when wolf spiders died after eating *Teleogryllus.* To further investigate if *Teleogryllus* toxicity was acquired from their environment, we offered wolf spiders *Teleogryllus* nymphs that had been collected from the same grassy areas and then reared on soy and agar diet for 25 days. No spiders died after eating diet-reared *Teleogryllus,* suggesting that this toxicity was acquired from the environment and not endogenous. Toxicity of *Teleogryllus* to wolf spiders was transient; wolf spiders did not die after eating *Teleogryllus* collected in the same areas one year later. The source of *Teleogryllus* toxicity remains unknown; possibilities include toxic compounds acquired through feeding on plants or animals in the field, a pathogen, or most likely, exposure to sublethal levels of pesticide.

Introduction

Wolf spiders (Araneae: Lycosidae) are generalist predators that attack a diverse range of prey (Hayes & Lockley, 1990; Nentwig, 1986; Nentwig & Wissel, 1986; Nyffeler & Benz, 1988), sometimes larger than the spider's body size (Nentwig & Wissel, 1986). As a consequence of their generalist predatory strategy, wolf spiders may sometimes be at risk of attacking noxious or dangerous prey (Fisker & Toft, 2004; Theodoratus & Bowers, 1999) that may have even lethal consequences. Wolf spiders are ubiquitous predators in many agroecosytems where they may contribute to biological control of crop pests (Kuusk & Ekbom, 2012; Marshall & Rypstra, 1999; Rendon *et al.*, 2015). An inability of wolf spiders to discriminate against fatally toxic prey could have serious consequences for their contribution to biological control; as wolf spiders are considered generalist predators that exhibit little selectivity in prey choice (Edgar, 1969; Persons & Uetz, 1997), toxic prey could potentially constrain the biological control capacity of wolf spiders in cotton crops.

The present study documents the first record of the ground cricket *Teleogryllus commodus* (Walker) (Orthoptera: Gryllidae) as a potentially toxic prey for *Tasmanicosa leuckartii* (Thorell) and *Hogna crispipes* (Koch) wolf spiders. In Australian cotton agroecosystems, wolf spiders can serve as biological control agents to suppress the cotton bollworm, *Helicoverpa* spp. (Lepidoptera: Noctuidae). These spiders are commonly found in cotton crops (Rendon *et al.*, 2015), and readily kill *Helicoverpa* spp. larvae and moths on the soil (Chapter 2, Chapter 3, Chapter 4). *Teleogryllus commodus* is abundant on the soil-plant interface of cotton crops in Australia, and is a common prey of wolf spiders (Chapter 2, Chapter 5). Our initial observations arose opportunistically during the course of a series of predation experiments in which wolf spiders died hours after feeding on a *Teleogryllus commodus* nymph collected from one location. Recognizing this as an unusual observation,

we carried out a series of studies to explore whether such toxicity was transient and therefore likely to be environmentally acquired.

Materials and methods

Collection of wolf spiders and crickets

This study was carried out at the Australian Cotton Research Institute (ACRI; 33ºS, 149ºE), near Narrabri, New South Wales, Australia, during the 2013 - 2014 and 2014 - 2015 cotton-growing seasons. Individuals of *Tasmanicosa leukartii*, *Hogna crispipes* and *Teleogryllus commodus* (hereafter referred to as '*Tasmanicosa*', '*Hogna*' and '*Teleogryllus*', respectively) were collected for experiments from December 2013 to March 2014 and from February 2015 to March 2015. Specimens of *Tasmanicosa* (cephalothorax width = 7.4 ± 1.2 mm, mean ± SD) and *Hogna* (cephalothorax width = 4.8 ± 0.6 mm, mean ± SD) were collected in and around Bt-cotton fields after sunset. Since *Teleogryllus* nymphs shelter inside soil cracks and are difficult to find and collect inside cotton fields, *Teleogryllus* nymphs (body length = 12.4 ± 2.7 mm, body weight = 0.09 ± 0.04 g; mean ± SD) were collected around the buildings at ACRI (hereafter 'field-collected' *Teleogryllus*). Surroundings of these buildings had been sprayed with bifenthrin (Maxxthor™ 100, Ensystex Australasia Pty Ltd) on 22 November 2013, and 21 November 2014, following manufacturer's instructions. Spiders and crickets were found by visual search using a headlamp (Petzl Tikka, 140 lumens), and were collected manually in clear 70 mL cylindrical plastic containers.

After collecting, all spiders and crickets were brought to the laboratory. Cephalothorax width of spiders and body length of crickets was measured to the nearest 0.1 mm using a manual caliper (resolution 0.1 mm). Sex and life stage (male, female or juvenile) of spiders was also recorded. Collected spiders were individually housed in cubical clear plastic containers (hereafter 'spider container'; 228 mm height x 238 mm length x 238 mm width, 8.5 L, Décor Tellfresh superstorer®) containing 2 L of moist soil, and were kept in a

controlled environment room (24.4 ± 0.5°C, mean ± SD) with a L14:D10 photoperiod for 24 hrs before being used in all experiments. All trials were run in this controlled environment room under the same conditions. All spiders and crickets were used in experiments only once, and then released back in the field.

Experiment 1: Spider mortality following cricket predation

This experiment was designed to compare mortality of *Tasmanicosa* and *Hogna* after feeding on field-collected *Teleogryllus* and commercially supplied (ARCade, http://frogs.org.au) house crickets *Acheta domesticus* (Linnaeus) (Orthoptera: Gryllidae; hereafter '*Acheta*'). Twenty-one day old *Acheta* nymphs were supplied on 9 February 2014 and were housed together in a single clear 8.5L plastic container with a wet cotton wick to provide moisture, and were fed *ad libitum* soy and wheat agar diet cubes (Teakle & Jensen, 1985) for 7 days before being used in experiments. *Acheta* nymphs (body length = 10.2 ± 0.8 mm, body weight = 0.05 ± 0.01 g; mean ± SD) were individually placed in clear plastic 70mL containers for 24 hrs before being used in experiments. *Teleogryllus* nymphs were collected from the field in the same area described above and were held individually in clear plastic 70 mL containers for 24 hrs before being used in experiments. *Tasmanicosa* (n=20) and *Hogna* (n=20) were randomly offered either one *Teleogryllus* or one *Acheta* nymph. Between 30 and 60 minutes after the dark phase commenced in the controlled environment room, one cricket (*Teleogryllus* or *Acheta*) was released inside each spider container. Cricket and spider mortality was assessed 24 hrs later. A contingency table was analysed using a Chi-square test for independence to ascertain whether spiders were more likely to die after eating *Teleogryllus* or *Acheta*. A binary logistic regression was done to determine whether spider stage (male, female, juvenile) or spider cephalothorax width were predictors of spider mortality after eating *Teleogryllus.*

Experiment 2: Toxicity of* Teleogryllus *isolated from field influences

This experiment was designed to test whether toxicity persisted if *Teleogryllus* were dissociated from potential influences in the field (e.g., diet, environmental toxins). One group of *Teleogryllus* early nymphs (body length 1.8 ± 0.2 mm, mean ± SD; hereafter 'diet-reared') were collected in the field on 5 March 2014, and housed together in a single clear 8.5L plastic container (hereafter 'diet container') with a wet cotton wick to provide moisture, and fed *ad libitum* soy and agar diet cubes for 25 days until the nymphs reached a body length of 12.2 ± 0.16 mm (mean ± SD). A separate group of field-collected *Teleogryllus* nymphs were collected on 28 March 2014 from the field in the same areas described above. One *Tasmanicosa* was randomly paired with one diet-reared (N=21) or one field-collected (N=21) *Teleogryllus* nymph. Both diet-reared and field-collected *Teleogryllus* nymphs were individually placed in clear plastic 70 mL containers for 24 hrs before being used in experiments. Between 30 and 60 minutes after the dark phase commenced in the controlled temperature room, one diet-reared or field-collected *Teleogryllus* nymph was released inside each spider container. Cricket and spider mortality was assessed 24 hrs later. A contingency table was analysed using a Chi-square test for independence to ascertain whether spiders were more likely to die after eating diet-reared or field-collected *Teleogryllus*.

Experiment 3: Yearly variation in cricket toxicity

Following compelling results in the 2013-2014 season, this experiment was carried out to assess whether toxicity of *Teleogryllus* persisted in the 2014-2015 season. All *Teleogryllus* nymphs were collected in the same areas as in 2014. One group of *Teleogryllus* nymphs was collected on 27 February 2015, and housed together in a single clear 8.5L plastic container with a wet cotton wick to provide moisture, and fed *ad libitum* soy and agar diet cubes for 3 days ('laboratory-fed'). Field-collected *Teleogryllus* nymphs were collected immediately before trials in the same areas as experiments in 2013-2014 season. One *Tasmanicosa* was randomly paired with one field-collected (N= 12, approximately 5 minutes after being collected from the field), or one laboratory-fed *Teleogryllus* nymph (N= 12). Between 30 and

60 minutes after the dark phase commenced in the controlled environment room, one laboratory-fed or field-collected *Teleogryllus* nymph was released inside each spider container. Cricket and spider mortality was assessed 24 hrs later. A contingency table was analysed using a Chi-square test for independence to ascertain whether spiders were more likely to die after eating laboratory-fed or field-collected *Teleogryllus*.

To compare weather variability between both years, total rainfall (mm), mean maximum temperature and mean minimum temperature measurements were obtained from http://www.weather.cottassist.com.au, between 1 December 2013 to 1 April 2014, and from 1 December 2014 to 1 December 2015.

***Experiment 4: Predation on* Helicoverpa *in the presence of toxic* Teleogryllus**

This experiment was carried out to determine whether the presence of toxic *Teleogryllus* interferes with *Helicoverpa armigera* (Hübner) (hereafter *'Helicoverpa'*) predation by wolf spiders. Larvae of *Helicoverpa* were supplied by the Commonwealth Scientific and Industrial Research Organization (CSIRO) Agricultural Flagship Bt Resistance Monitoring Group. Larvae were reared in individual wells in trays with soy and agar diet (Downes *et al.*, 2009; Teakle & Jensen, 1985); and kept in a controlled environment room (24.4 ± 0.5ºC, mean ± SD) with a L14:D10 photoperiod. A laboratory experiment was set up in which one 5th instar *Helicoverpa* larva (weight = 0.41 ± 0.06 g, mean ± SD) was paired with (i) one *Tasmanicosa* (N=27), (ii) one *Hogna* (N=19), (iii) one *Tasmanicosa*, and one field-caught *Teleogryllus* nymph (N=27), and (iv) one *Hogna* and one field-collected *Teleogryllus* nymph (N=27). Spiders and crickets were collected between 12 December 2014 and 11 March 2014. After being collected in the field, *Teleogryllus* were individually housed in clear plastic 70 mL containers for 24 hrs before being used in experiments. Between 30 and 60 minutes after the dark phase commenced in the controlled temperature room, one *Teleogryllus* and one 5th instar *Helicoverpa* larva were simultaneously released into each spider container. Cricket, spider and *Helicoverpa* mortality was assessed 24 hrs later. A

contingency table was analysed using a Chi-square test for independence to ascertain whether *Tasmanicosa* and *Hogna* were more likely to kill *Helicoverpa* in the presence or absence of *Teleogryllus.*

Experiment 5: The effect of toxic crickets on spider behaviour

To observe *Tasmanicosa* hunting behaviour, trials described in experiment 4 in which *Tasmanicosa, Teleogryllus* and *Helicoverpa* were paired together were recorded for 24hrs. Continuous video recording began immediately after *Helicoverpa* and *Teleogryllus* were released, and ended 24 hrs later; our recording system comprised a 1/3" CCD monochromatic infra-red camera (CCS- Sony Go Video) with a 4 mm C mount lens positioned above each container, which recorded to a 2TB DVR4-100 hard drive recorder. One infrared (940nm) illuminator (IR-covert) was placed 10 cm to the side from each container. To improve video contrast, each animal was dusted with fluorescent dye (HCA Colours Australia, VM311 Pink for *Helicoverpa* larvae and *Teleogryllus*, VM317 Yellow for *Tasmanicosa*). From each video the latency to attack *Teleogryllus*, time consuming *Teleogryllus,* and behaviours unique to spiders that later died were determined.

Results

Experiment 1: Spider mortality following cricket predation

All *Tasmanicosa* spiders ate all the field-collected *Teleogryllus* and *Acheta* nymphs, and 40% of *Tasmanicosa* died after eating *Teleogryllus.* All *Hogna* spiders ate all the *Acheta* crickets, and all but one ate the *Teleogryllus* cricket. 30% of *Hogna* died after eating *Teleogryllus.* No spiders died after eating *Acheta*. Both spider species were more likely to die after preying on *Teleogryllus* than on *Acheta* (*Tasmanicosa,* χ^2= 4.971, df= 1, p= 0.026; *Hogna,* χ^2= 4.242, df= 1, p= 0.039). Spider mortality after feeding on *Teleogryllus* was not predicted by spider stage or spider cephalothorax width (binary logistic regression, all *Tasmanicosa* and *Hogna* $p > 0.05$).

Experiment 2: Toxicity of* Teleogryllus *isolated from field influences

Comparing field-collected and diet-reared *Teleogryllus*, when *Tasmanicosa* was paired with a field-collected *Teleogryllus* nymph, 4.7% rejected *Teleogryllus*, and 28% died after preying on *Teleogryllus*. When *Tasmanicosa* was paired with a diet-reared *Teleogryllus*, 19% rejected *Teleogryllus*, and none died after preying on *Teleogryllus*.

Experiment 3: Yearly variation in cricket toxicity

Unlike the results in 2014, in 2015 no *Tasmanicosa* died after eating either field-collected *Teleogryllus* (that had been collected from the field within 5 minutes before experiments) or laboratory-fed *Teleogryllus* (that had been maintained in the laboratory for 3 days prior to experiments). Environmental variables such as temperature and rainfall were similar between both years; the total rainfall from 1 December 2013 – 1 April 2014 was 201.80 mm, the average high temperature was 34.3°C, and the average minimum temperature was 18.0°C. The total rainfall from 1 December 2014 – 1 April 2015 was 210.2 mm, the average high temperature was 33.5°C, and the average minimum temperature was 17.7°C.

***Experiment 4: Predation on* Helicoverpa *in the presence of toxic* Teleogryllus**

In the absence of *Teleogryllus*, 96.2% of *Tasmanicosa* killed *Helicoverpa* (N= 27), whereas in the presence of *Teleogryllus*, 72.3% of *Tasmanicosa* killed *Helicoverpa* (N = 27). Thus *Tasmanicosa* was less likely to kill *Helicoverpa* when *Teleogryllus* was present (χ^2 = 5.91, df = 1, p = 0.015). In the absence of *Teleogryllus*, 89.4% of *Hogna* killed *Helicoverpa* (N = 19), and in the presence of *Teleogryllus*, 86.1% of *Hogna* killed *Helicoverpa* (N = 27). There was no evidence that the tendency of *Hogna* to kill *Helicoverpa* was influenced by the presence of *Teleogryllus* (χ^2 = 0.11, df = 1, p = 0.73).

Videorecorded trials with *Tasmanicosa*, *Teleogryllus* and *Helicoverpa* showed that 11% of *Tasmanicosa* died after eating *Teleogryllus* and did not kill *Helicoverpa*. Furthermore, 7.4% of *Tasmanicosa* died after eating *Helicoverpa* followed by *Teleogryllus* (Figure 1). When *Hogna* was paired with both *Teleogryllus* and *Helicoverpa*, 3.7% of *Hogna* died after

eating *Teleogryllus* and did not kill *Helicoverpa,* and 11.1% killed *Teleogryllus* but were still alive and did not kill *Helicoverpa*, none killed *Teleogryllus* after eating *Helicoverpa*, and no *Hogna* rejected both prey.

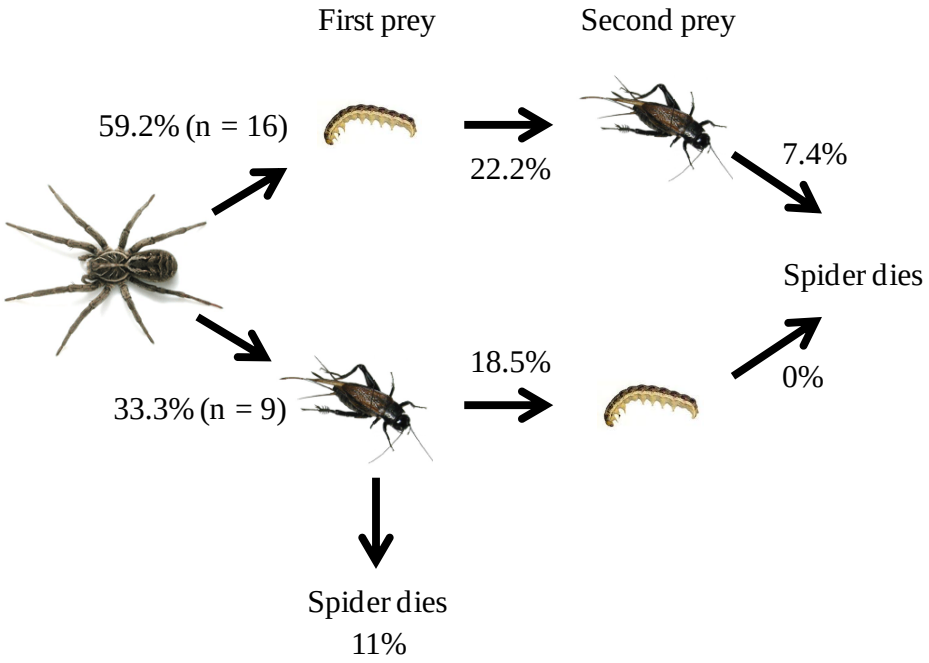

Figure 1. Predation sequence of *Tasmanicosa* paired with *Helicoverpa* and *Teleogryllus* (videorecorded trials). Direction of arrows indicates predation sequence. Percentages indicate how many spiders out of the original n = 27 followed the predation sequence indicated by the arrow.

Experiment 5: The effect of toxic crickets on spider behaviour

The latency for *Tasmanicosa* to attack *Teleogryllus* was 2.2 ± 1.92 hr (mean ± SD) after trials started. *Tasmanicosa* remained immobile consuming *Teleogryllus* for 2.00 ± 0.61 hr. Some behaviours observed in *Tasmanicosa* were specific to those that died after eating *Teleogryllus.* These behaviours included: (1) erratic sudden running: *Tasmanicosa* were initially immobile, and then suddenly ran around the container, sometimes sideways or in a zigzag pattern (latency after attack: 3.67 ± 2.56 h); (2) Leg curling: *Tasmanicosa* flexed all joints so that legs I curled towards their body when immobile (latency after attack: 4.10 ± 2.83 h); (3) Curled leg walking: *Tasmanicosa* walked with their legs I curled, pushing their body with legs II, III and IV (latency after attack: 4.30 ± 2.73 h); (4) Sudden jumps: initially

immobile spiders suddenly jumped and then returned to an immobile state (latency after attack: 4.92 ± 3.57 h); (5) Tipping sideways: spiders standing upright turned their entire body sideways for a few seconds (latency after attack: 5.30 ± 3.40 h); (6) Inverting: spiders standing upright turned their whole body upside-down for a few seconds (latency after attack: 5.37 ± 3.32 h), (7) Tremors: initially immobile spiders briefly shook their legs or bodies (latency after attack: 6.35 ± 3.01); and (8) Complete paralysis: the spider is completely immobile for more than one minute, lasting until the end of the trial (latency after attack: 7.47 ± 4.38 h) After this point, it was not possible to determine from video recordings whether the spider was alive but paralysed, or already dead.

Discussion

Teleogryllus *toxicity*

This is the first study to report toxicity of crickets to wolf spiders. Toxicity may be produced endogenously by some insects such as stink bugs (Hemiptera; Krall *et al.*, 1999; Staples *et al.*, 2002) or earwigs (Dermaptera; Gasch *et al.*, 2013; Gasch & Vilcinskas, 2014), which secrete noxious compounds to deter predators. However, in the order Orthoptera, there are no reported cases of endogenously produced toxins, or specific glands that might produce toxins. It seems far more likely that *Teleogryllus* in our study acquired toxicity from their environment. There are multiple mechanisms by which *Teleogryllus* could have acquired toxicity in the field.

First, *Teleogryllus* might have acquired toxicity by sequestering noxious compounds from plants or simply by having noxious compounds from such plants in their gut when eaten by spiders. Plant toxin sequestration is common in Lepidoptera and Coleoptera (Opitz & Muller, 2009), presumably as an antipredatory strategy (Mason *et al.*, 2014). A few species of crickets are known to sequester plant toxins (e.g., Acrididae: *Romalea guttata* [Jones *et al.*, 1989]; Acrididae: *Schistocerca emarginata* [Sword, 2001]; Pyrgomorphidae: *Poekilocerus*

bufonius [Euw et al., 1967]). However, as no herbicides were used in the area, we expect that the same plants and weeds were present in the study site on both years, which would not explain why *Teleogryllus* toxicity was only observed in one year. Another possibility is that, as an omnivore, *Teleogryllus* can accumulate toxic compounds by feeding on arthropods toxic to wolf spiders such as Collembola (Fisker & Toft, 2004).

Second, mortality of wolf spiders might have resulted from the presence of a pathogen that could be transferred across taxa, from *Teleogryllus* to wolf spiders. (Reinganum *et al.*, 1970) reported a virus that affects *Teleogryllus* spp (CrPV). Symptoms of infected crickets include impaired coordination and paralysis of their hind legs. This virus can remain latent in the body of a cricket without killing it, and late-instar nymphs are less susceptible to development of paralytic symptoms and death. In the present study, all the crickets appeared healthy. This virus can also infect other insects such as fruit flies (Plus & Scotti, 1984), but it is not known if predators can also become infected after feeding on infected prey. The hypothesis of spiders becoming infected with a virus from *Teleogryllus* is unlikely, given that spiders did not die after eating diet-reared *Teleogryllus* nymphs in the 2013-2014 season. If infected *Teleogryllus* nymphs had been brought into the laboratory, it is likely that the virus would have persisted in the diet-reared colony, therefore still infecting *Teleogryllus* and spiders.

Third, *Teleogryllus* might have acquired toxicity through toxic chemicals in the environment. In some cases, wolf spiders are not affected by eating prey exposed to pesticides in concentrations applied to agricultural fields. For example, *Pardosa birmanica* did not die when offered a diet of mixed prey sprayed with acetochlor (Tahir *et al.*, 2011). Similarly, *Pardosa pseudoannulata* did not die or modify its hunting behaviour after eating leafhoppers that had been exposed to imidacloprid (Widiarta *et al.*, 2001). However, there is evidence that doses of pesticide that are sublethal for herbivores may prove lethal to the predators that consume them (Pekar, 2012). For example, *Acheta* crickets injected with small quantities of

the pesticide azadirachtin remain active, but are lethal prey for the wolf spider *Schizocosa episina* (Punzo, 1997). The type of pesticide, its method of application, and concentration may have variable nontarget effects on predators feeding on prey exposed to the pesticide (Wilson *et al.,* 2015).

The location from which crickets were collected had been sprayed previously with Bifenthrin, a neurotoxin used to target soil insect pests, including crickets. As a pyrethroid pesticide, bifentrin has a very high impact on reducing spider populations after application (Wilson *el al.* 2015). It is possible that *Teleogryllus* in this area were exposed to sub-lethal doses of bifenthrin that were not sufficient to kill the crickets but made them lethal prey for wolf spiders. In mole crickets (*Scapteriscus* sp.), bifenthrin poisoning induces erratic movements and tremors similar to those we observed in wolf spiders that later died. Mole cricket nymphs show a lethal time 50 of 6.5h after being injected with 2.5 Ug/UL of bifenthrin (Kostromytska *et al.*, 2011). Spider densities can also be reduced by the application of bifenthrin (Larson *et al.*, 2012). The persistence of bifenthrin is highly variable, depending on temperature and humidity, and it can remain on the soil from 7 days to 8 months (Material Safety Datasheet, Ensystex Australasia Pty Ltd). Although rainfall and temperature conditions were similar between the two seasons, this high variability in persistence could explain why *Teleogryllus* from this site were toxic to wolf spiders in the 2014-2015 season but not in the 2014-2015 season.

***Implications for biological control of* Helicoverpa**

Teleogryllus is a common inhabitant in and around cotton fields, and has been observed as prey for wolf spiders (Chapter 2, Chapter 4). When paired only with field-collected *Teleogryllus* in the 2014-2015 season, mortality was high in both *Tasmanicosa* (40%) and *Hogna* (30%) and neither spider species showed any discrimination against *Teleogryllus* as prey. This high mortality rate suggests that lethally toxic *Teleogryllus* are not common in the field, as persistent exposure to such toxic prey would result in either greatly

reduced wolf spider abundance or evolved resistance in the form of prey discrimination. This group of toxic *Teleogryllus* appears to represent a spatially and temporally isolated population, not commonly encountered or widespread in and around cotton fields.

Even if it is an uncommon scenario, the fact that *Teleogryllus* can acquire toxic compounds from the field can still affect biological control. The presence of *Teleogryllus* decreased *Helicoverpa* predation by *Tasmanicosa* by causing spiders to die after eating *Teleogryllus.* The areas where *Teleogryllus* were collected are approximately 50m away from a cotton field; although it is unlikely that wolf spiders would travel such a long distance (Ahrens & Kraus, 2006), it is possible that other isolated populations of *Teleogryllus* that live inside or closer to cotton fields could be acquiring the same toxic compound as the population used in these experiments. Exposure to lethally toxic *Teleogryllus* can diminish the abundance of wolf spiders in a particular area, therefore allowing *Helicoverpa* to survive.

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GENERAL DISCUSSION

The main aim of this thesis was to examine the impact of wolf spiders as native predators of ground-dwelling stages of *Helicoverpa* spp[1]. that represent a risk for the evolution of Bt-resistance in cotton crops. In this discussion, I refer back to the research questions raised in the introduction (Table 1; Macfadyen *et al.*, 2015), and discuss how each chapter addresses and answers these questions. Additionally, I discuss some of the benefits and limitations of each method in assessing the impact of wolf spiders on *Helicoverpa* spp. (Table 2, copied with permission from Macfadyen *et al.*, 2015), which can guide future studies.

[1] Note on terminology: for consistency with chapter terminology, *Helicoverpa* spp. refers to both *Helicoverpa armigera* and *Helicoverpa punctigera*, while '*Helicoverpa'* refers to *Helicoverpa armigera*, used in laboratory and glasshouse experiments. When the distinction is needed, *Hogna crispipes* and *Hogna kuyani* are referred to by genus and species, while '*Hogna'* refers to *Hogna crispipes* used in laboratory and glasshouse experiments.

Table 1: Questions to assess the impact of a predator on a pest prey, and the chapters in this thesis that address the corresponding question (adapted from Macfadyen *et al.*, 2015).

Question	**Chapter**
Q1: Does the predator species kill the pest species?	2, 3
Q2: How many pests does the predator kill and how quickly?	2, 3, 4, 5
Q3: How do predator populations respond to changes in prey density?	1, 2
Q4: How does the predator search for prey?	2, 3, 5
Q5: What are the indirect impacts of predator behaviours on pest populations and herbivory?	3, 4
Q6: Are predator species present in the crop at the same time as pest species?	1, 2
Q7: Do other species in the community impact the predator-prey interaction?	4, 5, 6
Q8: Do other abiotic factors impact the predator-prey interaction?	1

1. Does the predator species kill the pest species?

The main questions asked were: (1) which species of wolf spiders kill *Helicoverpa* spp. in cotton fields and enclosures? (2) Do wolf spiders kill larvae, pupae, or moths? I employed several methods to address these questions: direct field observations (Chapter 2), gut-content analysis (Chapter 2), predation in small enclosures (Chapter 2, 3), and predation in larger glasshouse enclosures (Chapter 3). I here address the main findings, advantages, and limitations and potential ways to improve each technique.

From direct observation in Bt cotton fields (Chapter 2) I could not assess if wolf spiders hunt *Helicoverpa* spp. on the soil. Wolf spiders (*Lycosa*) have been observed eating *Helicoverpa* spp. larvae in cotton fields (Bishop, 1978), but these encounters were very rare. The difficulty in finding wolf spiders eating *Helicoverpa* spp. in the field is also confounded by the fact that 5th instar larvae or emerging adults are difficult to find on the soil, as they quickly burrow underground to pupate, or climb to the plants before flight (Chapter 3). Furthermore, general predation events were very rarely observed in the field. Given the limitations of direct observation, gut-content analysis therefore offered a tool to determine predation that could not be observed in the field.

Gut-content analysis through ELISA revealed that 2.1% of wolf spiders collected in Bt cotton fields tested positive for the presence of IgG. The presence of positive IgG marks in spiders suggest that some spiders did kill *Helicoverpa* spp. in the field; however such interpretation should be approached with caution, since spiders can also pick up IgG traces from the soil or by touching marked *Helicoverpa* spp. There are two possible explanations for the high proportion of IgG negative spiders: that most wolf spiders did not kill IgG-marked *Helicoverpa* spp. when encountered, which was not supported by field container predation trials; or as mark-recapture surveys indicated, that wolf spiders that ate IgG-marked *Helicoverpa* spp. had a low likelihood of being recaptured.

Despite the limitations of field and molecular techniques, predation can still be evaluated in controlled enclosures. Before this thesis, it was unknown which wolf spider species would kill *Helicoverpa* spp. on the soil of cotton fields. Predation trials in field containers (Chapter 2) allowed confirming that the three largest wolf spider species (*Tasmanicosa leuckartii, Hogna crispipes, Hogna kuyani*) did in fact kill *Helicoverpa* spp. 5th instar larvae. With a cephalothorax width of less than 3mm, the most abundant species reported in Chapter 1, *Venatrix konei,* is too small to hunt and kill a large 5th instar *Helicoverpa* spp. larva or moth (pilot studies, personal observation), and therefore was not included in visual surveys or predation trials. *Tasmanicosa leuckartii* was abundant in pitfall traps in complex plots (minimum-tilled with winter crop), commonly observed in visual surveys, had the largest body size of all wolf spiders, and was an early colonizer in cotton fields. For these reasons, *Tasmanicosa* was an excellent model to evaluate predator effectiveness on ground-dwelling stages of *Helicoverpa.*

Predation trials in laboratory containers revealed that a high proportion of *Tasmanicosa* killed both 5th instar larvae as they descend from the plant to pupate in the soil, and emerging moths (Chapter 3). Predation was not observed on underground pupae, which indicated that this is the only ground-dwelling *Helicoverpa* stage safe from attack by *Tasmanicosa.* However, there is evidence that *Helicoverpa* spp. pupae can be attacked underground by other wolf spider species (*Helluo insignis;* Room, 1979). Bt-resistant individuals must be eradicated at any point before they mate and lay eggs. In this scenario, *Tasmanicosa* killed *Helicoverpa* from after 5th instar larvae descends from the plant after foraging, to when moths emerge from its underground pupal chamber; therefore *Tasmanicosa* can be an effective biological control agent during key stages of the developmental cycle of *Helicoverpa* spp.

2. How many pests does the predator kill and how quickly?

In glasshouse enclosures (Chapter 3), the presence of a predatory spider reduced the number of *Helicoverpa* moths emerging by 66% on average compared to no spider. Additionally, in some cases a single spider killed all six *Helicoverpa.* These enclosures had a higher density of *Helicoverpa* larva than that expected in cotton fields (6 larvae per 0.24m^2, or 25 larvae / m^2), therefore spiders have the capacity of reducing *Helicoverpa* numbers even when larvae are present in high densities. In smaller laboratory containers (Chapter 3), one *Tasmanicosa* killed on average 2.5 *Helicoverpa* 5th instar larvae, but could kill up to six larvae in eight hours. This rate of predation is due to the fact that wolf spiders are opportunistic hunters, and do not always consume their prey entirely before killing another accessible prey (Framenau *et al.*, 1999). Furthermore, this result is supported by the finding that *Tasmanicosa* killed multiple prey in a food web (Chapter 5). In chapter 4, only the larval stage of *Helicoverpa* was exposed in glasshouse enclosures, and over a period of three days, one *Tasmanicosa* or one *Hogna* were capable of killing approximately half of the six 5th instar larvae initially released. No other studies have looked at the efficacy of predators that hunt ground-dwelling stages *Helicoverpa* spp., and the results of this thesis provided a numerical estimate on the impact of wolf spiders on *Helicoverpa* spp in enclosures.

3. How do predator populations respond to changes in prey density?

The main value of generalist predators in biological control is that their abundance is not tied closely to that of the pest prey (Symondson *et al.*, 2002). Wolf spiders were abundant in Bt cotton plots, where *Helicoverpa* spp. larvae and moths are rare (Chapters 1,2). Factors such as cotton stage, presence of alternative prey and field disturbances are more likely to affect wolf spider populations than fluctuations in *Helicoverpa* spp. densities. In the absence of pest prey, alternative prey can sustain a high abundance of wolf spiders within the cotton fields (Ishijima *et al.*, 2006) such that there is a high chance of a resistant *Helicoverpa* spp. encountering a wolf spider either when descending to pupate or when emerging as a moth.

4. How does the predator search for prey?

In the field, spiders were found mainly immobile, and were not observed actively pursuing prey (Chapter 2). *Tasmanicosa* is most likely to find *Helicoverpa* spp. on the soil, but *Hogna crispipes* and *Hogna kuyani* may also encounter and kill *Helicoverpa* spp. larvae that are on the cotton plant (Chapter 2, 4). In laboratory enclosures, *Tasmanicosa* oriented towards *Helicoverpa* larvae or moths when they moved, even if the larva or moth was located on the opposite end of the container (over 200 mm; Chapter 3). *Tasmanicosa* usually attacked *Helicoverpa* whenever it made physical contact with the larvae (Chapter 5), suggesting that encounters with *Helicoverpa* will likely lead to attack. Although wolf spiders can travel several metres in one night (Chapter 2), the mostly stationary behaviour of spiders suggest that instead of chasing its prey, wolf spiders follow a "sit-and-move" strategy (Samu *et al.*, 2003), whereby spiders undertake brief bouts of movements between stationary sites, and prey attack is triggered by prey movement perceived from stationary sites.

Although the observations in laboratory containers suggest that *Tasmanicosa* is mainly a visual predator (Persons & Uetz, 1997), it is possible that in certain distances and substrates *Tasmanicosa* can perceive vibrations (Wrinn & Uetz, 2008) from larvae or moths that might trigger local searching or an attack. Pupae were never dug up and attacked by spiders, which suggests that *Tasmanicosa* may not be able to detect pupae moving underground, or that being unable to see them, wolf spiders do not respond to moving pupae. Although there is evidence that wolf spiders also respond to chemical cues left by their prey (Persons & Rypstra, 2000; Persons & Uetz, 1996; Punzo & Preshkar, 2002), this mechanism was not explored as a mechanism for *Tasmanicosa* to find *Helicoverpa.*

5. What are the indirect impacts of predator behaviours on pest populations and herbivory?

Besides consumptive effects; i.e., the direct impacts of *Tasmanicosa* predation on *Helicoverpa,* Chapter 3 also examined nonconsumptive effects on the behaviour and development of *Helicoverpa.* Nonconsumptive effects can have greater impacts on prey than consumptive effects; this is because one predator is limited by the number of prey it can kill and handle in a period of time, but this single predator can have simultaneous nonconsumptive effects on multiple prey (Beckerman *et al.,* 1997; Preisser *et al.*, 2005; Werner & Peacor, 2003). In this study enclosure type largely influenced nonconsumptive effects; the presence of a risk spider modified the behaviour and development of *Helicoverpa* in small laboratory containers, but not in large glasshouse enclosures.

In the presence of a risk spider (with glued chelicerae, Chapter 3) in a laboratory container, *Helicoverpa* spent less time on the cotton ball, yet unexpectedly, cotton bolls lost more mass. This result is interpreted as more intensive foraging as a result of predation risk. Furthermore, *Helicoverpa* might leave the cotton bolls sooner if a predator is detected, given that the cotton plant foliage is the first microhabitat where larvae are at risk. Contrasting this result, numerous studies have reported decreased plant damage by herbivores in the presence of spiders (Beckerman *et al.*, 1997; Carter & Rypstra, 1995; Hlivko & Rypstra, 2003; Whitehouse *et al.*, 2011). However, intensive plant foraging in response to predation risk was also reported in another study in which wood crickets foraged more intensively on strawberry leaves and gained more weight in the presence of spider cues (Bucher et al., 2014). Similarly, the presence of wolf spiders has been associated with diminished production in cucurbit crops due to herbivory (Snyder & Wise, 2001). Effects on plant production were not investigated in larger glasshouse enclosures, but foraging behaviour and mobility of *Helicoverpa* larva did not change in the presence of a risk spider in glasshouse enclosures, suggesting that maybe a ground predator such as *Tasmanicosa* might not be detected in higher and more complex plant canopies, therefore diluting any potential effects of negative trophic cascades.

Intraguild interactions between wolf spiders also led to nonconsumptive effects on *Helicoverpa* mortality (Chapter 4). Recorded laboratory trials showed that in the presence of *Hogna, Tasmanicosa* is less effective at killing *Helicoverpa* compared to when *Hogna* is absent, but overall *Helicoverpa* mortality is not affected as *Hogna* still killed *Helicoverpa* in similar proportions in the presence or absence of *Tasmanicosa.* Spider mortality in glasshouse enclosures was not correlated with *Helicoverpa* mortality, suggesting that even if one spider does not kill the other, just the mere presence of another spider is enough to modify spider predatory behaviour. The behavioural effects stemming from intraguild interations between spiders do not reduce biological control, but these behavioural effects restrain biological control in a way such that the effect of each individual spider is not additive.

6. Are predator species present in the crop at the same time as pest species?

A combination of pitfall trapping (Chapter 1) and direct observations (Chapter 2) confirmed that wolf spiders are present in cotton fields before *Helicoverpa* spp. moths emerge from winter diapause, are abundant throughout the cotton growing season, and remain inside the fields as *Helicoverpa* spp. larvae descend to diapause at the end of the season. Resistance management in cotton has different objectives depending on the plant growth stage: before planting, it is important to assess the type and area of non-Bt refuge to be planted; during the growing season, the crop has to be monitored for pest and damage thresholds; and after harvest, the focus is on mechanically destroying all surviving pupae by 'pupae busting' (Deguine *et al.*, 2008). An advantage of incorporating wolf spiders in resistance management strategies flows from the staggered phenology of diverse species in the field (Chapter 1): wolf spiders can kill resistant *Helicoverpa* spp. before, during, and after the cotton growing season.

In addition to studying field phenology, time budget analysis in laboratory containers (Chapter 3) also showed that *Tasmanicosa* and *Helicoverpa* were active during the same photoperiods. Besides determining if wolf spiders and *Helicoverpa* spp were synchronized

chronologically, it is also relevant to determine whether they co-existed spatially. Mark-recapture surveys (Chapter 2) showed that wolf spiders crossed the edges where *Helicoverpa* spp. were released, thereby confirming that wolf spiders and *Helicoverpa* spp. were active at the same time, and in the same area.

7. Do other species in the community impact the predator-prey interaction?

Predator-prey interactions between wolf spiders and *Helicoverpa* in cotton fields never occur in isolation. Instead, ground trophic webs are influenced by other elements such as alternative prey and competitor predators, which in turn may also become intraguild prey. The next step after looking at predator-prey interactions between *Tasmanicosa* and *Helicoverpa* was to increase the trophic web complexity, and assess how the presence of intraguild predators (Chapter 4) and alternative prey (Chapter 5) influenced *Helicoverpa* mortality.

When multiple species of predators are present in an agroecosystem, intraguild interactions can disrupt the pressure that each species individually exerts over a pest prey (Snyder & Tylianakis, 2012). A negative result from antagonistic intraguild interactions may be that one or both predator species reducing its efficacy at killing this pest, either from behavioural modifications, or simply by being killed by a dominant predator. Both *Tasmanicosa leuckartii* and *Hogna crispipes* are abundant in complex plots (Chapter 1), where the coexistence of these two spider species is possible in this structurally complex habitat. The next question is, does the presence of these two spider species have an additive, neutral, or negative effect in *Helicoverpa* mortality?

The presence of two spiders in glasshouse enclosures (Chapter 4) did not have an additive effect on *Helicoverpa* mortality. Glasshouse experiments revealed that the presence of two spiders of the same species (*Tasmanicosa* + *Tasmanicosa*, *Hogna* + *Hogna*, one smaller than the other) did not improve biological control on *Helicoverpa* beyond that exerted

by just one spider (*Tasmanicosa* or *Hogna*). When comparing spider abundance (two spiders of the same species) with spider richness (two spiders of different species), two spiders of the same species had a similar effect on *Helicoverpa* mortality compared to two spiders of different species. Previous studies have shown that intraguild interactions can interfere with biological control when multiple spiders are combined (Finke & Denno, 2004; Hogg & Daane, 2014). There was, however, a subtle effect of increasing spider diversity; in trials with *Tasmanicosa* + *Hogna*, *Helicoverpa* survival was significantly lower than in trials with *Tasmanicosa* only. This result suggests that increasing spider diversity may have a positive, yet not additive, effect on *Helicoverpa* control.

Efficacy of diverse predators has been attributed to predator specificity and niche separations (Finke & Denno, 2005; Finke & Snyder, 2008; Finke & Snyder, 2010). Even though *Tasmanicosa* and *Hogna* are likely to kill another spider that is smaller in size, differences in the temporal niches of these two species may dilute intraguild predation. Pitfall trap sampling suggests that *Hogna* persists in the cotton fields later in the season than *Tasmanicosa* (Chapter 1); without *Tasmanicosa*, the disruptive effects of intraguild predation may be decreased and *Hogna* can kill *Helicoverpa* larvae as they descend to overwinter.

An addition to the trophic web between wolf spiders and *Helicoverpa* includes the presence of alternative prey. Alternative prey may interfere with *Helicoverpa* predation if spiders are more likely to find and attack alternative prey (Koss & Snyder, 2005). From direct observations in the field (Chapter 2), we observed that the most commonly found prey was the ground cricket *Teleogryllus commodus* (Orthoptera: Gryllidae). Interestingly, no other study on wolf spider prey has reported crickets as common prey in the field. Furthermore, commonly reported prey such as Hemiptera and Diptera (Hayes & Lockley, 1990; Nyffeler & Benz, 1988) were not observed as prey of wolf spiders in the field.

In 4-way trophic webs (Chapter 5) *Helicoverpa* was more likely than *Teleogryllus* to be killed by *Tasmanicosa.* In laboratory enclosures, *Teleogryllus* was the least common prey in the presence of alternative prey, which contrasted with observations from field surveys (Chapter 2), where *Teleogryllus* was the most commonly found prey. Being the most common prey in the field, wolf spiders should have an efficient hunting strategy to catch and subdue *Teleogryllus.* It is possible that prey observed in the field is confounded by the actual abundance of *Teleogryllus,* and that wolf spiders hunt *Teleogryllus* often simply because there is many of them. But in a confined container, despite being equally available, *Teleogryllus, Helicoverpa* and *Hogna* were a more common prey to *Tasmanicosa* than *Teleogryllus* was. The mechanism underlying lower proportions of *Teleogryllus* predation is most likely its more effective escape behaviour (Dangles *et al.*, 2006)*; Teleogryllus* was easily encountered, but not easily caught, and therefore was a less vulnerable prey for *Tasmanicosa* than *Hogna* or *Helicoverpa.* More importantly, food web laboratory trials show that the presence of alternative prey did not reduce predation on *Helicoverpa.*

The diversity of alternative prey is important for sustaining predator populations for multiple reasons. For example, predators rely on alternative prey to sustain populations when pest densities are low (Harwood & Obrycki, 2005). Wolf spiders were abundant in cotton fields in November and December, after the first *Helicoverpa armigera* moths have emerged from diapause, and before the first generation of *Helicoverpa armigera* descends to pupate (Baker *et al.*, 2011). During this period when there are very few ground-dwelling stages of *Helicoverpa,* wolf spiders in cotton fields rely on a diverse arthropod community as alternative prey. Alternative prey may also provide spiders with a balanced nutrient intake, necessary for optimal development and reproduction (Uetz *et al.*, 1992).

In addition to impacting on predator populations and influencing predation outcomes, the presence of alternative prey can affect the development of insecticide resistance in the

target pest. For example, predator-prey simulations between the ladybeetle *Coleomegilla maculata* (predator) and the Colorado potato beetle (pest) showed that the presence of the European corn borer (alternative prey) delayed Bt resistance by up to 40 generations (Mallampalli *et al.*, 2005). In the present system, the direct impact of prey diversity (including intraguild predators) on the evolution of Bt-resistance by *Helicoverpa* remains to be studied.

There was an unexpected way in which alternative prey disrupted *Helicoverpa* predation; in 2014, 30-40% of *Tasmanicosa* and *Hogna* died after eating *Teleogryllus.* This resulted in *Tasmanicosa* killing *Helicoverpa* less often after consuming toxic *Teleogryllus.* There are no reports of crickets (order Orthoptera) being toxic prey for spiders. Since *Teleogryllus* acquired its toxic compounds from the field, this finding unveils a potential nontarget effect of chemicals and pesticides on beneficial predators. The source of this toxic compound remains unknown, and it is a research question worthwhile pursuing with future controlled experiments.

8. Do other abiotic factors impact the predator-prey interaction?

One method for enhancing populations of native predators is by implementing farming practices that provide suitable microhabitats such as minimum-tillage (Stinner & House, 1990; Tillman *et al.*, 2004). Given the conflict with adequate 'pupae busting', minimum-tillage plots rely on complementary measures for biological control of *Helicoverpa* spp. to prevent Bt-resistance. Minimum-tillage cotton plots with winter crops ('complex plots') had higher wolf spider diversity than tilled plots without winter crops ('simple plots'), especially early in the season (Chapter 1). This is reflected by a more even distribution of dominant species, and a higher abundance of rare species present in complex plots. Moreover, abundance of a large predator, *Tasmanicosa,* was higher in complex plots.

Despite having a lower diversity, simple plots can still benefit from biological control from wolf spiders. Wolf spiders were similarly abundant in simple plots and complex plots (Chapter 1). Furthermore, *Hogna crispipes* quickly colonised tilled fields, indicating resilience to tillage practices. The high abundance of *Hogna crispipes* can complement pupae busting in disturbed simple plots.

Efficacy of diverse predators has been attributed to spatial separation (Finke & Denno, 2006; Finke & Snyder, 2010), arguing that in structurally complex plots, such as one with retained stubble, intraguild interactions between spiders can be minimized (Finke & Denno, 2006). From a spatial perspective, *Tasmanicosa* is most often a ground hunter, whereas *Hogna crispipes* and *Hogna kuyani* can be found on tall grasses, weeds or the lower part of cotton plants. This spatial separation can reduce the frequency of antagonistic interactions among these spider species, thereby improving their individual impact on *Helicoverpa* spp. It would be interesting to investigate whether multiple spider species are more effective at controlling *Helicoverpa* spp. in complex plots than in simple plots. The effect of structural complexity on intraguild interactions, and its carryover effect on *Helicoverpa* spp. mortality remains an open question to follow up from this thesis.

It is unknown how much influence minimum-tillage and cover crop strategies have in wolf spider assemblages in the long term. This is particularly important when comparing seasons with drastically different weather conditions, such as drought and flooding. It is also possible that the size of the plots studied do not reflect the effect of the "farmscape" on biological control; that is, the general structure of the vegetation and planting regimes spanning several kilometres around the cotton plots (Smukler *et al.*, 2010). Due to the wandering nature of wolf spiders and the small size of the plots, it is possible that the plots that we studied served as temporary sources and sinks for the wolf spiders encountered. A review by Schellhorn *et al.*, (2014) discusses how landscape spatial arrangements, structural

connectivity, source-sink relationships, and the temporal heterogeneity should be integrated to assess the potential for an agroecosystem to sustain native predators. This thesis provided a "snap-shot" of wolf spider assemblage differences throughout a single growing season, but care must be taken when extrapolating these patterns, as the spider population can also be affected by year-to-year climate variability, and changes in the surrounding large-scale landscape.

An additional abiotic factor that may affect predator populations is sprayed insecticides. In chapter 6, bifenthrin applications are discussed as a potential source for *Teleogryllus* toxicity to wolf spiders, but this hypothesis remains to be tested. Spiders are highly susceptible to bifenthrin (pyrethroids), while *Helicoverpa armigera* shows increasing levels of resistance to bifenthrin (Wilson *et al.*, 2015). *Teleogryllus* and *Helicoverpa* can be exposed to sublethal concentrations of bifenthrin in the field, which can then become lethal to wolf spiders when consuming prey exposed to this insecticide. The effects of bifenthrin and other sprayed insecticides on wolf spiders through ingested prey should be further investigated.

Benefits and limitations of the techniques used to measure wolf spider impact on *Helicoverpa* spp.

Each of the methods assessed in this thesis effectively answered each of the research questions. However, each method on its own had limitations on assessing the impact of wolf spiders as predators; the multiple methods used in this thesis complemented each other to provide a more complete overview of the effectiveness of wolf spiders. I here discuss how some of the methods I used present similar challenges and benefits from those stated in Table 2 (Macfadyen *et al.*, 2015), and identify additional challenges to be addressed when evaluating the impact of wolf spiders on *Helicoverpa* spp.

Table 2. Benefits and challenges associated with measuring different components of the ecology and impact of a predator on a pest prey (copied with permission; Macfadyen *et al.*, 2015).

Method	Benefits	Challenges
Measuring species richness (Q6)	Identification of a species is often the 1st step towards managing the species May give some indication of the stability of pest control services	Each sampling technique will not sample each species efficiently Rare species require much sampling to record Some species may not be involved in pest control services
Measuring species abundance (Q6)	May give some idea of the magnitude of pest control services Ignores species that have low abundance (but may still have high impact)	Species that are in high abundance may not always be important for providing pest control services More individuals ≠ greater levels of pest control Each sampling technique will not give an equivalent estimate of each species abundance
Measuring impact–exclusion cage studies (Q2, Q3, Q4)	Illustrates the combined impact of natural enemy community	Difficult to separate the impact of individual species
Measuring impact–laboratory feeding studies (Q1, Q2, Q5)	Gives an indication of maximum prey consumption ability and preferences	Highly artificial environment. The natural enemy does not have to find prey in a complex environment
Measuring impact–direct mortality (e.g., gut content analysis, parasitism rate) (Q1, Q2, Q8, Q7)	Quantifies direct mortality to a pest species	Initial development costs high for molecular techniques Parasitism rate easy to measure but difficult to interpret

There were some differences in wolf spider species assemblages determined from direct observations (Chapter 2) compared to pitfall trap collections (Chapter 1), confirming that each technique differentially samples each species. For example, *Hogna kuyani* was the most abundant spider recorded in visual surveys during the peak flower cotton stage (February, Chapter 2), yet *Hogna kuyani* was very uncommon in pitfall trap collections during peak flower (Chapter 1). Similarly, *Tasmanicosa leuckartii* became progressively less common in pitfall traps, yet it was still abundant in visual surveys from January until March. One consistent result in both visual surveys (personal observation) and pitfall traps was that *Hogna kuyani* was rarely found in the field early in the season (Oct-Dec), which is the reason why this species was not used in laboratory and glasshouse predation trials. Previous studies have reported discrepancies between pitfall trapping and visual sampling (Andersen, 1991; Andersen, 1995; Lin *et al.*, 2005); indicating that a single sampling method can misreport whether predators and prey are present in the same time and space. Even though the

discrepancy between sampling methods in this study might reflect year-to-year variability, differences in collection methods, or differences in the cotton fields, both sampling methods confirmed that wolf spiders coexist with *Helicoverpa* spp. in the same timeframe and space. Multi-year surveys in various fields can help determine how the estimated phenology and abundance of different wolf spider species can vary between studies using pitfll trapping and visual surveys.

The results from pitfall trap surveys confirmed that species that are abundant may not always be important in pest control (Table 2); *Venatrix konei*, the most abundant wolf spider, has negligible impact on the control of ground-dwelling *Helicoverpa* spp. due to its small body size. For this reason, *V. konei* was excluded from visual surveys. In contrast, a species rarely sampled in pitfall traps, *Hogna kuyani*, was very abundant in visual surveys, and furthermore, was more commonly found with prey, and killed *Helicoverpa* spp. in predation trials in field containers. When evaluating the effect of farming practices on spider abundance and richness, it is important to note the limitation of pitfall trap sampling. As a passive technique, it may not accurately show the population assemblage and species abundance, but it combines factors such as spider activity levels on the ground surface, and ability to avoid and escape the traps (Brennan *et al.*, 1999; Cheli & Corley, 2010; Topping & Sunderland, 1992). Since the pitfall traps were shaded and had moisture inside, they could also act as a shelter and might even be more attractive for ground arthropods in fallow soils with little cover. Based solely on pitfall trap information, the impact on *Helicoverpa* spp. of *V. konei* could have been overestimated, while the impact of *H. kuyani* could have been greatly underestimated.

Despite its limitations, pitfall trap surveys were a valuable technique for providing information on wolf spider species richness in these cotton fields, which was previously unknown. An advantage of determining species richness throughout the cotton season was

that, in complement with predation trials, it confirmed that different species of wolf spiders which kill *Helicoverpa* spp. are present throughout the cotton growing season (stability of pest control services, Table 2).

One challenge of measuring species abundance is making the assumption that more individuals exert greater levels of pest control (Table 2). Experiments in glasshouse enclosures confirmed that a higher abundance of individuals of the same species did not lead to increased *Helicoverpa* mortality (Chapter 4). However, an increase in wolf spider diversity showed to have a positive (yet not additive) effect compared to the impact of only one *Tasmanicosa.* Despite the potential difficulty on separating the impact of individual species (Table 2), the experimental design used in Chapter 4 allowed making comparisons of the impacts between individual and combined species.

According to Table 2, laboratory predation trials can give an estimate on prey preference. However, predation outcomes cannot be attributed to prey preference for multiple reasons. In food web laboratory trials (Chapter 5), it cannot be concluded that *Tasmanicosa* had a lower "preference" for *Teleogryllus*; when observing the prey's behaviour, predation outcomes can be attributed to the prey's ability to escape, rather than the predator actively choosing to avoid this prey. Unless all of the different prey's characteristics are uniformly controlled (e.g., chance of escape, defences), it is not possible to affirm that laboratory predation trials give an indication on prey preference. Furthermore, *Teleogryllus* was a common prey in the field, therefore confirming that the low proportion of *Teleogryllus* consumed in laboratory trials was not necessarily due to "prey preferences".

The purpose of laboratory and glasshouse enclosures was not to mimic spider and *Helicoverpa* densities in the field, but to assess the mechanisms that can influence spider predation on *Helicoverpa*. Even though predation trials were carried out in a highly artificial environment and may not correspond to field settings, laboratory predation trials provided

useful information on whether wolf spiders kill *Helicoverpa* in the presence of intraguild predators and alternative prey. Moreover, there was no evidence that spiders reject *Helicoverpa* as prey on larger enclosures.

Measuring direct mortality of pests in the field provides information on predatory behaviour in a natural setting, but here are multiple challenges to measuring impact of predators in the field. An IgG sandwich ELISA was chosen as a gut-content analysis method due to its cheaper cost, the short time it requires to set up, and its longer detectability time compared to PCR and Mab ELISA (Agusti *et al.*, 1999a; Agusti *et al.*, 1999b; Furlong *et al.*, 2014).Despite these advantages, the IgG external marking method that we used for this ELISA technique had limitations because of cross-contamination and the incidence of false positives in confined containers. Marking *Helicoverpa* spp. only internally through diet might have reduced the incidence of cross-contamination. As tray-reared larvae burrow in their diet it as they feed, it may be also necessary to wash larvae to discard traces of IgG in their pseudopodia. A potential disadvantage of internal marking to be further investigated is that since *Helicoverpa* spp. larvae feed and excrete continuously, there is a risk that an internal IgG mark would be rapidly digested and excreted, therefore limiting its detectability. As stated in Table 2, gut content analysis have high initial costs for developing and optimizing a protocol, which in this case are reflected on optimizing a larval IgG-marking technique. Further studies are still needed to test different larval marking techniques which can reduce the likelihood of false positives.

An alternative gut –content analysis method is using PCR to detect DNA remains of *Helicoverpa* spp. (both larvae and moths) present at any given time in any given section of the studied plot. However, *Helicoverpa* spp. DNA segments are rapidly digested by spiders, resulting in a very short detectability time (Agusti *et al.*, 1999b). Spiders that test positive for *Helicoverpa* spp. remains through PCR are likely to have hunted in the previous hours, but

this method can underestimate predation events that happened 24 hrs before. Even though it involves initial costs for optimizing a protocol, gut-content analysis through PCR could be used and improved by designing multiple primers that can detect shorter specific sections of *Helicoverpa* spp. DNA, therefore increasing its detectability time range.

An additional challenge to measuring direct mortality is that direct observations and gut-content analysis can greatly underestimate predation rate in the field. This is because direct predation events are rare to observe, and spiders that consumed IgG marked *Helicoverpa* spp. can have low recapture rates. The likelihood of recapturing spiders that ate IgG-marked *Helicoverpa* in the field can be improved by increasing the number of IgG-marked larvae released in the field, and expanding the area for collecting spiders. The challenge of underestimating predation rates is not stated in Table 2, yet it is crucial to address this limitation when evaluating the impact of predators in the field.

Each method has several limitations for assessing predator impact, which can lead to different estimates of pest prey consumption. This study therefore emphasizes the importance of using multiple methods to complement each other; observations compared and contrasted by multiple methods in this thesis allowed to adequately assess the importance of wolf spider species in controlling *Helicoverpa* spp.

Summary of wolf spider effects

Summarizing the main findings from all the research chapters and considering the limitations of each technique employed, multiple strenghts and constrains from wolf spiders as biological control agents for *Helicoverpa* were identified:

Strenghts of wolf spiders as biological control agents:

1) Wolf spider diversity can be enhanced by environmentally-friendly farming techniques such as minimum-tillage, winter crops, and retained stubble.

2) Species such as *Hogna crispipes* have high colonizing abilities and resilience to soil disturbance, and can therefore still contribute to kill *Helicoverpa* spp. in tilled or fallow fields.
3) The three largest wolf spider species in the cotton fields studied (*Tasmanicosa leuckartii, Hogna crispipes, Hogna kuyani*) all killed 5th instar *Helicoverpa* spp. Larvae in small enclosures.
4) *Tasmanicosa leuckartii*, *Hogna crispipes,* and *Hogna kuyani* have staggered phenologies throughout the cotton season. This can minimize antagonistic intraguild interactions by temporal separation, and ensure that *Helicoverpa* spp encounters wolf spiders from the moment overwintering moths emerge, to when the last larvae descend to diapause underground.
5) *Hogna crispipes* and *Hogna kuyani* can encounter *Helicoverpa* spp. on the soil or on the plant canopy.
6) *Tasmanicosa* killed both *Helicoverpa* late-instar larvae and emerging moths in similar proportions, therefore eradicating *Helicoverpa* at the life stages critical for control of Bt-resistance.
7) As wolf spiders are generalist predators and rely on multiple prey, wolf spider abundance is not dependant on *Helicoverpa* spp. abundance, therefore wolf spiders are present in high densities in Bt cotton fields.
8) Even in the presence of alternative prey (*Teleogryllus*), *Tasmanicosa* and *Hogna* still killed *Helicoverpa.*

Constrains of wolf spiders as biological control agents:

1) In glasshouse enclosures, intraguild interactions limit the individual predatory capacity of each wolf spider species on *Helicoverpa* larvae, suggesting that, in simple settings, increasing spider abundance will not necessarily augment biological control.

2) In small laboratory enclosures, the presence of spiders is correlated with higher foraging effort from *Helicoverpa* larva, resulting in higher mass loss in cotton bolls.
3) Wolf spider predation of *Helicoverpa* in field settings is rare to detect, but this result is confounded by experimental limitations.

The goal of this thesis is not to suggest that biological control from wolf spiders can replace existing mechanisms for destroying ground-dwelling *Helicoverpa* spp. to control Bt resistance. Rather, the main recommendation from this study to is emphasise that wolf spiders should be accounted for as an important component of biological control in cotton agroecosystems. Sustainable IPM strategies should plan for long-term plant protection, and not just immediate crop damage. From this perspective, conservation of native wolf spiders serves as a complementary tool that can prolong the efficacy of Bt-cotton.

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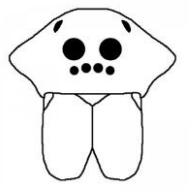
APPENDIX I: Wolf spiders (Araneae: Lycosidae) found at the Australian Cotton Research Institute (Narrabri, NSW)

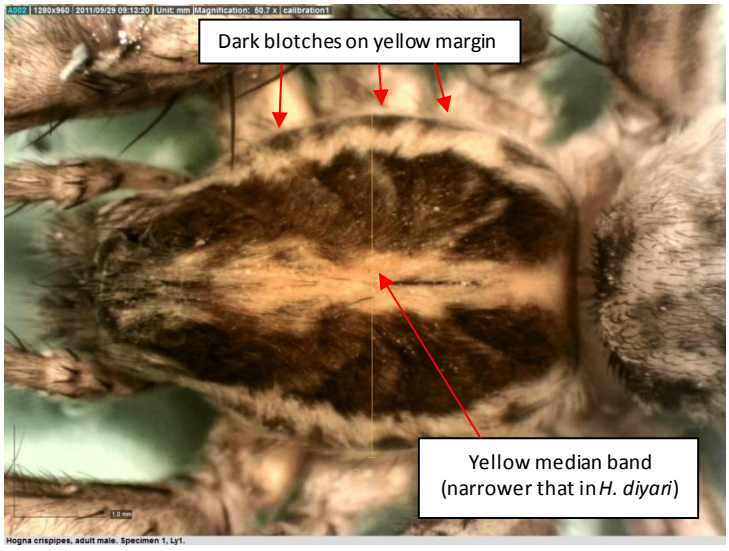
Family Lycosidae distinctive characteristic: 4:2:2 eye pattern



Genus, species	Cephalothorax	Abdomen	Male palp	Female epigyne	Fangs	Legs
***Hogna crispipes* Koch** Fig. 1a,b, 2a,b. (Framenau et al., 2006)	Dorsal: Yellow median band (narrower that in *H. diyari*) Dark blotches on yellow margin	Dorsal: Irregular dark grey with yellowish marks, indistinct pattern.	Tip of cymbium without claw (Compare to *Venatrix*) No basoembolic apophysis (compare to *Artoria, Artoriopsis*)	Inverted T-shaped.	Smooth, without tubercules (compare to *Venatrix*)	Indistinct pattern / colour
***Hogna kuyani* Framenau** Fig. 3a,b,c,d; 4a,b. (Framenau et al., 2006)	Dorsal: Dark reddish brown, dense cover of silver setae. No light median band	Dorsal: Grey, indistinct light and dark patches Ventral: light yellow-gray.	Median apophysis with ventral process that points basally, terminal apophysis sickle-shaped, embolus with long thin tip (similar to *H. crispipes*).	Inverted T-shaped, longer median septum than *H. crispipes.*	Smooth, without tubercules (compare to *Venatrix*)	Indistinct pattern / colour
Venatrix konei* Hickman (= *goyderi) Fig. 5a,b,c,d; 6a,b,c,d. (Framenau et al., 2006; Framenau & Vink, 2001)	Dorsal: Brown carapace with narrow light median band. Irregular light dorsal band or spots.	Dorsal; Dark brown, light lanceolate mark. Ventral; irregular yellow spots.	Claw-like setae on tip of cymbium (characteristic of *Venatrix*) Terminal apophysis forms a roof over embolus. Small median apophysis	Triangular opening, hoods touching centrally, median septum weakly sclerotized.	Tubercule on male fang (Characteristic of *Venatrix*)	Indistinct pattern / colour
***Venatrix fontis* Framenau** Fig. 7a,b,c,d; 8a,b,c. (Framenau *et al.*, 2006; Framenau & Vink, 2001)	Dorsal: Brown, narrow light median band	Dorsal: Lanceolate mark and lateral dark stripes (different from *V. konei*)	Median apophysis with lobe-like ventral process and broad lateral process.	Inverted T-shaped, without median transverse part.	Tubercule on male fang (Characteristic of *Venatrix*)	Leg I longest (Characteristic of species).
***Venatrix speciosa* Koch** Fig 9a,b,c,d; 10a,b,c. (Framenau & Vink, 2001)	Dorsal: Dark brown with light median band narrowing posteriorly. White setae on median band and margin.	Males: Dorsal characteristic light band with dark oval in middle. Two light lateral lines on dark venter. Females: Dorsal indistinct dark colouration	Median apophysis with two separate parts	Inverted T-shaped, wider than longer.	Tubercule on male fang (Characteristic of *Venatrix*)	Indistinct pattern / colour

Artoria spp. Fig. 11a,b,c. (Framenau, 2002; Framenau, 2010)	Dorsal: Brown to black, light median band.	Dorsal: Brown to dark grey with light lanceolate heart mark.	Basoembolic apophysis (characteristic of subfamily Artoriinae) Median apophysis (bifurcate or spoon-shaped and narrow base characteristic of *Artoria*) No claw on tip of cymbium (compare to *Venatrix*)	Variable, a simple opening covered by a sclerotized plate, median part usually oval or inverted-T shaped.	Smooth, without tubercules (compare to *Venatrix*)	Indistinct pattern / colour
***Artoria victoriensis* Framenau** Fig. 12a,b,c. (Framenau et al., 2006)	Dorsal: Brown, with distinct light brown median and submarginal bands; head region black, dark grey radial pattern. White setae	Dorsal: Dark grey; yellowish-brown lanceolate mark in anterior half, irregular lateral yellow patches	Median apophysis resembles upside-down sock in ventral view	White center, with sclerotized posterior ring reaching medially into the center	Smooth, without tubercules (compare to *Venatrix*)	Distinct dark grey annulations on light brown.
***Artoriopsis whitehouseae* Framenau** Fig 13a,b,c; 14a,b,c. (Framenau, 2007)	Dorsal: Dark brown with indistinct darker radial pattern; distinct median and marginal bands formed by white setae.	Dorsal: Pattern unique within the genus; reduced to a single, irregular light band on a dark grey surface	Basoembolic apophysis present (subfamily Artoriinae). Tegular (=median) apophysis with ventrally directed tip.	Female undescribed	Smooth, without tubercules (compare to *Venatrix*)	IV>I>III>II, retrolateral spine on the patella of leg I.
Artoriopsis expolita Fig. 15a,b,c,d; 16a,b,c. (Framenau, 2007)	Dorsal: Indistinct radial pattern with marked light brown median and submarginal bands.	Dorsal: Olive grey, distinct yellow lanceolate cardiac mark in anterior half which bisects a black diamond-shaped patch in the center	Basoembolic apophysis present (subfamily Artoriinae). Tegular apophysis with hook-shaped terminal structure	Trapezoid-shaped, median septum wider posteriorly.	Smooth, without tubercules (compare to *Venatrix*)	Indistinct pattern / colour
Tasmanicosa leuckartii* Thorell *(= Lycosa) Fig. 17a,b,c; 18a,b,c. (McKay, 1975)	Dorsal: Characteristic radial pattern (compare to *Hogna*) Ventral: dark sternum.	Ventral: Dark brown, large pale patch on underside.	Not described.	Narrow median septum, deep lateral furrows.	Smooth, without tubercules (compare to *Venatrix*)	Dark underside of coxae, patellae.
Anomalosa oz Fig 19a,b,c,d; 20a,b,c. (Framenau, 2006)	Dorsal: Shiny dark brown/ black. Indistinct radial pattern, narrow light median band. Ventral: dark brown sternum.	Dorsal: Dark grey, light median band	Membranous and reduced tegular apophysis. Prolateral tegular lobe larger than tegular apophysis.	Heart-shaped, simple sclerotized plate with posterior incision.	Smooth, without tubercules (compare to *Venatrix*)	Annulated femur, yellow coxa.
Possibly ***Hogna* spp.** Fig 21a,b,c..	Dorsal: Dark brown, wide median band	Dorsal: Brown, indistinct pattern.	No claw-like setae on tip of cymbium, no basoembolic apophysis, long median apophysis.			

a)



b)

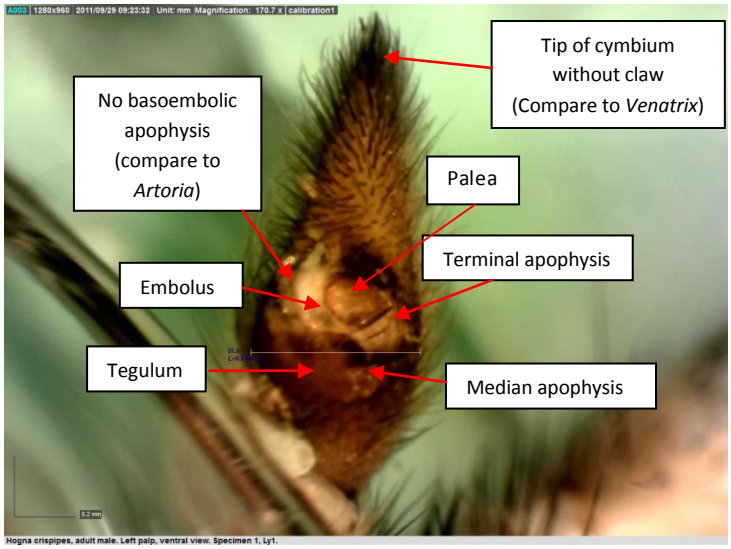


Figure 1. *Hogna crispipes* male, a) cephalothorax (dorsal); b) left palp (ventral)

a)

b)

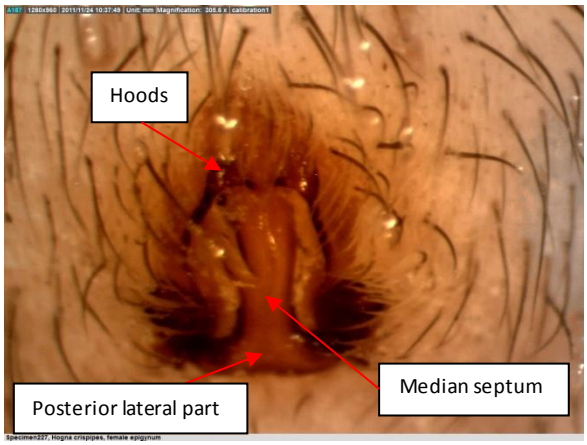
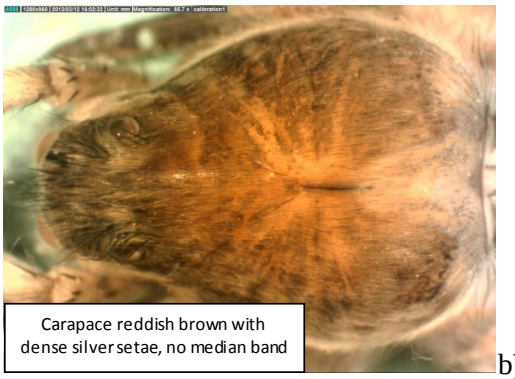


Figure 2. *Hogna crispipes* female, a) dorsal cephalothorax and abdomen; b) epigyne

a) 

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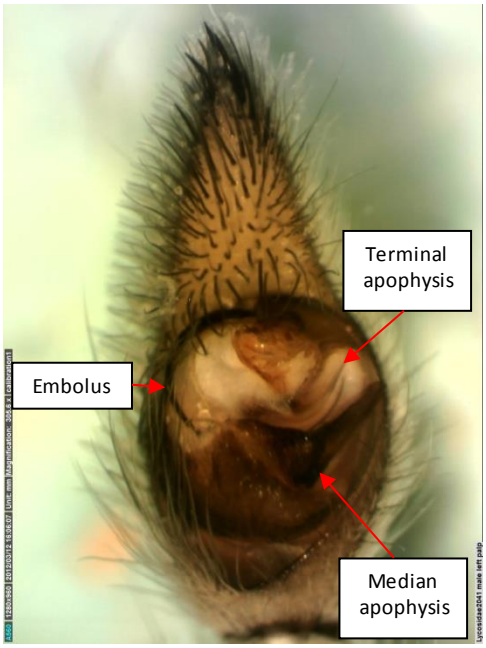
c) 

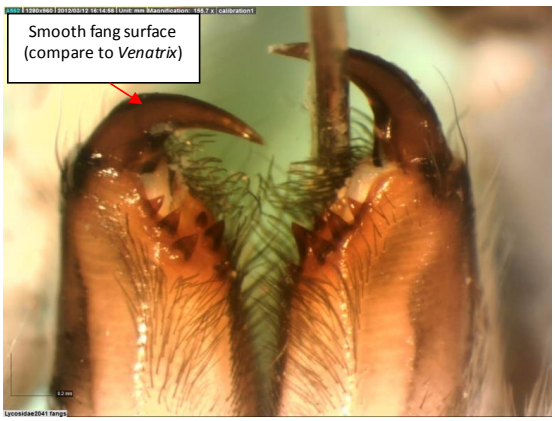
d) 

Figure 3. *Hogna kuyani* male, a) dorsal cephalothorax; b) ventral cephalothorax; c) left palp; d) ventral chelicerae and fangs

a) 

b) 

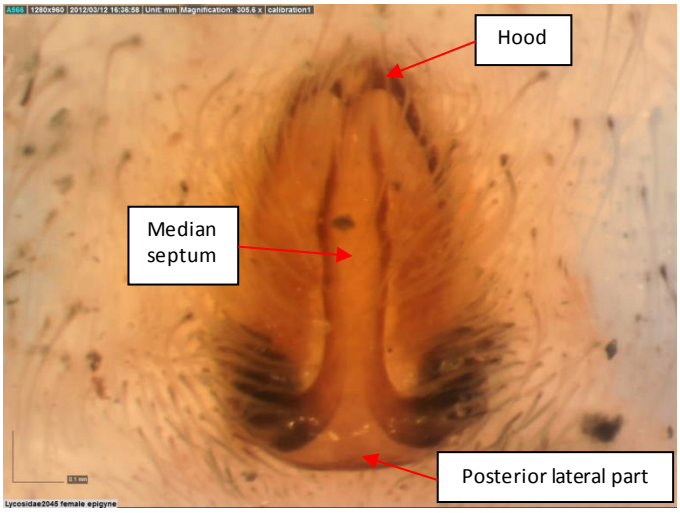
c) 

Figure 4. *Hogna kuyani* female, a) dorsal cephalothorax; b) ventral cephalothorax; c) epigyne.

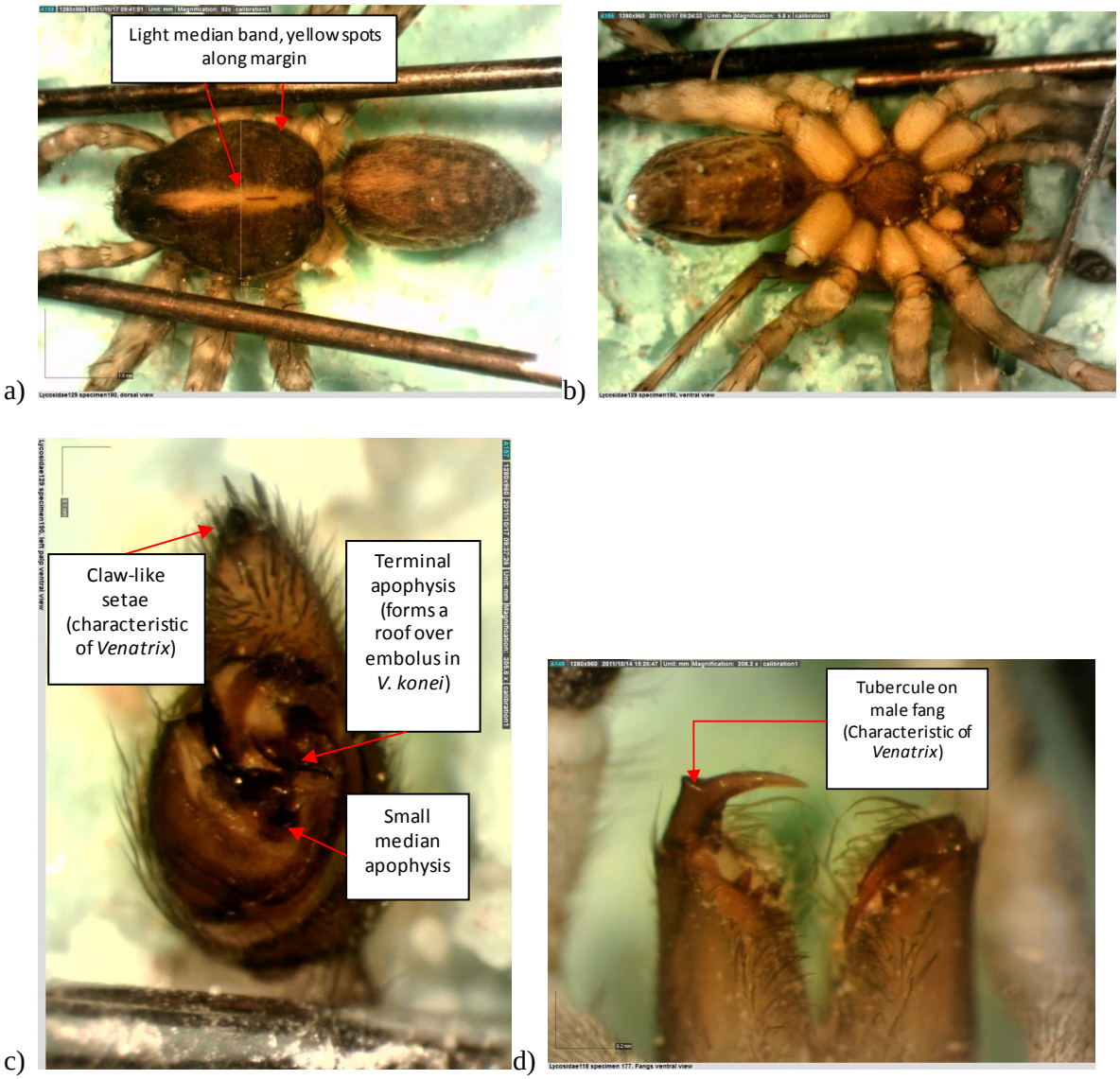


Figure 5. *Venatrix konei (= goyderi)* male, a) dorsal cephalothorax and abdomen; b) ventral cephalothorax and abdomen; c) left palp; d) ventral chelicerae and fangs.

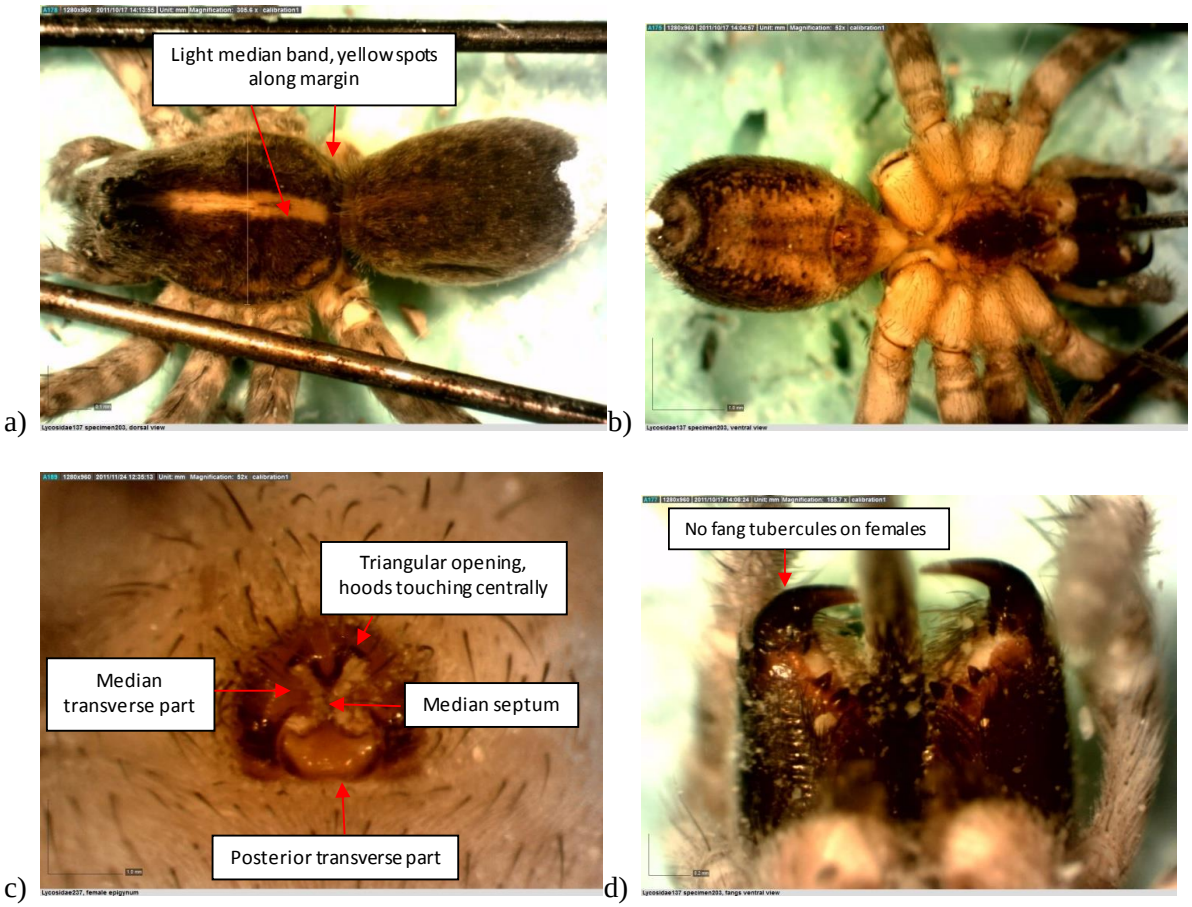


Figure 6. *Venatrix konei* (= *goyderi*) female, a) dorsal cephalothorax and abdomen; b) ventral cephalothorax and abdomen; c) epigyne; d) ventral chelicerae and fangs.

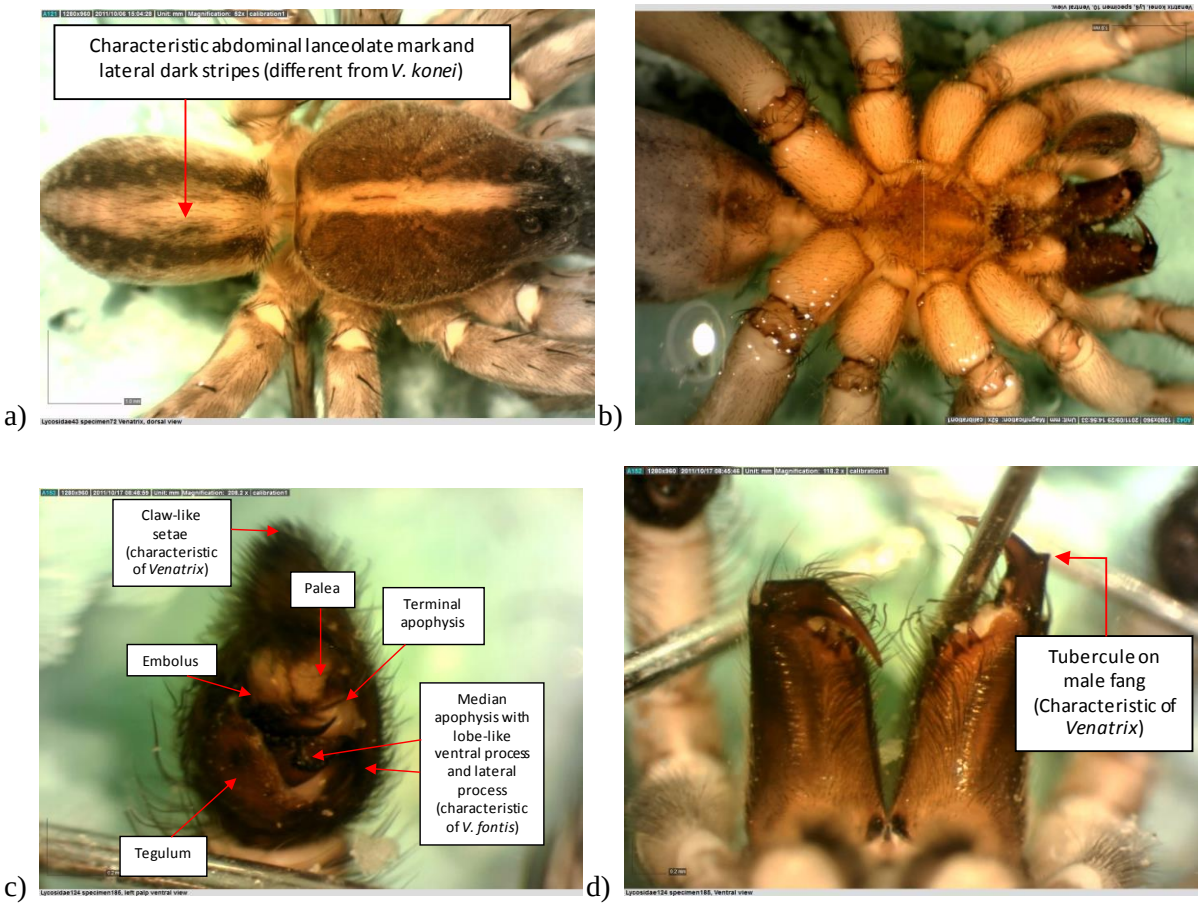
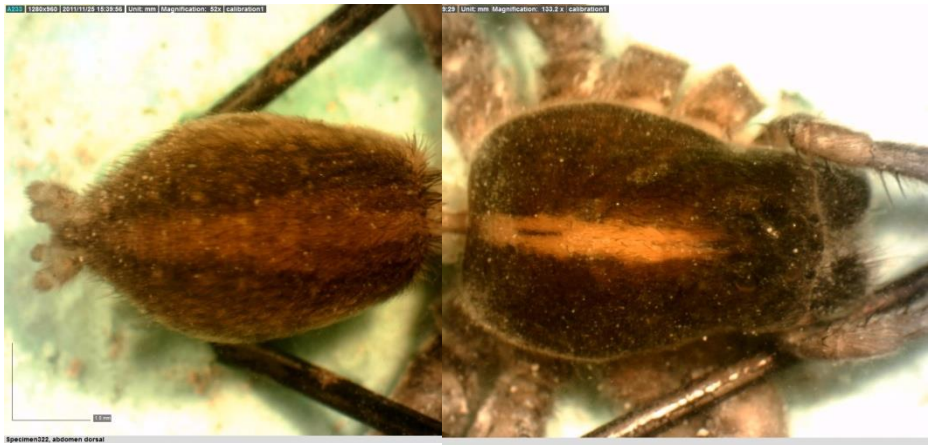


Figure 7. *Venatrix fontis* male, a) dorsal cephalothorax and abdomen; b) ventral cephalothorax, c) left palp, d) ventral chelicerae and fangs.

a)



b)

c)

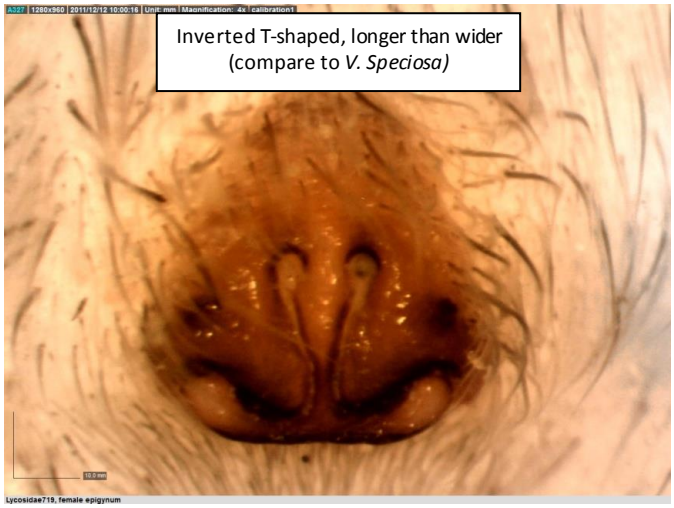


Figure 8. *Venatrix fontis* female, a) dorsal cephalothorax and abdomen; b) ventral cephalothorax and abdomen; c) epigyne.

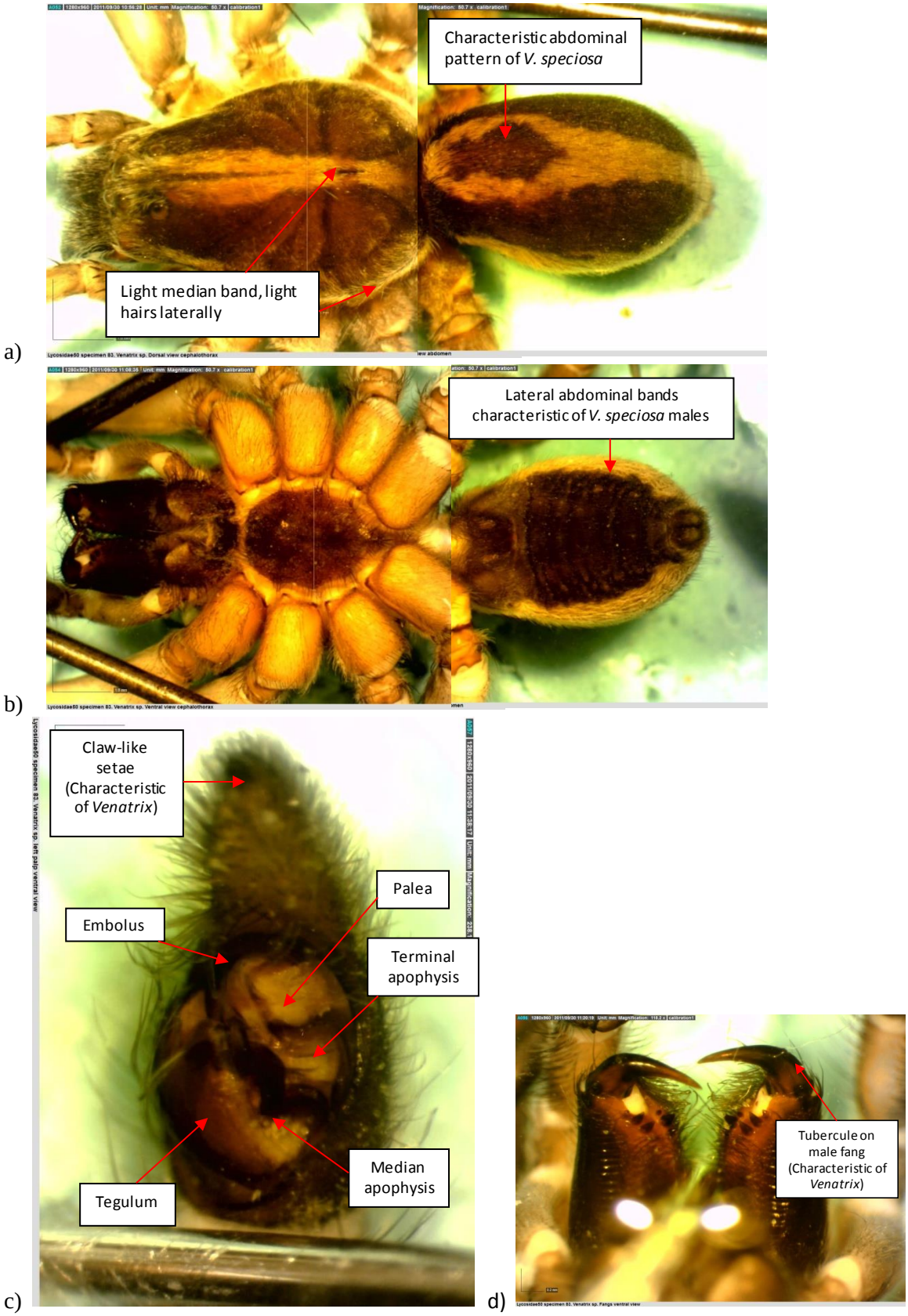
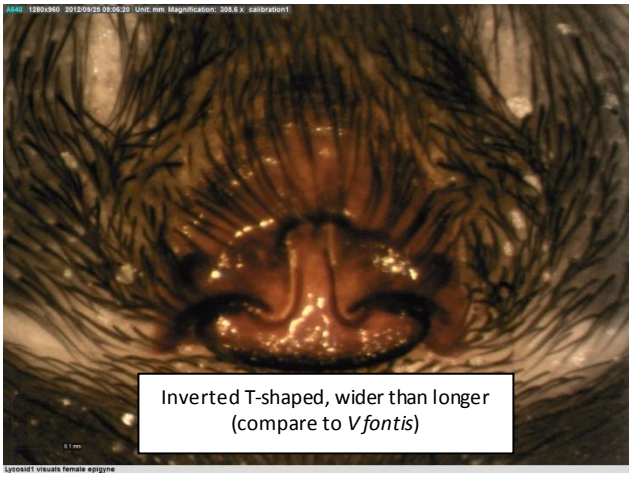


Figure 9. *Venatrix speciosa* male, a) dorsal cephalothorax and abdomen; b) ventral cephalothorax and abdomen; c) left palp; d) ventral chelicerae and fangs.

a)

b)



c)

Figure 10. *Venatrix speciosa* female, a) dorsal cephalothorax and abdomen; b) ventral cephalothorax and abdomen; c) epigyne.

a) 

b) 

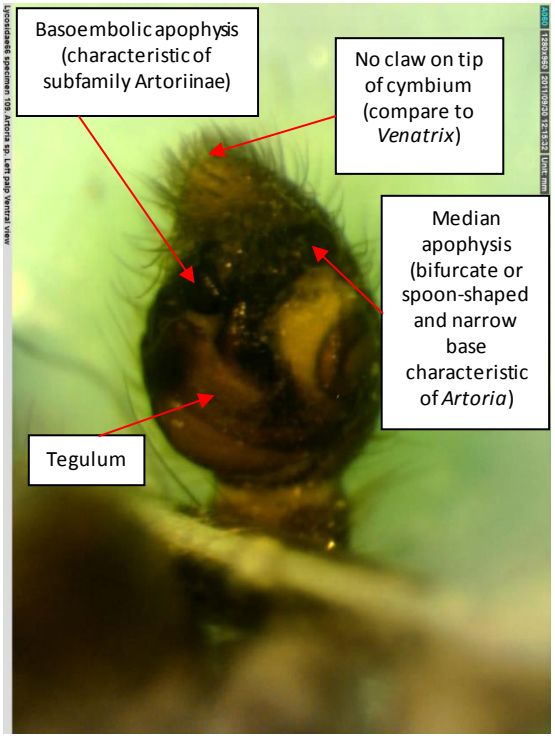
c) 

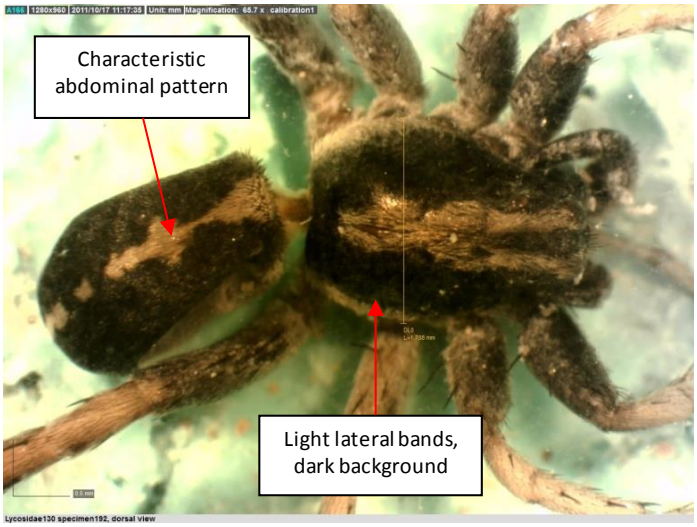
Figure 11. *Artoria* sp., male, a) dorsal cephalothorax and abdomen; b) ventral cephalothorax and abdomen; c) left palp.

a)

b)

Figure 12. *Artoria victoriensis*, female, a) dorsal cephalothorax; b) epigyne.

a)



b)

c)

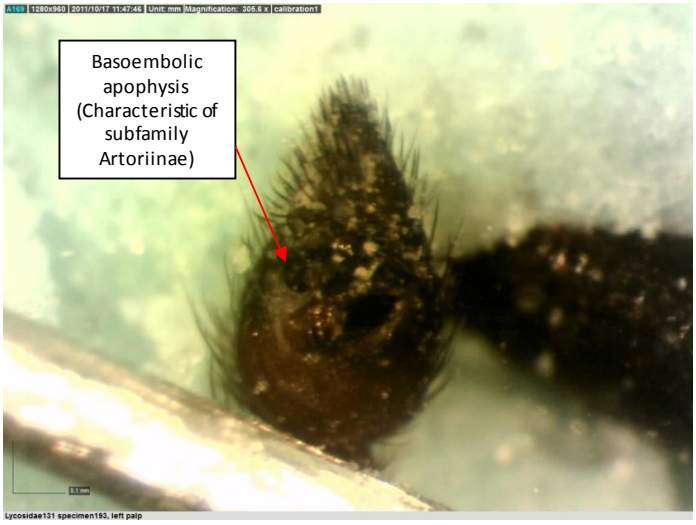
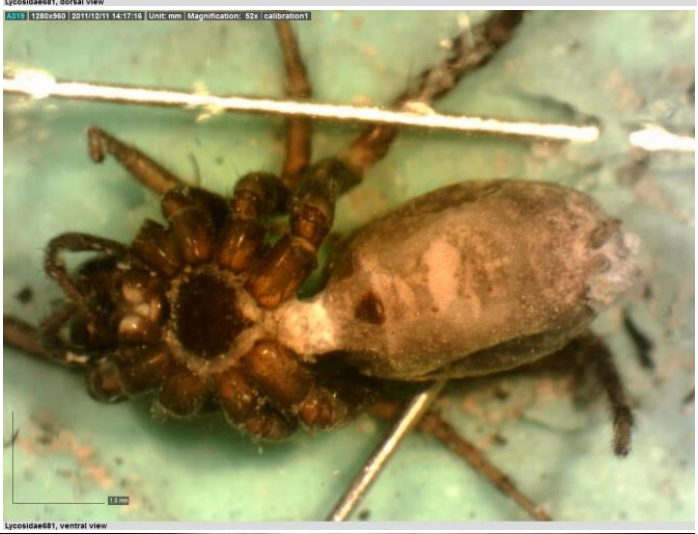


Figure 13. *Artoriopsis whitehouseae* male, a) dorsal cephalothorax and abdomen; b) ventral cephalothorax and abdomen; c) left palp.

a)

b)



c)

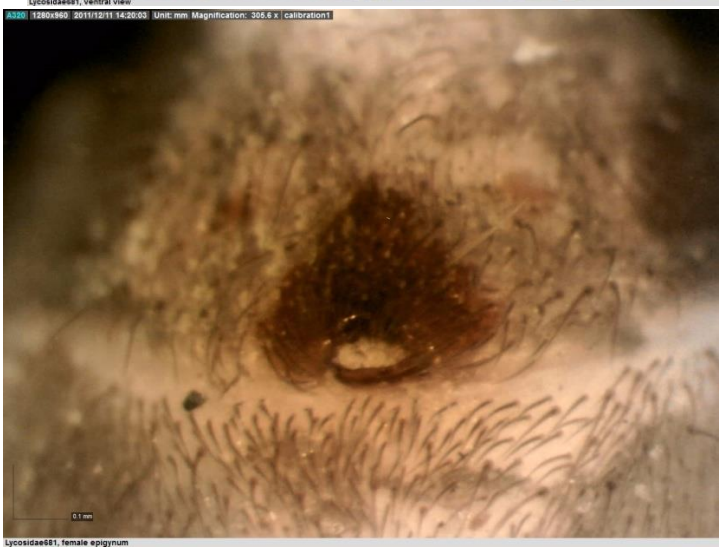
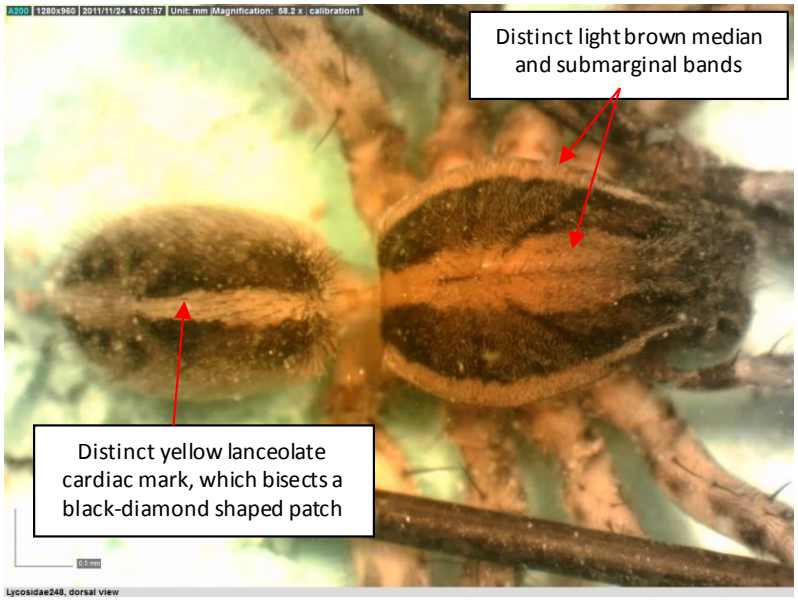


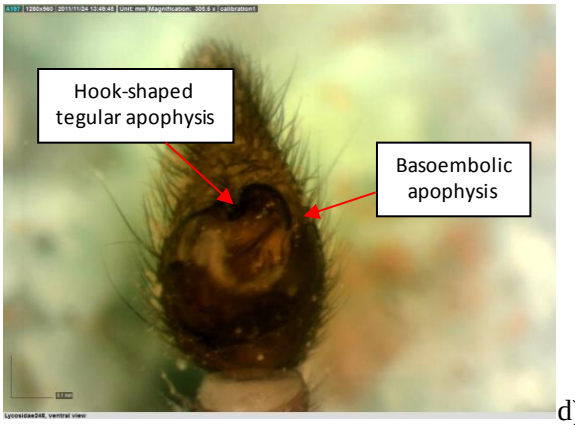
Figure 14. Possibly female of *Artoriopsis whitehouseae* (undescribed), a) dorsal cephalothorax and abdomen; b) ventral cephalothorax and abdomen; c) epigyne.

a)



b)

c)



d)

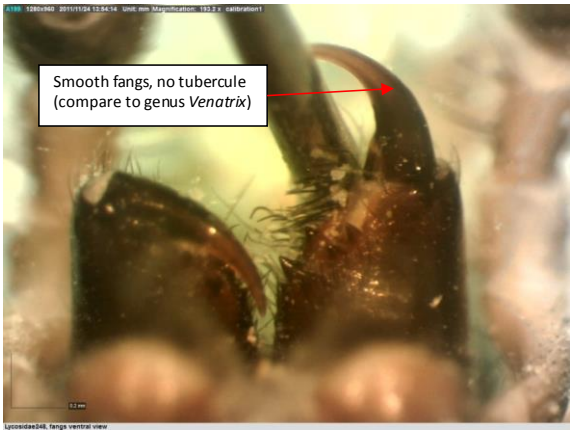
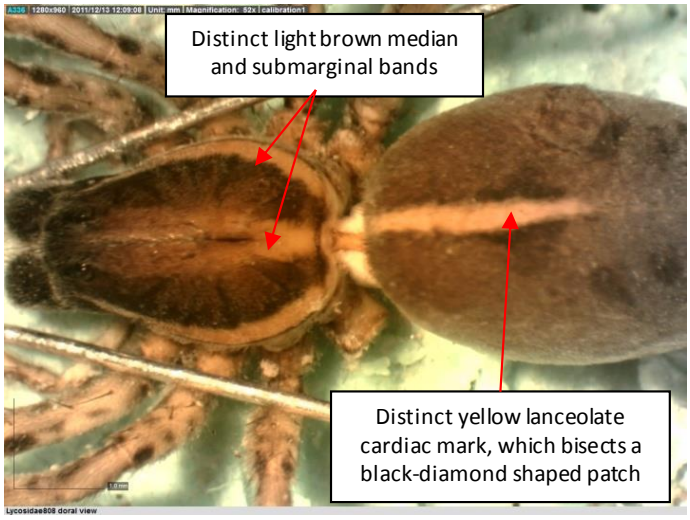


Figure 15. *Artoriopsis expolita* male, a) dorsal cephalothorax and abdomen; b) ventral cephalothorax and abdomen; c) left palp; d) ventral chelicerae and fangs.

a)



b)

c)

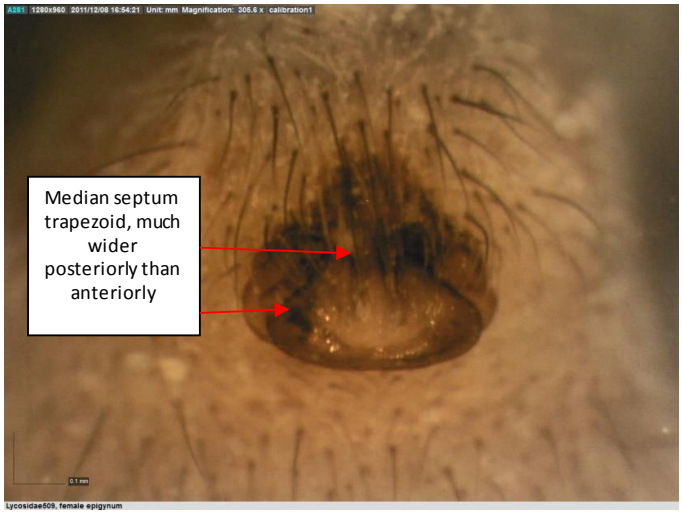
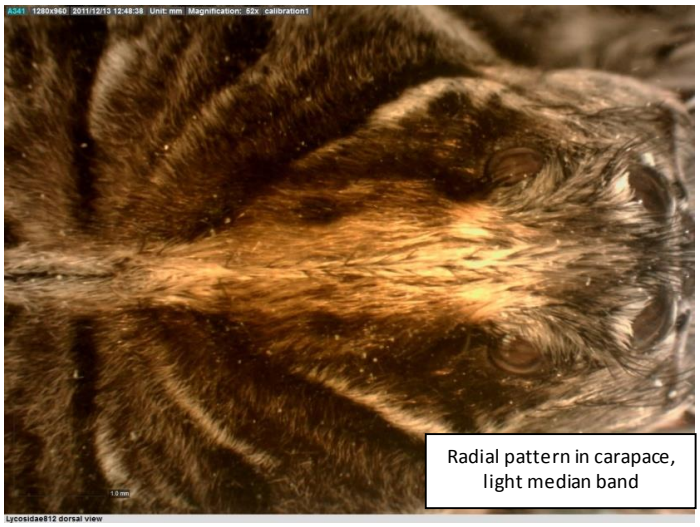
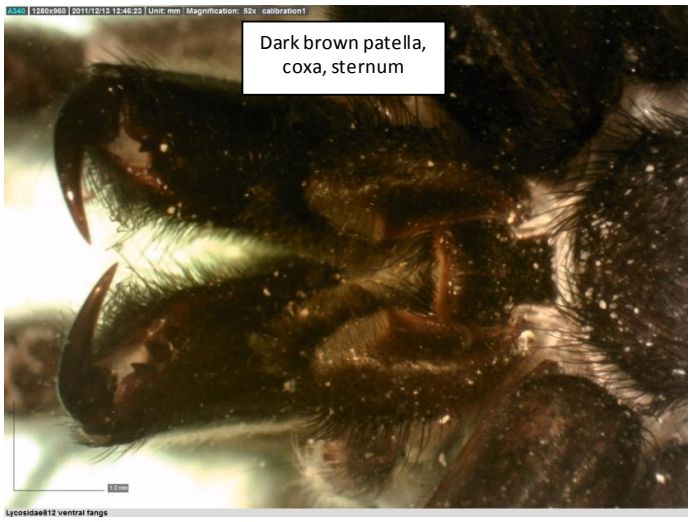


Figure 16. *Artoriopsis expolita* female, a) dorsal cephalothorax and abdomen; b) ventral cephalothorax and abdomen; c) epigyne.

a)



b)



c)

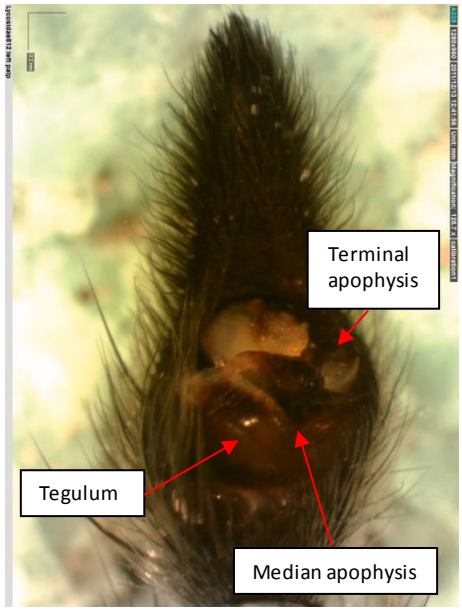
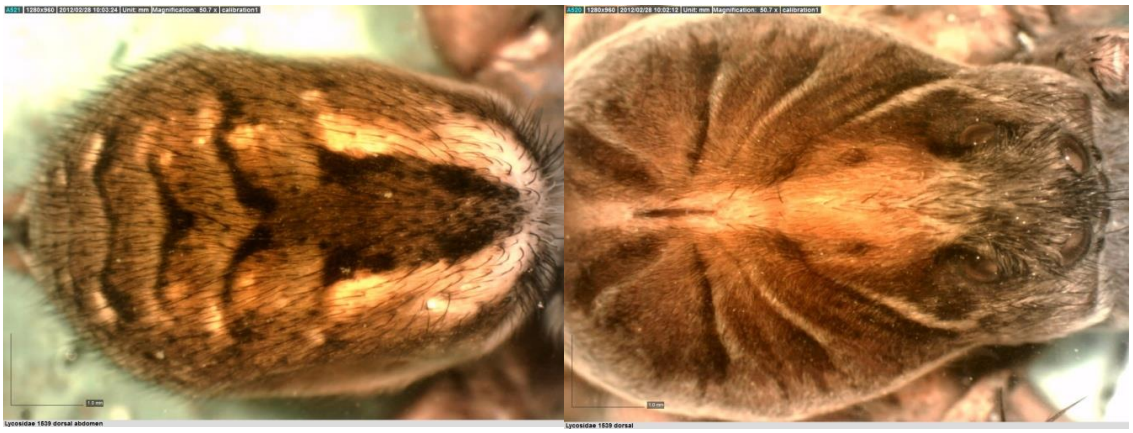


Figure 17. *Tasmanicosa leuckartii*, male, a) dorsal cephalothora; b) ventral cephalothorax; c) left palp.

a)



b)

c)

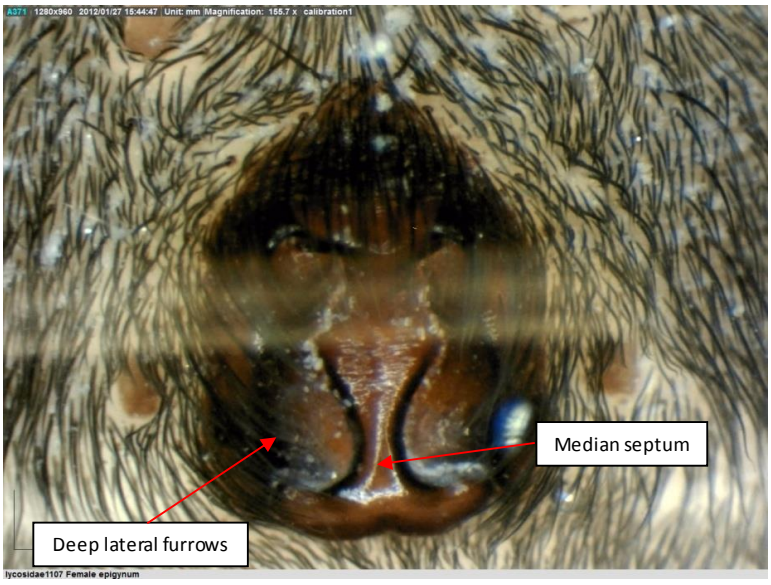
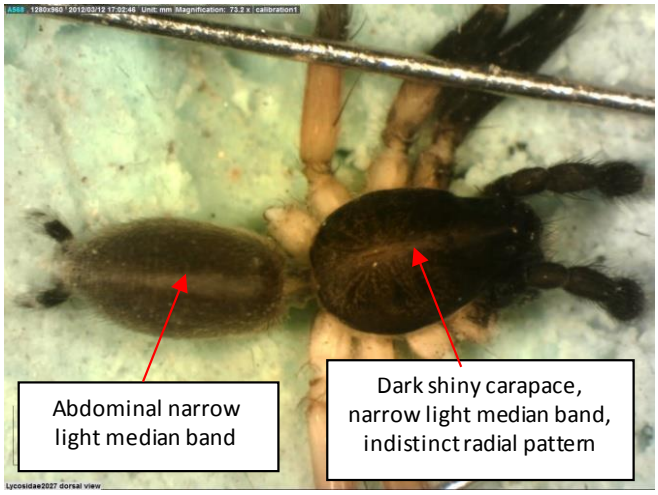
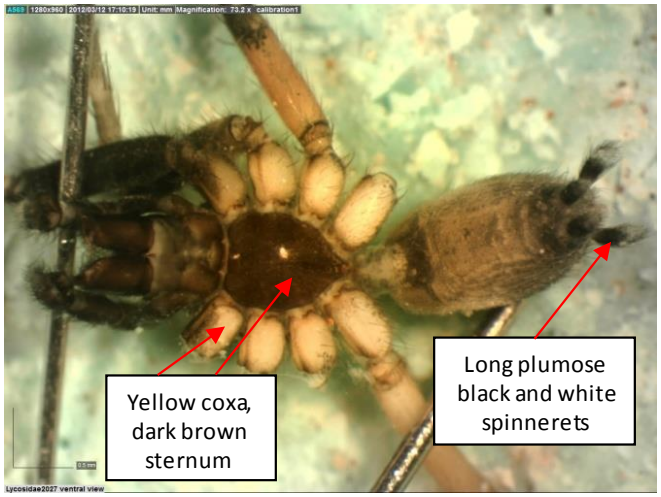


Figure 18. *Tasmanicosa leuckartii* female, a) dorsal cephalothorax and abdomen; b) ventral cephalothorax and abdomen; c) epigyne.

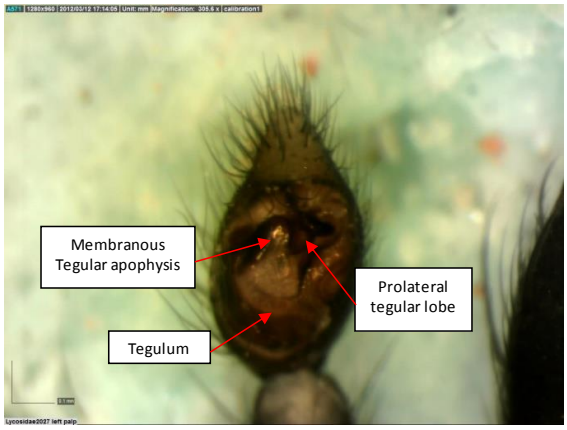
a)



b)



c)



d)

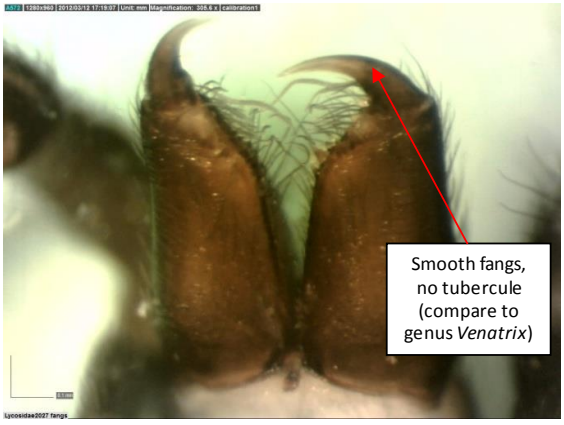


Figure 19. *Anomalosa oz* male, a) dorsal cephalothorax and abdomen; b) ventral cephalothorax and abdomen; c) left palp; d) ventral chelicerae and fangs.

a)

b)

c)

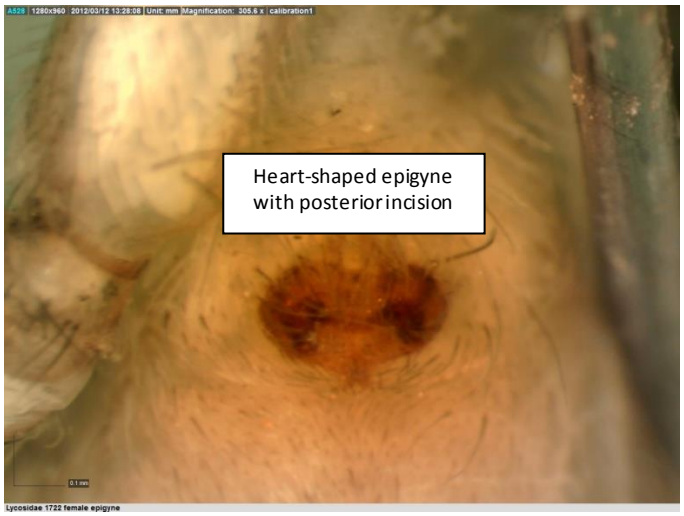
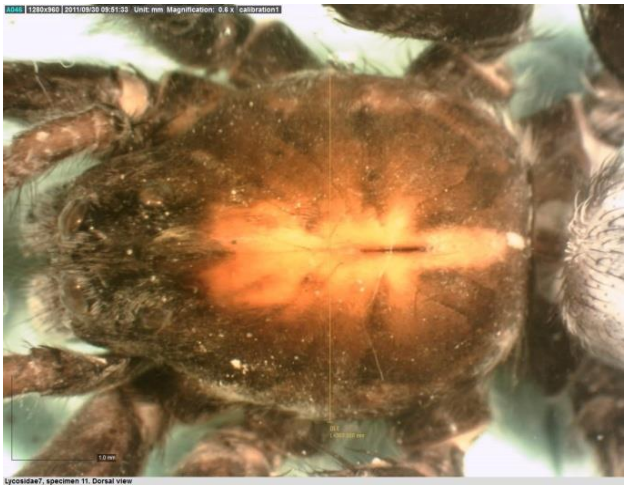


Figure 20. *Anomalosa oz* female, a) dorsal cephalothorax and abdomen; b) ventral cephalothorax and abdomen; c) epigyne.

a)



b)

c)

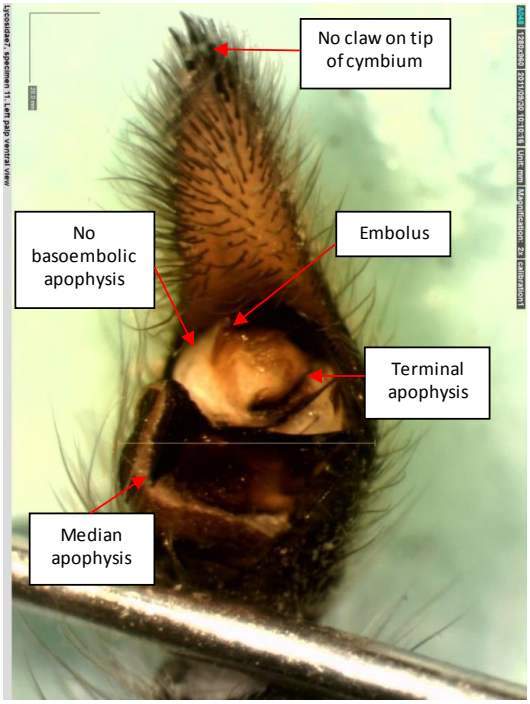


Figure 21. Undescribed possibly *Hogna* spp. male, a) dorsal cephalothorax; b) ventral cephalothorax, c) left palp.

1

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APPENDIX II: Author permission to reproduce tables and figures.

From: Rendon-Castaneda, Dalila (Agriculture, Myall Vale)
Sent: Sunday, 21 June 2015 6:44 PM
To: Macfadyen, Sarina (Agriculture, Black Mountain)
Subject: RE: Access to your PhD thesis

Next Previous

Hi Sarina,

I hope all is well. I wanted to ask you for another favour...is it ok if I use tables and figures from your paper "Assessing the impact of arthropod natural enemies on crop pests at the field scale" on my thesis? It fits my introduction quite perfectly. Thanks so much for your help!

Best regards,

-Dalila

RE: Access to your PhD thesis

Macfadyen, Sarina (Agriculture, Black Mountain)

You replied on 22/06/2015 2:49 PM.

Sent: Mon 22/06/2015 8:37 AM
To: Rendon-Castaneda, Dalila (Agriculture, Myall Vale)

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However, If you come to turn parts of your thesis into a paper – to re-publish in a journal you would need to seek permission from the publisher of the article. That's assuming you don't change it. If you alter it enough to suit your needs then you are not causing copyright issues and can just cite as before.

Cheers,

Sarina

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