Part 1 - Summary Details

Cotton CRC Project Number: 1.01.03

Project Title: Mortality of *Helicoverpa* in Bollgard II® cotton fields and implications for Bt resistance management

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Cotton CRC Program: The Farm

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Part 3 – Final Report

Background

In the mid-1990's the Australian cotton industry adopted Ingard®, a transgenic variety that contains a Bt toxin (Cry1Ac) which is specific to the group of insects including the target *Helicoverpa armigera*, but excluding natural predators and parasitoids of this pest. In the 2004/05 season, Bollgard II® replaced Ingard® as the transgenic variety of cotton available to Australian growers. It improves on Ingard® by incorporating an additional insecticide protein (Cry2Ab) to combat *H. armigera.* In its first available season Bollgard II® was well adopted, with an average of 70% of the planted area throughout the industry.

We know from artificial selection experiments in the laboratory that *H. armigera* can develop Bt resistance. In addition, CSIRO Entomology has worked on resistance by natural populations of *H. armigera* to transgenic cotton for many years, and presently maintains strains that are resistant to Cry1Ac or Cry2Ab. In both cases, the forms of genes (alleles) that confer resistance were isolated from field populations (CSE102C and CSE104C).

To help prevent the development of Bt resistance in field populations of *H. armigera* and thus prolong the utility of Bollgard II®, growers that use this tool must follow a resistance management plan (RMP). The RMP involves, in part, the growing of dedicated refuge crops aimed at producing sufficient Bt-susceptible *H. armigera* moths such that there is a high probability of them mating with resistant moths that may arise from the transgenic crop. The RMP is largely based on information from studies of the ecology and population genetics of *H. armigera*, and the outputs of computer simulation models that use biological information to predict the likelihood of resistance developing under different scenarios. These models assume that any individuals which are resistant to the toxins in Bollgard II® survive to successfully reproduce in cotton landscapes.

However, natural enemies that frequent Bollgard II® crops may kill Bt resistant individuals. Such mortality is not represented in current models which predict the temporal aspects of development of resistance, and is currently unquantified in the field. It would also be useful to know if such mortality differs between Bollgard II® crops and the common refuge crops (unsprayed conventional cotton, pigeon pea).

Some studies have characterized the arthropod fauna in fields of Bollgard II® versus sprayed conventional cotton. Bollgard II® crops are believed to contain a higher abundance and diversity of arthropods because broad-spectrum synthetic insecticides are not usually applied to this crop to control *Helicoverpa*. These arthropods include species that are natural enemies (predators or parasitoids) of a range of species including *Helicoverpa*. However they can also include secondary pests of cotton, such as mirids, which are controlled with synthetic chemistries (e.g., Regent®) that also kill other sucking pests, thrips, and hymenoptera. Some of these arthropods include natural enemies of *Helicoverpa* (e.g., mirids attack eggs of *Helicoverpa*). Therefore, improved abundance and diversity of natural enemies in Bollgard II® fields may prevent Bt resistant larvae from reaching adulthood, but the frequency of use of synthetic insecticides to control secondary pests such as mirids might also affect this situation.

In unsprayed refuges, such as pigeon pea, the abundance and diversity of arthropods can be very high indeed, thus creating a dilemma between using refuges to produce susceptible moths in the face of biological control. How does the survival of *Helicoverpa* in Bollgard II® crops compare with survival in these other crops? If we understood the degree and nature of mortality of *Helicoverpa* better across the landscape, we could improve the accuracy of the simulation models and refuge options used to drive the current RMP for transgenic cotton. For example, if Bt resistant individuals have a low probability of survival relative to the survival of all genotypes produced in refuges, then these retreats will be more effective than anticipated. Alternatively, if insecticide use (for sucking pests) in Bollgard II® crops enables resistant individuals to survive more frequently than eggs laid in refuges, then the efficacy of these retreats in limiting the evolution of resistance may be diminished.

Since the introduction of Bollgard II®, consultants and growers have occasionally noticed worrying numbers of large larvae of *Helicoverpa* in peak flowering crops. Such observations have raised concerns as to the potential damage such individuals might cause to crops, and if the larvae survive to pupation, what contribution they might make to Bt resistance development. The temptation has been to spray, without a good understanding of the probability of survival irrespective of spraying. This project sought to co-ordinate the collection and rearing of surviving larvae in Bollgard II® fields as a first step toward understanding the mechanisms underlying survival. Larvae that survived to healthy moths in the lab were donated to the Bt resistance monitoring program to ascertain their susceptibility to Bt toxins. Such work is thus a specific sub-set of the general thrust of the project to evaluate factors influencing the survival of *Helicoverpa* in Bollgard II® crops.

Whilst a substantial effort has been made to identify optimum refuge crops to use within the RMP for Bollgard II®, the majority of this effort coincided with the deployment of Ingard® cotton. The landscape has changed substantially since then, with fewer and different pesticides being applied to transgenic cotton crops. It behoves the cotton industry to monitor the ongoing performance of key refuge crops (e.g. pigeon pea) as we enter this new era in use of transgenic crops. In addition, whilst we believe we understand which refuge crops are most productive, we still have very little appreciation of how effectively the susceptible moths generated in refuges mate with moths arising from transgenic crops. As stated above, Project CSE107C is examining these aspects of refuge efficiency, but resources to enable such work have been significantly limited because of the recent drought. The technician appointed to this project was therefore deployed to assist CSE107C as available.

Objectives

Our major objective was to determine how natural enemies in Bollgard II® versus refuge crops affect the probability that *Helicoverpa armigera* will survive from hatching until adulthood, thus helping to refine the RMP for Bt cotton. Part of this objective is testing how the application of pesticide in Bollgard II® fields to control sucking pests affects natural enemy communities and, in turn, survival of *H. armigera*. Our secondary objectives are to co-ordinate the collection of surviving Helicoverpa larvae from Bollgard II® crops and rear them for inclusion in the Bt resistance monitoring program and, to increase the scale of work on evaluating refuge crop efficacy, in particular the extent of cross-mating that occurs between Helicoverpa moths from different crop origins. Except for the work on survivors, these aims were outlined in the original proposal.

TABLE 1: The objectives, milestones, and performance indicators of CRC Project 1.01.03. The text highlighted in bold font is different to the original objectives as explained in the main text.

No.	Objective	Milestone	Performance Indicator	Yr 1	Yr 2	Yr 3
1	Develop field methods necessary to complete objective 3	Trial several potential methods for manipulating arthropod abundance	Established rigorous method for altering natural enemy abundance	✓		
2	Establish local variability in spraying rates of Bollgard II® for sucking pests	Consult growers about previous rates of Regent application on Bollgard II®	Incorporated rigorously defined natural enemy "treatments" of Bollgard II® into experiment design	✓		
3	Determine degree to which natural enemies limit survival of surrogates for Bt resistant *H. armigera* in the field	Adopt field methods developed in year 1 to determine survival in Bollgard II® crops	Reliable estimates of survival of *H. armigera* obtained in Bollgard II® crops		✓	✓
4	Examine variation in the communities of natural enemies of *H. armigera* in Bollgard II® crops	Adopt standard field methods to determine arthropod communities in Bollgard II® crops	Rigorous assessment of variation in natural enemies in crops		✓	✓
5	Verify variation in the communities of natural enemies of *H. armigera* in different caged treatments	Adopt standard field methods to determine the communities of arthropods in the two types of cages	Confirmation that methods developed in year 1 are in effect throughout the study		✓	✓
6	Evaluate the survival of natural, late season egg lays of *Helicoverpa* in Bollgard II® crops **Co-ordinate the collection of surviving Helicoverpa larvae from Bollgard II®**	Surveys of field populations completed **Requests for material and collection protocols disseminated to all valleys**	Survivorship of late season egg lays calculated, advice given (final year) on treatment needed **Survivors reared to moths and donated to Bt resistance monitoring program**	✓	✓	✓
7	Evaluation of refuge crop efficacy & inter-crop mating by *Helicoverpa*	Surveys of field populations completed	Input made to CSE107C program, refuge performance documented	✓	✓	✓
8	Timely submission of final report	Completion of final report	**Final report submitted by 30 September 2008**[1]			✓

The work on survivors (objective 6) is a modified version of an original objective to evaluate the survival of natural, late-season egg lays of *Helicoverpa* in Bollgard II® crops. From late November 2005 until January 2006, we sampled two fields of Bollgard II® at the Australian Cotton Research Institute (both unsprayed with Heliocides) weekly for egg lays and larvae of Helicoverpa. We also requested that researchers on site alert us of any surviving larvae on Bollgard II®. We did not locate any grubs on these crops despite weekly sampling and a high abundance of grubs on adjacent conventional crops. During early January we received several reports from consultants and growers that Helicoverpa were surviving on Bollgard II® elsewhere in the valley. We therefore altered our approach to survey commercial farms for surviving Helicoverpa larvae on alerts from consultants rather than monitoring of egg lays. This developed into co-ordinating an industry wide collection of survivors on Bollgard II® cotton for rearing in the laboratory and incorporation into the Bt resistance screening program. We also encouraged submission of tissue from host plants of the larvae for analyses for expression of Cry proteins.

Our revised objective 6 does not enable us to determine the proportion of eggs laid, or the proportion of surviving larvae, that were able to survive to adulthood on Bollgard II® but rather asks whether larvae that reach a certain size on Bt plants are able to survive to adulthood (and potentially reproduce) when taken off Bollgard II® and reared on non-Bt diet. Given the difficulty in tracking natural egg lays through to larvae, this approach was considered a good compromise to our original question. It also enabled collaboration with the Bt resistance monitoring program to determine if surviving larvae were resistant to Bt.

In addition, the performance indicator for objective 8 was modified; we received an extension on this final report from 30th September 2008 until 31st October 2008 since it is linked to a CRDC presentation[1].

Mortality of Helicoverpa in a Bollgard II® landscape

Novel methods and tests of assumptions

In the first season of the project we developed the methods necessary to enable us to experimentally evaluate, in the second and third years of the project, how natural enemies affect the mortality of Helicoverpa larvae in a Bollgard II® landscape. We outline below the novel methods used for our study and some pilot studies that were performed to verify the critical assumptions of our main experiment.

(i) The design of our experimental cages

We used closed tents that excluded arthropods to create a low natural enemy environment and open tents that allowed a freer exchange of arthropods (but not larvae) to create a high natural enemy environment. Our cages were modified from those used by Dr John Stanley for his PhD work on Helicoverpa predation.

Each cage had a steel frame of 6 mm diameter mild steel rod, from which a rectangular shaped screen tent (100 cm long x 100 cm wide x 200 cm high) was suspended on elastic straps (Figure 1a). The two sides that ran parallel to the crop rows had a zipper along the whole length. A suspended fabric floor was joined around the stems of the plants with Velcro and then sealed with a non-acetate, silicone sealant (Figure 1b). This prevented access or escape of arthropods via soil fissures which would be possible if the tent walls were sealed onto the cracking-clay soils of the fields used in this study. Suspending the screen tent on elastic straps helped to protect the tent from tearing by the wind.

The top of the steel frame formed a cross that was hinged in the centre by a bolt. The legs of the frame were vertical tubular sections of steel pipe. Each leg was pushed about 30 cm into the ground allowing alignment of the tent floor with the stems of the cotton plants. The tent was attached to the frame by elastic along 5 positions per vertical seam and at the tip. These elastic straps were tied to the tent by strong cloth loops sewn into the tent seams during manufacture. A loop was also sewn at the centre of the roof panel to lift the fabric which helped shed rain water.

A commonly available polyester curtain material called ‘woven voile’ (terylene - 772 holes per cm^2 with 0.20 mm^2 holes) was used for the walls, floor and ceiling of the screen tents. The panels of the tent were sown together with polyester thread and the openings; the slit in the floor and the side door slits were lined with 25 mm wide Velcro. To create a cage in which natural enemies could freely exchange we removed 15 squares (7 x 7 cm) of material from the two sides of the tent that were at right angles to the crop row (see Figures 1a and 1c for details of location and arrangement of the “holes”). Open tents could be effectively closed by securing a 100 x 200 cm window to the Velcro on the edge of the holed face of the cage.

Figure 1: The tents (100 x 100 x 200 cm) used in our field experiments were “open” (with holes to enable natural enemy exchange) or “closed” (with no holes to exclude natural enemies).

(a) The tents were secured by elastic to a steel frame that was positioned over it and pegged into the ground.

(b) The split floor of the tent was closed with Velcro around the base of each plant and sealed with silicone.

(c) Open tents could be effectively closed by securing a window (100 x 200cm) to the Velcro on the edge of the holed face of the tent.

(ii) Processing arthropod samples collected from the experimental tents

As part of the processing phase of our main experiment we counted the numbers of arthropods, including larvae that are present within our tents at the end of a 7 day experimental period. We needed to rear any larvae recovered from inside the tents to maturity to examine levels of parasitism, and therefore developed a method to record the arthropods and larvae present within tents without killing them.

If the tents were open, we covered the two sides that were holed with a window of cloth adhered to the tent edges with Velcro. We then used secateurs to cut the plants below the base of the sealed bottom of the tent, untied the elastic straps from the metal frame, and transported the removed tent to a cool room that was set at 2 degrees Celsius.

The tent was hung from its peak such that the base barely touched the floor. At least one hour later, when all insects were cold-narcotized, we placed the floor of the hanging tent over a white tub (100 cm long x 100 cm wide x 10 cm high) that was position on a yellow sheet of plastic (200 x 200 cm). If the tents were open, we removed the two windows from the sides of the tent that were holed (Figure 2a). We then carefully opened the split in the floor to empty the contents from the tent (Figures 2b and 2c). The inside of the tent, and yellow plastic sheet, were inspected for arthropods, which were relocated to the white tub.

Figure 2: The tents were hung in a cool room set to 2 degrees Celsius before being emptied for processing.

(a) A window is being removed from a tent that is hung in the cool room over a tub placed on a yellow sheet.

(b) The split floor of the tent is being opened from the underside of the floor to enable its contents to be removed.

(c) The contents are emptied into the tub. Then the inside of the tent and yellow sheet are searched for arthropods that are placed into the tub.

Each plant was then individually lifted and shaken over the tub 10 times to dislodge any arthropods, including larvae. Any dislodged plant material that remained in the tub after the plants were shaken was lightly brushed. All larvae were removed with forceps and placed individually within 24 well trays that were half filled with artificial diet. The tub containing the remaining arthropods was then transported to a bench outside of the controlled temperature room that was at ambient temperature. The same observer sorted the arthropods by noting the number of individuals of each species.

(iii) Did we create high versus low natural enemy environments?

During the first phase of our main experiment we attempted to eradicate arthropods from closed and open tents 7 days prior to introducing larvae. By applying the same treatment to both tent types, and allowing natural enemies to recolonise open tents only, we controlled for any effect of the removal on our results.

Before erecting the tent over an area of row we shook all plants in the 1m section. Once the tent was sealed at the floor we shook the plants a further 10 times to dislodge any remaining arthropods, and with a handheld spray pack immediately applied Pyrethrum (Amgrow Organix, Envirogreen Pty Ltd) at a rate of 100ml/4L on the inside walls of the tent and the base of the plants. We did not apply the spray directly to the plants since a preliminary study determined that this process affected the development rate of Helicoverpa larvae. We also removed by hand any large arthropods that may not have been killed by the spray. Seven days after this eradication process we infested tents with larvae. We processed the tents 7 days after this larval infestation.

We used MANOVA on data collected during the first year of the study to test the assumption that closed tents exclude most arthropods thereby creating a low natural enemy environment relative to the open tents. We sampled every fortnight from December until the end of February, and combined data for the first and second, third and fourth, and fifth and sixth time periods; this corresponded with samples for December, January and February respectively. The factors included in the initial model were time, tent, and crop. We assigned arthropods to one of 8 categories according to their size and role as predators or parasitoids of Helicoverpa larvae as per “The Cotton Pest and Beneficial Guide” by Pyke and Brown (Table 2). Each of the groups was included as a dependent variable in the analysis.

TABLE 2: The assignment of arthropods to groups according to their size and role as predators or parasitoids of Helicoverpa larvae.

Function	Category	Species or groups included in the category
NON-PREDATORS	Arthropods <5mm	Jassid, Apple Dimpling Bug, Green Mirid nymph, Brown Mirid nymph, Flea Beetle, Shiny Beetle, Pollen Beetle, Cotton Seed Beetle, Rutherglen Bug, Hoverfly Larvae, Green Vegetable Bug nymph, Brown Smudge Bug
	Arthropods 5mm or >	Green Mirid adult, Brown Mirid adult, Green Lacewing, Brown Lacewing, Green Vegetable Bug adult
PREDATORS	Arthropods <5mm	Lacewing Larva, Red and Blue Beetle, Big Eyed Bug, Pirate Bug
	Arthropods 5mm or >	Glossy Shield Bug, Predatory Shield Bug, Damsel Bug, Common brown earwig
	Spiders	Yellow Night Stalker, Orb Weaver, Jumping, Lynx, Crab, Wolf, Tangle web, Unknown
	Ants	Ants < 2mm, Ants > 2mm
	Lady beetles	3-Banded, Common Spotted, Variable, Hippo, Transverse, Striped, Minute Two-Spotted
PARASITOIDS	Larval parasitoids	Microplitus, Tachinid, Heteropelma, Netelia

The effect of tent type on arthropod abundance did not vary for the different crops (Wilks' Lambda, F = 1.4, P = 0.11) but there was a significant interaction between tent type and time throughout the season (Wilks' Lambda, F = 3.6, P < 0.001). This interaction reflects the higher relative abundance of arthropods mid-season which created a difference between closed and open tents that shifted over time. Analysis on each time period separately demonstrated a highly significant difference in arthropods among tent types for all times (Wilks' Lambda, F > 11.78, P < 0.0001). In all cases, there were at least 3 times as many individuals per functional group in the open tents compared to the closed tents (Table 3). We conclude based on this evidence that our closed tents excluded the majority of natural enemies and served to create an environment that was relatively low in the abundance of potential predators and parasites compared to open tents.

TABLE 3: The mean ± SE no. of individuals per category in closed tents versus open tents. F = function, NP = non-predator, P = predator, Pa = parasitoid.

		Mean ± SE number of individuals per 1m tent								
		Time 1			Time 2			Time 3		
F	**Group**	**Closed**	**Open**	**C:O**	**Closed**	**Open**	**C:O**	**Closed**	**Open**	**C:O**
NP	Arthropods <5mm	4.0 ± 0.6	13.1 ± 1.8	**3.3**	5.3 ± 1.9	19.4 ± 3.6	**3.6**	2.4 ± 0.6	9.2 ± 1.2	**3.8**
NP	Arthropods 5mm or >	0.0 ± 0.0	0.5 ± 0.1	-	0.4 ± 0.3	1.4 ± 0.4	**3.5**	0.0 ± 0.0	0.7 ± 0.2	-
P	Arthropods <5mm	0.8 ± 0.4	6.6 ± 1.2	**8.3**	0.4 ± 0.1	3.1 ± 0.8	**7.8**	0.1 ± 0.1	0.8 ± 0.2	**8**
P	Arthropods 5mm or >	0.3 ± 0.2	1.2 ± 0.6	**4**	0.1 ± 0.1	1.1 ± 0.3	**11**	0.4 ± 0.2	2.3 ± 0.8	**5.8**
P	Spiders	1.3 ± 0.4	4.4 ± 0.6	**3.4**	1.0 ± 0.3	7.2 ± 0.7	**7.2**	1.2 ± 0.2	7.6 ± 0.9	**6.3**
P	Ants	0.2 ± 0.1	0.6 ± 0.3	**3**	0.1 ± 0.1	0.5 ± 0.3	**5**	0.1 ± 0.1	0.4 ± 0.3	**4**
P	Lady beetles	0.3 ± 0.2	1.4 ± 0.3	**4.7**	0.1 ± 0.1	1.2 ± 0.2	**12**	0.1 ± 0.1	0.4 ± 0.1	**4**
Pa	Larval parasitoids	0.0 ± 0.0	0.9 ± 0.3	-	0.2 ± 0.1	1.4 ± 0.3	**7**	0.0 ± 0.0	1.1 ± 0.2	-

(iv) Do natural enemies in open tents represent communities in the open field?

In our main experiment we undertook to exclude natural enemies from open tents to provide a procedural control for the closed tents. Pilot work in 2005/06 demonstrated that 7 days after our exclusion procedure, the natural enemy communities inside the open tents were similar to those in the open field.

In 2006/07, we verified that natural enemies in open cages were representative of communities in the open field by comparing the samples obtained in our tents during the processing phase of our main experiment, and beat sheets performed near the tents. For our main experiment we set up tents in groups that comprised 3 open tents and 1 closed tent in four sequential plots that were positioned 8 m apart (see methods below for more details). For the analyses we averaged 10 x 1 m beats, each of which was performed within 4 m of a set of four experimental tents in a crop. This value was compared with the average for the 3 open tents per group.

We used MANOVA with time (1st, 2nd, 3rd, 4th, 5th, 6th sampling period), crop, and location (tent, field) as factors. All categories in Table 2, except larvae parasitoids, were included as a dependent variable. We did not include larval parasitoids in our analysis because they are not reliably detected using a beat sheet.

There was no significant interaction between time, crop or location (Wilks' Lambda, F = 0.7, P = 0.9), and no significant interaction between crop and location (Wilks' Lambda, F = 1.3, P = 0.2) or time and location (Wilks' Lambda, F = 1.5, P = 0.06). There also was no significant main effect of whether sampling took place in open tents versus nearby in open fields (Wilks' Lambda, F = 1.4, P = 0.3). For most categories the relationship between the mean number of individuals in the open tent: open field was close to 1. The exception was the number of predatory arthropods that were at least 5mm; in this category there were almost twice as many individuals within tents compared to the open field.

We conclude based on this evidence that natural enemies recolonised open tents throughout the 7 day period leading up to infestation with larvae, such that the number of individuals within these structures was similar to that in the surrounding open field.

TABLE 4: The mean ± SE no. of individuals per category in open tents versus nearby in open fields. F = function, NP = non-predator, P = predator. Data were averaged across crop and time.

		Mean ± SE number of individuals per 1 m sample		
F	**Categories**	**Open tent**	**Open field**	**tent:field**
NP	Arthropods <5mm	13.9 ± 1.4	14.8 ± 1.4	**0.9**
NP	Arthropods 5mm or >	0.9 ± 0.2	0.7 ± 0.1	**1.2**
P	Arthropods <5mm	3.5 ± 0.6	3.9 ± 0.4	**0.9**
P	Arthropods 5mm or >	1.5 ± 0.4	0.8 ± 0.3	**1.9**
P	Spiders	6.4 ± 0.5	6.3 ± 0.5	**1.0**
P	Ants	0.5 ± 0.2	0.4 ± 0.1	**1.3**
P	Lady beetles	1.0 ± 0.1	0.9 ± 0.1	**1.1**

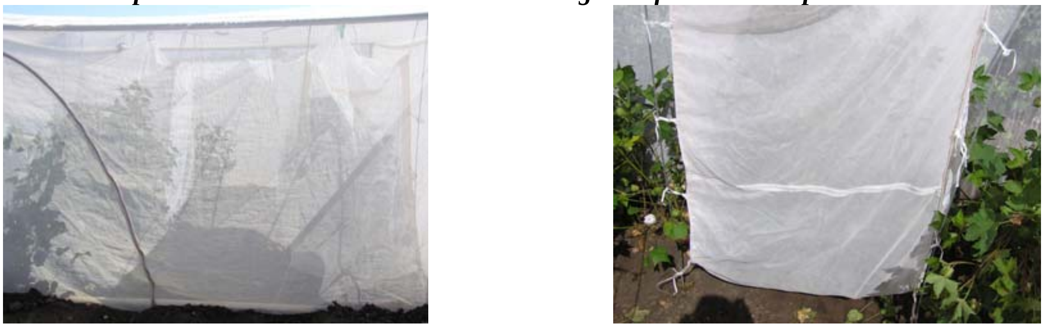
(v) Do the experimental larvae move out of the holed tents?

An important assumption of our main experiment is that the larvae placed inside our open tents do not exit during the 7 day period that they are exposed to natural enemies. We used two approaches to test this assumption.

Experimental test

We placed an open tent and closed tent inside a larger closed cage (300 long x 300 cm wide x 210 cm high) that covered three rows of conventional cotton (Figure 3a). We removed by hand all plants in the middle row of cotton that ran through the large cage. One of the experimental tents was set in the centre of the inner row of cotton and the other tent was set in the centre of the outer row of cotton. Both tents were positioned with 1 m of cotton either side (Figure 3b).

Figure 3: The set-up used to test whether larvae were moving out of the holed experimental tents.



(a) One holed tent and one closed tent was set-up inside a larger cage positioned over conventional cotton.

(b) Within the larger cage, both types of tents were positioned with 1m of cotton either side.

To all of the cotton in the large cage, including that inside the 1 m experimental cages, we applied the same procedures used to eradicate arthropods during phase 1 of our experiment (see iii above). This process was necessary to reduce the probability that larvae were consumed by predators, thereby enabling us to accurately determine the numbers of individuals that moved out of the holed cages. As per our main experiment, we then introduced 20 individuals to both the open and closed cages and 7 days later processed the tents using the methods outlined in (ii) above. We repeated the experiment at two time periods (time 1 and 2) on attractive maturing cotton using 2 larger cages as replicates each time. We used only 3rd instar larvae because previous work had established that they are more likely than neonates to move large distances.

A two-factor ANOVA showed no significant interaction between time and tent type (df = 1,4, F = 0.5, P = 0.5), thus this term was removed from the model. When in the low natural enemy environment of the larger cage, there was no significant variation in the number of larvae in open tents versus closed tents (mean no. larvae ± SE = 14.7 ± 1.5 closed, 15.0 ± 1.8 open: ANOVA, df = 1,5, F = 0.1, P = 0.8). This result supports the assumption that the larvae placed inside our open tents do not exit during the 7 day period that they are exposed to natural enemies.

‘Beats’ of plants that neighboured experimental cages

Immediately prior to removing our tents for processing, we used standard methods to perform a beat of the plants that were within 1 m either side. Throughout the 2006/07 season we performed 224 beats (2 m total), 168 around open tents and 56 around closed tents. On 3 occasions (1.3%) we detected *H. armigera* larvae. All cases were recorded in our unsprayed conventional cotton treatment and 2 cases were next to open tents, whereas 1 case was next to a closed tent. This finding supports that assumption that the larvae placed inside our open tents do not exit during the 7 day period that they are exposed to natural enemies.

Methods for the main experiment

Our main experiment was conducted in 2006-07 and 2007-08. In both years we followed the same procedures. The study was comprised of two components: a manipulative field experiment and among crop samples of natural enemy communities in replicated fields. All of the work was conducted on fields that were located within a 10 km radius of the Australian Cotton Research Institute in the Namoi Valley. All of the crops were fertilized and irrigated on beds 1 m apart with agronomic practices that followed commercial “best practice”.

Both components focussed on four crop types: pigeon pea, unsprayed conventional cotton, unsprayed Bollgard II® cotton and commercially sprayed Bollgard II® cotton. We selected pigeon pea and unsprayed conventional cotton because they are the two most common refuge crops grown since the introduction of Bollgard II® in 2003/04. By including an unsprayed Bollgard II® crop we could examine the implications of spraying for mites and sucking pests in Bollgard II® on natural enemy communities, and in turn Helicoverpa mortality. In both study years the commercial fields of Bollgard II® were sprayed in mid-December with Abamectin (30ml/ha), mid-January with Fipronil (62.5ml/ha), and in early February with Fipronil (40ml/ha) and Abamectin (30ml/ha). This regime conformed to the standard practices for other commercial fields in the Lower Namoi as determined by a recent survey conducted by Dr Mary Whitehouse.

(i) The manipulative field experiment

Each year the manipulative field experiment was conducted in one field per crop type. In both Bollgard II® treatments, we replaced one-metre lengths of one row of Bollgard II® seedlings with an isogenic variety of conventional cotton shortly after emergence. These plots allowed us to create a situation whereby Bt susceptible larvae placed on conventional plants "survive" in a Bollgard II® field. The plots were 8 m apart with 12 plots per row in 4 separate rows that were 20 m (rows) apart (total of 48 plots per field). There was at least 100 m of buffer before the first and last plot in a row. We used the popular pigeon pea variety "Quest", and the popular cotton varieties Sicot 71R as our conventional crop and Sicot 71BR as our Bollgard II® cotton crops.

The experiment was performed in three phases. The first phase involved setting up the tents. We positioned the steel frame over the 1 m plot and pushed the legs into the soil. We then tied the peak of the tent to the cross of the metal frame. The plants were shaken 10 times to dislodge any arthropods. The split in the floor of the tent was then inserted over the plants and secured with the Velcro. The rest of the tent was tied to the metal frame with the elastic tags, before the join in the floor of the tent was sealed with a non-acetate, silicone sealant. We shook the plants a further 10 times to dislodge any remaining arthropods, and with a handheld spray pack immediately applied Pyrethrum at a rate of 100ml/4L on the inside walls of the tent and the base of the plants. We also removed by hand any large arthropods that may not have been killed by the spray.

We initiated phase 2 of the experiment 7 days after this eradication process. We used a soft brush to place 20 *H. armigera* larvae near the terminals of the plants inside the tent. We infested tents with larvae of two size classes: neonates and 3rd instar. The neonates were fed for 24hrs on artificial diet because our previous work showed that mortality was otherwise significantly high even in our closed cages that contained few natural enemies. Since the larvae were exposed to natural enemies for a 7 day period (see below), starting with these two size classes covered the bulk of the larval period of *H. armigera*. In all crop types we set up tents in groups that comprised 3 open tents and 1 closed tent in four sequential plots. For neonate larvae and 3rd instar larvae we set up 6 groups of 4 tents (3 open, 1 closed) from December through to the end of February, at approximately two weekly intervals (i.e., 24 plots for neonates and 24 plots for 3rd instar larvae/crop). Before infesting with larvae, the plants within each tent were searched and all Helicoverpa eggs and larvae were removed.

Phase 3 of the experiment involved processing the material inside the tents 7 days after larval infestation. This process is outlined in detail under "Novel methods and tests of assumptions part (ii)" above. In brief, we used secateurs to cut the plants below the base of the sealed bottom of the tent, and transported the removed tent to a cool room. When all insects were cold-narcotized, we emptied the contents from the tent in a tub. Each plant was lifted and shaken to dislodge any arthropods, including larvae. All larvae were removed and placed individually in 24 well trays with artificial diet. They were subsequently reared under standard conditions to determine rates of parasitism. The tub containing the remaining arthropods was then transported to a bench outside of the controlled temperature room that was at ambient temperature. The same observer sorted the arthropods by noting the number of individuals of each species.

(ii) Among-crop variation in natural enemy communities

It was not practical to replicate the manipulative experiment in a number of fields. However we were able to replicate our sampling of natural enemy communities across fields. In both years of the study we replicated across 5 separate fields per crop type, including the fields that we used for the manipulative experiment.

We performed 10 x 1 m 'beats' (all plants in a 1 m length of a row of cotton were subjected to a standard beat-sheet routine) per field on 3 occasions throughout the season: mid December, mid January, and mid February. In both study years the replicate commercial fields of Bollgard II® were subjected to the same spray regime within 3 days of each other. All sampling for each time period was completed within a 5 day period and sampling on the same day was randomised across crop type.

Results for the main experiment

Our previous work showed that the closed tents were successful in excluding the majority of natural enemies. We therefore assumed that any mortality in the closed tents was due to abiotic reasons and used this value to correct for mortality in the open tents that was not related to natural enemies.

We used ANOVA to test the null hypothesis that there is no difference in mortality of larvae in different crop types irrespective of time throughout the season. For each group of 4 tents, we averaged the total larvae present from the 3 open tents minus the number of individuals that were parasitised (as determined through rearing) and added the number of missing larvae from the closed tent to this value. Hereafter we refer to this estimate as "survival". We sampled every fortnight from December until the end of February, and combined data for the first and second, third and fourth, and fifth and sixth time periods; this corresponded with samples for December, January and February respectively. The factors included in the initial model were year, time, larvae stage, and crop. Our estimate of survival was the dependent variable in the analysis. The initial analysis showed that survival of larvae in different crop types differed depending on life stage (crop x larvae stage interaction, ANOVA, df = 3, 48, F = 2.8, P = 0.04). Therefore we performed subsequent analyses on separate data sets for neonates and third instar larvae.

(i) Neonatal larvae

The mean ± SE corrected survival of Helicoverpa introduced as neonatal larvae was 11.2 ± 0.6 with a range of 5.0 – 19.7. The impact of crop type on the survival of third instar larvae was not consistent across the season (crop x time interaction, df = 6, 24, F = 3.4, P = 0.015). We subsequently performed ANOVA on separate data sets for each time period with year and crop as factors. In all cases there was no significant interactive or main effect of year on survival of neonatal larvae (P > 0.05).

In December there was no significant difference among crops in the survival of neonatal larvae (mean ± SE corrected survival = 9.6 ± 1.0, unsprayed Bollgard II$^{®}$; 11.4 ± 1.5, sprayed Bollgard II$^{®}$; 10.8 ± 1.3, unsprayed conventional; 13.8 ± 1.3, unsprayed pigeon pea: ANOVA, df = 3, 8, F = 3.0, P = 0.098).

In January and February there was a significant difference among crops in the survival of neonatal larvae (in both cases ANOVA, df = 3,8, F > 7.3, P < 0.011). During both of these time periods there was a higher survival of neonates in sprayed Bollgard II$^{®}$ and unsprayed pigeon pea compared with unsprayed Bollgard II$^{®}$ and unsprayed conventional cotton (Table 5).

TABLE 5: The summary statistics from a Tukey-Kramer Honestly Significant Difference posthoc test on the ANOVA with year and crop as factors and survival of neonates as the dependent variable. Values are presented separately for January and February.

		Summary Statistics from T-K HSD test		
Time	**Comparison**	**Mean difference**	**Critical difference**	**P-value**
January	BG, BG(s)	-5.08	4.47	**0.031**
	BG, CONV	1.83	4.47	0.372
	BG, PP	-5.67	4.47	**0.019**
	BG (s), CONV	6.91	4.47	**0.007**
	BG (s), PP	-0.58	4.47	0.771
	CONV, PP	-7.50	4.47	**0.005**
February	BG, BG(s)	-8.91	3.96	**0.001**
	BG, CONV	0.83	3.96	0.641
	BG, PP	-9.75	3.96	**0.001**
	BG (s), CONV	9.75	3.96	**0.001**
	BG (s), PP	-0.83	3.96	0.641
	CONV, PP	-10.58	3.96	**0.001**

(ii) Third instar larvae

The mean ± SE corrected survival of Helicoverpa introduced as 3rd instar larvae was 11.9 ± 0.5 with a range of 5.0 – 23.7. The impact of crop type on the survival of third instar larvae was consistent across the season and among years (for all interaction terms, F < 15.4, P > 0.2). There was no significant difference among years in the survival of 3rd instar larvae (mean ± SE corrected survival = 11.8 ± 0.8, 2006-07; 12.0 ± 0.7, 2007-08: ANOVA, df = 1, 24, F = 0.1, P = 0.8).

However, the survival of third instar larvae differed throughout the season (mean ± SE corrected survival = 13.6 ± 1.1, December; 10.2 ± 0.8, January; 11.1 ± 0.6, February: ANOVA effect, df = 2, 24, F = 4.7, P = 0.02), with a significant decline over time (T-K HSD, mean diff. > critical diff. for December vs. January and February, P < 0.05).

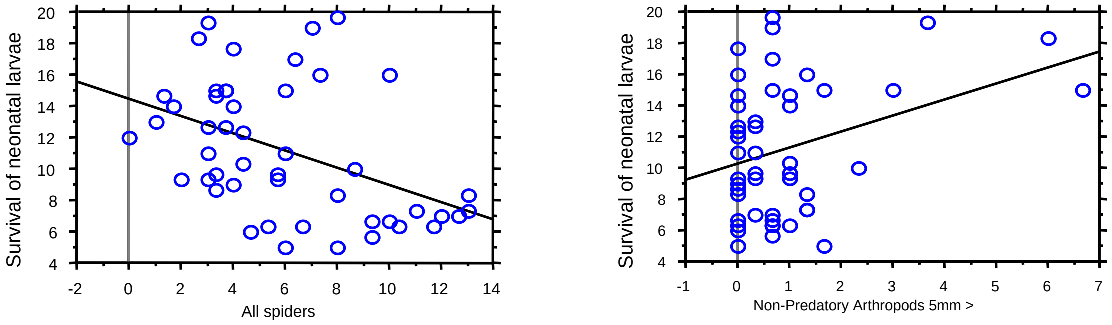
The survival of third instar larvae also varied among crop types (mean ± SE corrected survival = 10.2 ± 0.9 unsprayed Bollgard II$^{®}$; 12.3 ± 0.7, sprayed Bollgard II$^{®}$; 10.6 ± 1.1 unsprayed conventional; 14.6 ± 1.1, unsprayed pigeon pea: ANOVA effect, df = 3, 24, F = 4.8, P = 0.01). In particular, survival was significantly higher in pigeon pea compared with unsprayed Bollgard II$^{®}$ (T-K HSD, mean diff. = 4.4., critical diff. = 2.7, P = 0.002) and unsprayed conventional cotton (T-K HSD, mean diff. = 4.0, critical diff. = 2.7, P = 0.005). The survival of third instar larvae did not differ significantly among the remaining pairwise comparisons (in all cases mean diff. < critical diff., P > 0.08).

Relationships between larvae survival and arthropod abundance

(i) Neonatal larvae

A regression that incorporated the 8 categories of arthropods listed in Table 2 determined a significant relationship with survival of neonatal larvae (R = 0.65, df = 7, 40, F = 4.1, P = 0.0018). This result was driven by a negative relationship between survival of neonatal larvae and "spiders" (t- value = -7.7, P = 0.034: Figure 4). There was also a significant positive relationship between survival of neonatal larvae and non-predatory arthropods 5 mm or greater (t- value = -2.6, P = 0.012: Figure 4). There was no significant relationship between survival of neonatal larvae and any of the other categories of arthropods (Table 6).

Figure 4: The regression plot showing the significant relationship between survival of neonatal larvae and (a) "all spiders" and (b) "non-predatory arthropods 5 mm or greater". See Table 2 for a list of species included in these categories.



(a) survival versus spiders

(b) survival versus non-predatory arthropods 5 mm >

TABLE 6: The summary statistics from a multiple regression of the relationship between survival of third instar larvae versus the 8 categories of arthropods as defined in Table 2.

		Mean ± SE number of individuals per 1 m sample		
F	**Categories**	**Coefficient**	**t-value**	**P-value**
NP	Arthropods <5mm	-0.01	-0.23	0.82
P	Arthropods 5mm or >	0.25	1.32	0.19
P	Arthropods <5mm	0.14	2.06	0.05
P	Ants	0.04	0.12	0.90
P	Lady beetles	0.59	1.28	0.21
P	Larval parasites	0.03	0.11	0.09

(ii) Third instar larvae

A regression that incorporated the 8 categories of arthropods listed in Table 2 determined a significant relationship with survival of third instar larvae (R = 0.68, df = 7, 40, F = 4.9, P = 0.0005). This result was largely driven by a significant negative relationship between survival of third instar larvae and "spiders" (t- value = -3.5, P = 0.0013: Figure 5). There was no significant relationship between survival of third instar larvae and any of the other categories of arthropods (Table 7).

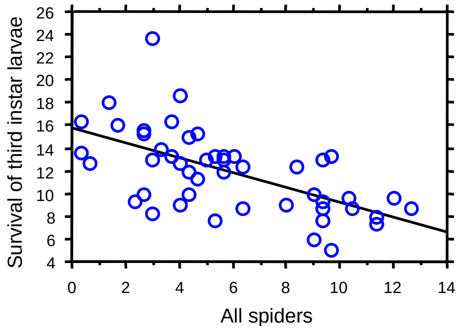


Figure 5: The regression plot showing the significant negative relationship between survival of third instar larvae and "all spiders". See Table 2 for a list of species included in this category.

TABLE 7: The summary statistics from a multiple regression considering the relationship between survival of third instar larvae versus the 8 categories of arthropods as defined in Table 2. F = function, NP = non-predator, P = predator. Data were averaged across crop and time.

		Mean ± SE number of individuals per 1 m sample		
F	**Categories**	**Coefficient**	**t-value**	**P-value**
NP	Arthropods <5mm	-0.01	-0.23	0.82
NP	Arthropods 5mm or >	-0.69	-1.66	0.10
P	Arthropods 5mm or >	0.25	1.32	0.19
P	Arthropods <5mm	0.14	2.06	0.05
P	Ants	0.04	0.12	0.90
P	Lady beetles	0.59	1.28	0.21
P	Larval parasites	0.03	0.11	0.09

Among crop variation in natural enemy communities

We used ANOVA with year, crop, and time as factors to examine variation in the communities of arthropods across the 5 replicate fields that we sampled using beat sheets in December, January and February. We performed the analysis on averages of the 10 beats per field. Separate analyses were performed on all categories in Table 2, except larvae parasitoids, which were included as the dependent variable. We did not include larval parasitoids in our analysis because they are not reliably detected using a beat sheet.

The abundance of ladybeetles, and predatory arthropods 5mm or greater, did not vary significantly among crop type (in both cases, ANOVA, df = 3, 96, F < 0.8, P > 0.50).

The abundance of spiders, non-predatory arthropods less than 5mm, non-predatory arthropods 5mm or greater, and predatory arthropods < 5mm varied significantly among crop types (ANOVA, df = 3, 96, F > 3.6, P < 0.016). For spiders and non-predatory arthropods less than 5mm, there were significantly more individuals in unsprayed Bollgard II® and conventional cotton fields compared to Bollgard II® fields that were sprayed for mirids and mites and unsprayed pigeon pea fields (Table 8). For predatory arthropods < 5mm there were significantly more individuals in pigeon pea compared to unsprayed Bollgard II®, sprayed Bollgard II® and unsprayed conventional cotton (Table 8).

The abundance of ants varied significantly among crop type but this relationship differed with time (crop x time interaction, ANOVA, df = 6, 96, F = 5.8, P < 0.0001). There was no significant difference in ant abundance in December (mean ± SE = 0.31 ± 0.14, unsprayed Bollgard II®; 0.06 ± 0.08, sprayed Bollgard II®; 0.27 ± 0.09, unsprayed conventional; 0.27 ± 0.19, pigeon pea: ANOVA, df = 3, 32, F = 0.94, P = 0.43) or January (mean ± SE = 0.49 ± 0.25, unsprayed Bollgard II®; 0.55 ± 0.39, sprayed Bollgard II®; 0.68 ± 0.38, unsprayed conventional; 0.19 ± 0.13, pigeon pea: ANOVA, df = 3, 32, F = 0.46, P = 0.71). In February there were significantly more ants in pigeon pea compared to any other crop (mean ± SE = 0.73 ± 0.33, unsprayed Bollgard II®; 0.13 ± 0.07, sprayed Bollgard II®; 0.44 ± 0.20, unsprayed conventional; 2.9 ± 0.83, pigeon pea: ANOVA, df = 3, 32, F = 0.35, P = 0.001).

TABLE 8: The summary statistics from a Tukey-Kramer Honestly Significant Difference posthoc tests on ANOVA with year, time, and crop as factors and categories of arthropods as the dependent variables. Values are presented separately for each category.

		Summary Statistics from T-K HSD test		
Category	**Comparison**	**Mean difference**	**Critical difference**	**P-value**
Spiders	BG, BG(s)	2.28	1.38	**0.001**
	BG, CONV	-1.13	1.38	0.109
	BG, PP	3.51	1.38	**<0.0001**
	BG (s), CONV	-3.40	1.38	**<0.0001**
	BG (s), PP	1.23	1.38	0.080
	CONV, PP	4.64	1.38	**<0.0001**
Non-Predatory Arthropods < 5mm	BG, BG(s)	11.17	6.34	**0.001**
	BG, CONV	1.58	6.34	0.623
	BG, PP	12.78	6.34	**0.001**
	BG (s), CONV	-9.59	6.34	**0.003**
	BG (s), PP	1.61	6.34	0.615
	CONV, PP	11.20	6.34	**0.001**
Non-Predatory Arthropods 5mm >	BG, BG(s)	-0.20	0.67	0.560
	BG, CONV	0.04	0.67	0.900
	BG, PP	-1.0	0.67	**0.006**
	BG (s), CONV	0.24	0.67	0.478
	BG (s), PP	-0.76	0.67	**0.027**
	CONV, PP	-1.00	0.67	**0.004**
Predatory Arthropods < 5mm	BG, BG(s)	-1.68	2.28	0.147
	BG, CONV	-0.55	2.28	0.631
	BG, PP	-7.30	2.28	**<0.001**
	BG (s), CONV	1.13	2.28	0.330
	BG (s), PP	-5.61	2.28	**<0.001**
	CONV, PP	-6.74	2.28	**<0.001**

Surviving larvae on Bollgard II®

During 2005/06, 2006/07 and 2007/08 there were reports from early-January until late-February of surviving larvae at threshold levels in Bollgard II® fields on some properties in Emerald, Lower Namoi, Upper Namoi, Darling Downs, Gwydir, Macquarie, Macintyre and St George. All affected fields were at mid-flowering to late-flowering. Over those three seasons we received 321, 231, and 764 larvae that were collected on Bollgard II®. The larvae were collected from a number of properties within each valley. We have not analyzed the numbers contributed by each valley or over years because this measure probably does not relate to the incidence of living larvae on Bollgard II® but instead reflects differences in the readiness of consultants or growers to submit larvae to the program.

Mr Gavin Whitburn, who holds a CCC CRC Summer Studentship, is currently working with CSIRO, CCA members and Monsanto to survey the proportion of Bollgard II® in each valley that reached threshold levels of Helicoverpa, and the proportion of threshold levels that were sprayed to control Helicoverpa. This survey also considers potential changes in these measures over time (2005-06, 2006-07, 2007-08). These data will be lodged with the CRC at the completion of the project.

We have presented below a summary of the information for each season on size, species, and emergence of the larvae submitted to the program (Table 9). We list the varieties of Bollgard II® from which larvae were collected to illustrate that survivors can occur in fields of a range of popular varieties.

All larvae that were successfully reared on artificial diet in the laboratory to healthy moths were assigned to the F_2 screening component of the Bt monitoring program. Of these “survivors” 124 and 108 alleles in 2005/06, 24 and 168 alleles in 2006/07, and 132 and 84 alleles in 2008/09 were screened against Cry1Ac and Cry2Ab for *H. armigera* and *H. punctigera* respectively. The results from CRDC Project CSE112 demonstrate that the larvae contributed to our program were not able to survive on Bollgard II® because they carried alleles conferring resistance to Bt toxins. A comprehensive analysis of these findings is presented in the final report for that project.

TABLE 9: A summary of the information for each season on the size, species, and emergence of larvae collected as survivors on Bollgard II® and submitted to the program. All of the varieties listed are Sicot. In 2007-08 we also recorded on which part of a plant (square, flower, boll) a surviving larvae was found. No. = Number; Small = 1st + 2nd + 3rd instar larvae; Large = 4th + 5th instar larvae.

	Season		
	2005-2006	**2006-2007**	**2007-2008**
Total larvae submitted	320	231	764
Varieties included	60BR, 71BR, 289BR	80BR, 71BR, 289BR, 61BR	43BR, 289BR, 71BR, 80BRF
No. 1st instars (% emerged)	185 (56)	2 (0)	12 (25)
No. 2nd instars (% emerged)	90 (44)	14 (7)	45 (55)
No. 3rd instars (% emerged)	10 (60)	40 (30)	187 (74)
No. 4th instars (% emerged)	23 (69)	76 (67)	254 (69)
No. 5th instars (% emerged)	12 (75)	91 (63)	211 (64)
% *Helicoverpa armigera*	50	30	75
% Small on boll, flower, square	-	-	47, 32, 18
% Large on boll, flower, square	-	-	76, 16, 5

There was a significant difference among years in the distribution of larvae of different sizes to the program ($\chi^2 = 787.1$, $P < 0.0001$). This difference is largely driven by one collection in 2005-06 of 260 first and second instar larvae from a field of Bollgard II® that was replanted after hail.

In 2006-07 and 2007-08 smaller larvae contributed to the program were significantly less likely to emerge as healthy moths ($\chi^2 > 17.0$, $P < 0.0013$) but there was no significant difference in emergence among size classes of larvae in 2005-06 ($\chi^2 = 7.9$, $P = 0.10$). The large collection of small larvae in 2005-06 were presumably from the same egg lay, and were concentrated on vegetative regrowth of the first planting which was unlikely to be expressing optimally. In contrast, most small larvae from 2006-07 and 2007-08 formed collections comprised mainly of medium to large larvae, and are presumably from a later egg lay. In this case, differential survival may reflect exposure of the second cohort to a plant that was recovering from a temporary dip in Bt expression.

There was a significant difference among seasons in the proportion of the collection that was *H. armigera* ($\chi^2 = 119.3$, $P < 0.0001$). In 2005-06 half of the collection was *H. armigera*, in 2006-07 the majority (70 %) of the collection was *H. punctigera*, and in 2007-08 the majority (75 %) of the collection was *H. armigera*. Aside from the one large collection mentioned above, in 2005-06 and 2006-07 we received few larvae from numerous valleys and our estimates of species composition from egg collections (as part of CSE112) are poor. In 2007-08 our collections focused on St George. The proportion of *H. armigera* in our sample of survivors is higher than that in the sample of eggs collected during the same time period as survivor collections (January and February) in St George. This finding suggests that in 2007-08 *H. armigera* may have differentially survived on Bollgard II®.

In 2007-08 we recorded on which part of the plant (square, flower, boll) a surviving larvae was found; few were recorded on leaves. There was a significant interaction between the size of the larvae and the part of the plant on which it was found ($\chi^2 = 54.4$, $P < 0.0001$). Small larvae (1st, 2nd and 3rd instars) were more evenly distributed among squares, flowers and bolls compared to larger larvae (4th and 5th instars) which were found most often on bolls compared with squares and flowers (see Table 9).

Qualitative ELISA tests of host plants

For a subset of larvae that were collected as survivors on Bollgard II® we have leaf samples from the host and surrounding plants. We used qualitative ELISA tests to analyze this material for the presence or absence of Cry1Ac and Cry2Ab. Note that our analyses were performed on a biased sample of because only plants supporting larvae were selected and tested.

In 2006/07 a total of 7 samples of the 168 tested scored negative for Cry1Ac or Cry2Ab. In one sample from the Lachlan valley the same leaf tested negative for Cry1Ac and Cry2Ab. In one sample from the Lower Namoi the leaf tested negative for Cry1Ac and positive for Cry2Ab. In 5 samples from St George the leaf tested positive for Cry1Ac and negative for Cry2Ab.

Our data suggest that of the samples taken from Bollgard II® plants that were hosts, or nearby hosts, of the collected surviving Helicoverpa larvae, at least one of the two Bt proteins was present in 99.4% of cases, Cry1Ac was absent and Cry2Ab was present in < 1% of cases, and Cry1Ac was present and Cry2Ab was absent in 2.9% of cases.

In 2007/08 a total of 4 leaf samples of the 295 tested scored negative for Cry1Ac or Cry2Ab. In the samples from the Macintyre valley the same leaf tested negative for both Cry1Ac and Cry2Ab. In two samples from the Lower Namoi and one sample from St George the plants tested positive for Cry1Ac and negative for Cry2Ab.

Our data suggest that 98.6% of the Bollgard II® plants that were hosts, or nearby hosts, of the sampled surviving Helicoverpa larvae, contained at least one of the two Bt proteins. Only one plant did not contain both proteins. No plants contained Cry2Ab only.

TABLE 10: The number of Bollgard II® host plants containing surviving larvae that scored positive for the* cry1Ac *or* cry2Ab *gene using qualitative ELISA. Data for each season have been summarised according to valley and separately for the two toxins. The total leaf samples include those that were the host of the larvae at the time of collection and those that immediately surrounded the host plant. We scored a sample as negative only if duplicate samples from that leaf proved negative.

Year	Valley	Total leaf samples	No. positive samples	
			Cry1Ac	Cry2Ab
2006/07	Gwydir	7	7	7
	Macintyre	68	67	67
	Lower Namoi	32	32	30
	St George	188	188	187
	Total	**295**	**294**	**291**
2007/08	Lower Namoi	4	3	4
	St George	159	159	154
	Lachlan	5	4	4
	Total	**168**	**166**	**162**

Assisting CSE107C staff in night collects of mating moths

Ms Trudy Staines, the technician appointed to this project, assisted CSE107C staff in night trapping surveys conducted to capture mating moths above Bollgard II® crops. As part of CSE107C the moths will be analysed using carbon isotope ratios to determine plant host origin, and thus determine the extent of cross-mating that occurs between Helicoverpa moths from different crop origins. Ms Staines also assisted CSE107C staff in regular surveys of invertebrates (pests and beneficial species) in refuge crops and their associated Bollgard II® crops within the Namoi Valley, to monitor their on-going efficacy. These results will be reported in full within the final report for CSE107.

Outcomes

Mortality of Helicoverpa in a Bollgard II® landscape

The main experiment performed in this project has provided empirical data on the relative field survival of Helicoverpa larvae in a number of crops that are common in the current Bollgard II® landscape. It also provided empirical data on the impact of standard commercial spraying practices in Bollgard II® on this survival through a direct comparison with unsprayed Bollgard II®. These data will be utilised to inform the industry's practise of IPM and contribute to the 'fine-tuning' of the current RMP for transgenic cotton.

Surviving larvae on Bollgard II® cotton

The co-ordination of collections of surviving Helicoverpa larvae from Bollgard II® crops for Bt testing is critical for understanding why threshold levels of this pest may sometimes occur and how this situation may be managed. In collaboration with CSE112 we have data which demonstrates that Bt resistance is not the mechanism allowing these larvae to survive on Bollgard II®. This knowledge contributes to our understanding of IPM, and the evolution of resistance. In particular it will directly contribute to our evaluation at REFCOM 2009 of the effectiveness of the current RMP for Bt cotton.

Assisting CSE107C staff in night collections of mating moths

The outcomes from this work will be reported in the final report for CSE107C.

Please describe any:

a) **Technical advances achieved:**

This project is not of a technical nature thus there were no commercially significant developments, patents applied for or granted licenses arising from this work.

b) **Other information developed from research (e.g., discoveries in methodology, equipment design, etc.):**

A number of novel methods were developed during this project and have been outlined in detail this report.

c) **Required changes to the Intellectual Property register:**

No changes to the IP register are required.

Conclusions

Mortality of Helicoverpa in a Bollgard II® landscape

- It is possible to use the ‘closed’ versus ‘open’ tents described herein to experimentally manipulate the exposure of larvae to arthropods.
- It is possible to use the methods described herein to non-destructively sample larvae and arthropods such that the former can be reared to examine rates of parasitism.
- Survival of Helicoverpa larvae differs significantly across the main crops employed in the current Bollgard II® landscape and the particulars of this trend differ among small and medium larvae.
- Survival of small larvae is greater in unsprayed pigeon pea and Bollgard II® cotton that is sprayed for sucking pests and mites compared with unsprayed conventional cotton and unsprayed Bollgard II® cotton. There is no significant difference in survival between unsprayed conventional cotton and unsprayed Bollgard II® cotton, and between unsprayed pigeon pea and sprayed Bollgard II® cotton.
- Survival of medium larvae is greater in unsprayed pigeon pea compared with unsprayed conventional cotton and unsprayed Bollgard II® cotton. There is no significant difference in survival for the remaining pairwise comparisons including unsprayed Bollgard II® cotton and Bollgard II® cotton that is sprayed for sucking pests and mites.
- For medium larvae the differential survival across crops is consistent across the period during which these crops are attractive (December until end February). However, for smaller larvae the differential survival across crops holds during mid and late season but early in the season there is no difference in survival across the four crop types.
- The similar levels of survival in Bollgard II® cotton and conventional cotton that are not sprayed is intuitive based on previous work demonstrating similar communities of natural enemies (and hence opportunities for predation and parasitism) in these crop types. The higher survival in pigeon pea (for both size classes) and Bollgard II® cotton that is sprayed for mites and sucking pests (for small larvae) suggests that these crops may have fewer natural enemies compared with unsprayed Bollgard II® and conventional cotton.
- The survival results also suggest that spraying Bollgard II® fields for mirids and mites may reduce the abundance of natural enemies (relative to unsprayed Bollgard II® fields), and that this process affects mortality of smaller larvae but not larger larvae. It is possible, for example, that the sprays reduce numbers of non-target predators that specialise on small larvae.
- These suggestions are supported by data collected across replicate fields.
- In particular, spiders appear to play a significant role in Helicoverpa mortality. For small and medium larvae there was a strong negative relationship between survival and the abundance of spiders in open tents but not with any of the other categories of predators (arthropods <5mm, arthropods 5mm or >, ants, ladybeetles) or larval parasites. Moreover, across replicate sampled fields of the different crops the abundance of spiders mirrored the mortality of larvae within open tents in the same crop types.
- For small larvae there was also a strong positive relationship between survival and the abundance of small non-predatory arthropods. In addition, across replicate sampled fields of the different crops the abundance of small non-predatory arthropods opposed the mortality of larvae within open tents in the same crop types. These results suggests that the presence of alternative small prey may improve survivorship of small larvae.

Surviving larvae on Bollgard II® cotton

- During the past three seasons, surviving larvae have been found in Bollgard II® fields on some properties in all of the main cotton growing valleys. These problems with survivors are restricted to fields that are at the mid to late flowering stage.
- In collaboration with CSE112 we have determined that Bt resistance is not the mechanism allowing these larvae to survive on Bollgard II®.
- Of the larvae submitted to the program, smaller individuals are less likely to emerge in the laboratory as healthy moths. This differential survival may reflect exposure of a second cohort of eggs, that were collected as small larvae, to a plant that was recovering from a temporary dip in Bt expression.
- There is a significant difference among seasons in the proportion of the collection of survivors that was *H. armigera.*
- Of the survivors that we sampled in the field, small larvae are more evenly distributed among squares, flowers and bolls compared to larger larvae which were found most often on bolls compared with squares and flowers.
- Qualitative ELISA tests demonstrate that of the samples taken from Bollgard II® plants that were hosts, or nearby hosts, of the collected surviving Helicoverpa larvae, at least one of the two Bt proteins was present in 98.6% of cases. Thus, the absence of Bt proteins in the host or surrounding plants is not the mechanism allowing these larvae to survive on Bollgard II®.

Assisting CSE107C staff in night collections of mating moths

- The conclusions from this work will be reported in the final report for CSE107C.

In-kind support

We would like to acknowledge the significant in-kind support provided to this project from Auscott Farms Narrabri, in particular Mel Crocker and Henry Taylor. From October until April in 2006-07 and 2007-08 we spoke several times a week with Mel or Henry to get updates on the agronomy of the fields that we worked on at Auscott. During that period we estimate a total contribution of their time at 30mins per week.

Extension Opportunities

Detail a plan for the activities or other steps that may be taken:

(a) To further develop or to exploit the project technology:

This project is not of a technical nature.

(b) For the future presentation and dissemination of the project outcomes:

The outcomes of the mortality of Helicoverpa experiment and survivors on Bollgard II® components of this project will be disseminated to industry via written articles in the Australian Cotton Grower and oral presentations at appropriate industry forums (e.g., field days, CCA AGM, etc). In particular, a presentation will be given at the REFCOM meeting in 2009, which is a key forum for discussing issues around Bt resistance. I will also give an informal presentation of the work to our collaborators at Auscott Narrabri which will present a general overview as well as results that are specific to this property.

(c) For future research:

Our ideas for future research in this area were detailed in an EOI to the CCC CRC in June 2007, which has been appended to this report.

Publications

Refereed articles in popular science magazines and industry publications

Baker G, Tann C, Downes SJ, 2008, Research comments: Entomology, Cotton Seed Distributors Trial Book, pp. 49-52

Downes SJ, Wilson L, Kauter G, Farrell T, 2008, Preamble to the Resistance Management Plan (RMP) for Bollgard II® for 2008-2009, Cotton Pest Management Guide (Ed T Farrell), pp. 39-46

Downes SJ, Rossiter L, Parker T, McKenzie F, Staines T, 2007, How to collect *Helicoverpa* for resistance testing. The Australian Cottongrower Dec-Jan 28:48-49

Baker G, Tann C, Downes SJ, 2007, Research comments: Entomology, Cotton Seed Distributors Trial Book, pp. 45-47

Downes SJ, Wilson L, Kauter G, Farrell T, 2007, Preamble to the Resistance Management Plan (RMP) for Bollgard II® for 2007-2008, Cotton Pest Management Guide (Ed T Farrell), pp. 36-44

Downes SJ, Mahon R, Parker T, Staines T, 2006, WANTED ALIVE: Large *Helicoverpa* larvae from Bollgard II® plants. The Australian Cottongrower Dec-Jan 27:8-10

Downes SJ, Wilson L, Kauter G, Farrell T, 2006, Preamble to the Resistance Management Plan (RMP) for Bollgard II® for 2006-2007, Cotton Pest Management Guide (Ed T Farrell), pp. 37-45

Presentations at scientific meetings and industry forums and seminars

Lu B, Downes SJ, Wilson L, Gregg P, Kauter G, Knight K, 2008, Survivors in Bt cotton: how do they survive, and what damage do they cause? Cotton Catchment Communities Conference, Narrabri, October

Downes SJ, Mahon R, Parker T, Lu B, 2008, The changing Bt resistance landscape. Australian Cotton Conference, Gold Coast [Invited Plenary Lecture]

Downes S, Mahon R, 2008, Bt resistance update, Cotton Consultants Australia Cotton Production Seminar, Narrabri, May

Downes SJ, 2008, Is Helicoverpa developing resistance to Bt-cotton?, *Australian Cotton Research Institute*, May

Downes S, Mahon R, 2008, Survivors on Bollgard II® cotton, Transgenic and Insect Management Strategy Resistance Roadshow, Emerald, Dalby, St George, Goondiwindi, Moree, Narrabri, Hillston, Warren, 26-30th May

Downes SJ, 2008, Bt resistance update and survivors on Bollgard II, Lower Balonne Field Day, 12th March

Lu B, Downes SJ, Wilson L, Gregg P, Kauter G, Knight K, 2007, Spray thresholds and mechanisms of survival for Bt-susceptible *Helicoverpa* living on Bollgard II® cotton. Cotton Catchment Communities Conference, Narrabri, August

Downes SJ, 2007, Adaptive resistance management of GM cotton, University of Melbourne, Department of Zoology, May

Downes S, Mahon R, 2007, Bt resistance update, Cotton Consultants Australia Cotton Production Seminar, Narrabri, June

Downes SJ, Mahon R, 2006, Monitoring for resistance to Bt-cotton in Australia: current status and future challenges. Combined meeting of the IX International Colloquium on Invertebrate Pathology and Microbial Control, XXXIX Annual Meeting of the Society of Invertebrate Pathologists and VIII International Conference on *Bacillus thuringiensis*, Wuhan, China [Invited presentation in the symposium on Monitoring and Managing for Bt-Resistance: The Challenges for the Next Decade]

Downes SJ, Mahon R, 2006, Resistance and refuge. Australian Cotton Conference, Gold Coast [Invited Plenary Lecture]

Online resources

No online resources have been developed from this project, although information from the survivors on Bollgard II$^{®}$ component of this project is included in the end of month and end of season Bt resistance monitoring reports which are on the CRC web site under “publications”.

http://www.cottoncrc.org.au/content/Industry/Publications/Pests_and_Beneficials/Insect_Resistance_Management.aspx

Part 4 – Final Report Executive Summary

To prolong the utility of Bollgard II® against *H. armigera*, growers that use this tool must follow a resistance management plan (RMP). This strategy is largely based on information from studies of the ecology and population genetics of *H. armigera*, and the outputs of computer simulation models that use biological information to predict the likelihood of resistance under different scenarios. These models assume that any individuals which are resistant to Bollgard II® survive to successfully reproduce in cotton landscapes.

In this project we developed novel methods to determine in the field how natural enemies in Bollgard II® versus unsprayed refuge crops affect the probability that *Helicoverpa armigera* will survive from hatching until adulthood. Part of this objective was testing how the application of pesticide in Bollgard II® fields to control sucking pests affects natural enemy communities and, in turn, survival of *H. armigera*. A secondary objective was to co-ordinate the collection of surviving Helicoverpa larvae from Bollgard II® crops and rear them for inclusion in the Bt resistance monitoring program.

Survival of Helicoverpa larvae differed significantly across the main crops employed in the current Bollgard II® landscape but the particulars of this trend differed among small and medium larvae. Survival of small larvae was greater in pigeon pea and Bollgard II® cotton that was sprayed for sucking pests and mites compared with conventional cotton and unsprayed Bollgard II® cotton. However, this trend held during mid and late season but early in the season there was no difference in survival across the crops. Survival of medium larvae was greater in pigeon pea compared with conventional cotton and unsprayed Bollgard II® cotton and this trend was consistent across the period during which these crops are attractive.

The similar survival in unsprayed Bollgard II® and conventional cotton is intuitive based on past work showing similar communities of natural enemies in these crops. The higher survival in pigeon pea (for both size classes) and Bollgard II® cotton that is sprayed (for small larvae) suggests that these crops may have fewer natural enemies compared with unsprayed Bollgard II® and conventional cotton. The survival results also suggest that spraying Bollgard II® fields for mirids and mites may reduce the abundance of natural enemies (relative to unsprayed Bollgard II® fields), and that this process affects mortality of smaller larvae. It is possible, for example, that sprays reduce numbers of predators that specialise on small larvae. These suggestions are supported by data on arthropod communities across replicate fields.

In particular, spiders appear to play a significant role in mortality. For small and medium larvae there was a strong negative relationship between survival and abundance of spiders in open tents but not with any other category of predators (arthropods <5mm, arthropods 5mm or >, ants, ladybeetles) or parasitoids. Moreover, across replicate fields the abundance of spiders mirrored the mortality of larvae in open tents in the same crops.

For small larvae there was a strong positive relationship between survival and the abundance of small non-predatory arthropods. In addition, across replicate fields the abundance of these arthropods opposed the mortality of larvae in open tents in the same crops. These results suggest that alternative small prey may improve survivorship of small larvae.

During the past three seasons, surviving larvae were found in Bollgard II® fields on some properties in all main cotton valleys. We determined (through collaboration with CSE112) that Bt resistance, or the absence of Bt proteins in the host or surrounding plants, is not the mechanism allowing these larvae to survive on Bollgard II®.

This information will be utilised in an upcoming forum to assist with reviewing the current Resistance Management Plan for Bt cotton.

For more information contact: Sharon Downes (Sharon.Downes@csiro.au), Geoff Baker (Geoff.Baker@csiro.au) or Rod Mahon (Rod.Mahon@csiro.au), CSIRO Entomology.

Research Project Expression of Interest

Please complete this form (strictly two pages only) for each project and email to **Lynda.George@cotton.crc.org.au**
Closing date for Expressions of Interest: **Friday 29th June 2007.**

Contact Details

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Project Details

Which CRC Program?	**Which CRC goal?**
Program 1 – The Farm	Enable the cotton industry to improve profitability and sustainability of production by reducing pesticide use by 50% through IPM for insects, weeds & diseases.

Brief Description of Project (½page max):

In the face of widespread reliance by the Australian cotton industry on Bt technology, a concerted effort is required to maintain expertise around Integrated Pest Management tactics for Helicoverpa and continue development of alternative chemical and biological control measures. This project aims to consolidate and extend previous work in this area. The proposed work is comprised of four parts, and though herein it is written as an integrated project, it may become more than one project or its components may become objectives within other larger projects.

1. Relative mortality of resistant versus susceptible Helicoverpa larvae. This work extends a current study by Sharon Downes which examines the impact of beneficials on the survival of *H. armigera* larvae in common crops within a Bollgard II landscape. As part of that work she examines the survival of 'resistant' larvae (i.e., Bt-susceptible larvae on non-Bt cotton plots) within Bollgard II crops. Baoqian Lu will examine the impact of beneficials on the survival of non-resistant Bollgard II survivors (i.e., Bt-susceptible larvae on Bt-cotton). The work proposed herein is to simultaneously determine the relative survival of non-resistant Bollgard II survivors versus 'resistant' Bollgard II survivors. This is a critical next step because, for example, slower development rate, increased mobility, and reduced vigour of susceptible larvae could increase exposure to enemies and enhance biotic mortality, thereby favouring survival of resistant larvae.

2. Foraging behaviour and movement of predators and parasitoids in Bollgard II. Good information exists on the role of key predators and parasites of Helicoverpa in conventional cotton systems where densities of larvae are often high. In contrast, the densities of Helicoverpa pests in Bollgard II crops are typically low. A key question is whether levels of predation and parasitism are maintained, increased or decreased, in Bollgard II crops. The proposed work would determine the foraging behaviour of common predators (using for e.g. the Halger ELISA method) and parasitoids (using sentinel larvae) of Helicoverpa in Bollgard II under the changed conditions of low prey/host densities. An essential component is determining the distances that these natural enemies will move into Bollgard II from surrounding sources (i.e., refuges and other non-cotton hosts). These studies will focus on the predators and parasitoids rather than the host. In particular, some of the predator work will be relevant to prey apart from Helicoverpa (e.g., mirids).

3. Natural enemy activity and thresholds for spraying Helicoverpa larvae in Bollgard II. Baoqian Lu will conduct work on threshold levels for spraying Helicoverpa larvae in Bollgard II fields. The value of this work will be significantly improved by incorporating information on how the composition and abundance of natural enemies of Helicoverpa larvae impact on these thresholds. A related issue for Bollgard II is how sprays for Helicoverpa impact on the development of secondary pests, and how sprays for sucking pests impact on Helicoverpa control.

4. Evaluating selective chemical options to control Helicoverpa. This component extends the current work by Lewis Wilson which examines the fit of new chemistry for Helicoverpa (or other pests) into IPM, including routine screening to examine gross non-target effects. It also involves the expansion of current work by Robert Mensah that examines the use of plant extracts and biopesticides in Bollgard II crops to manage Helicoverpa species.

N.B. There is potential overlap between some work in 1 and 3 and a recently commenced PhD study by Baoqian Lu. It will be necessary to track Baoqian's progress on these issues throughout the funding process to ascertain whether they remain 'gaps' in our knowledge and/or if some of this work can be a collaborative effort with Baoqian. The current portfolio for Baoqian's work is larger than a single PhD. It will be equally important to identify the elements that he does not examine and consider whether they should be covered as new objectives within this and related EOI's.

Approximate budget:
(Note: we are only seeking indicative budgets at this stage, especially in regard to in-kind. We do not expect these estimates to be approved by providing organisations.)

	2008/09 $	**2009/10 $**	**2010/11 $**
Cotton CRC	**190,000**	**195,000**	**200,000**
Other sources			
In-kind	**200,000**	**205,000**	**210,000**

List any collaboration with other organizations or projects:

This project has been developed from the CRC Insect Ecology and Management Workshop in Toowoomba, 21-22 June 2007. Collaborators identified at that Workshop (and the components of the project they would be involved in; see above) are: Sharon Downes (CSIRO Ent.) (components 1 and 3); Dave Murray (QDPI) and Mary Whitehouse (CSIRO Ent.) (component 2); Robert Mensah (NSW DPI) (components 3 and 4); Lewis Wilson (CSIRO PI) (components 3 and 4); and Peter Greg (UNE/CRC) (component 3). The proposed work by Robert Mensah within component 4, component 2 by Mary Whitehouse, and components 3 and 4 involving Lewis Wilson, are linked with the EOI around Mirid IPM co-ordinated by Moazzem Khan. Component 2 of Peter Gregg's EOI around refuges falls within the topic of this application but has been retained in the former to streamline administration.

Science and industry outcomes in relation to CRC Program goals (1/2 page max.)

The long-term sustainability of Bt-cotton is threatened with development of resistance by the targeted Helicoverpa pests. In the face of this risk, and with an increasing reliance on Bt technology, it is critical that the industry maintains conventional control techniques, skills, and products. The unexplained occurrence of apparently susceptible Helicoverpa larvae on some fields of Bollgard II, and the unexpectedly high baseline frequency of alleles conferring resistance to Cry2Ab in this species, highlights and adds to this need. All components of this proposal address issues that are critical to preserving IPM tactics for Helicoverpa species, and were highlighted as priority areas for future research through a recent process of industry prioritisation.

Component 1 provides important information on the level of control of Helicoverpa larvae due to natural enemies in Bollgard II crops. It also addresses the issue of differential survival of resistant versus susceptible larvae which is critical for understanding the potential for the evolution of resistance of Bollgard II cotton. Component 2 provides important new information on the performance of key predators and parasitoids of Helicoverpa in a low prey/host environment. This information could, for example, inform the merit of augmenting certain enemies within specific crops. Component 3 will provide growers with sound recommendations on thresholds for spraying Helicoverpa in Bollgard II that consider the impacts of natural enemies, and the potential consequences for flaring secondary pests. Component 4 is critical to maintaining capacity to selectively apply 'soft' chemistry to control Helicoverpa (or other pests). In addition, the plant extract and biopesticide work will enable the development of new tools that support beneficial insect activity, and thus will enable growers to use IPM on Bollgard crops to avoid flaring secondary pests.

Sharon Downes has budgeted for the work in component 1 elsewhere through a different configuration of key tasks (see component 3 on the EOI around the technical expertise of Ms Trudy Staines). The work proposed in component 2 would benefit from parallel studies conducted on the Darling Downs and elsewhere (i.e., Namoi/Gwydir) and has been budgeted at $50K per annum for 3 years to support technical staff at QDPI. The work by Mary Whitehouse as part of component 2 has been budgeted within the EOI on Mirid IPM. The QDPI study within component 2 has the potential to form the basis of a postgraduate student project. Robert Mensah's work within component 4 will support the salary of Ms Kylie May at around $75K per annum for three years. Lewis Wilson's work within components 3 and 4 will require $65K per annum for 3 years which includes a contribution to the salary of Ms Simone Heimoana.

Proposed pathway to adoption and any commercial potential (1/2 page max.)

For most of the work the main pathway to adoption would be to provide the results from the work to growers in the form of advice and recommendations on IPM. The work on relative mortality of resistant versus susceptible Helicoverpa larvae also has potential to influence the current RMP for Bollgard II cotton. Variations to that plan, as approved by the APVMA and OTGR, would be the main pathway to adoption.

Assistance from the CRC's Insect and Weed National Priority Team, and the new Regional Extension Officers, will be sought to disseminate the information. In addition, the research and its implications will be communicated to stakeholders through relevant articles in industry magazines such as *The Australian Cotton Grower* and *Spotlight*, as features on the web sites of the CRDC and CRC, and via presentations at various relevant meetings (i.e., CCA Cotton Production Seminar, REFCOM meetings, CSD Science Review, CRC Science Forum, ACGRA conference, etc). The work will also be disseminated as papers in refereed scientific journals and at relevant national and international conferences.

There is no commercial potential for any of the component parts of this project.