If you are participating in the presentations this year, please provide a written report and a copy of your final report presentation by 31 October.
If not, please provide a written report by 30 September.

Part 1 - Summary Details

Please use your TAB key to complete Parts 1 & 2.

CRDC Project Number: **DAN 196**

Project Title: Helicoverpa insecticide resistance monitoring, management and biochemical/molecular research

Project Commencement Date: 1st July 2008 **Project Completion Date:** 30th June 2011
CRDC Program: 3 Crop Protection

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1. Background

Helicoverpa armigera and *H. punctigera* are key economic pests of cotton, pulse and grains crops, and chemical insecticides have traditionally provided the key management tool for control of these pests. While *H. punctigera* has maintained a susceptible status, *H. armigera* has repeatedly demonstrated the ability to develop resistance to conventional insecticidal chemistries, putting at risk the effectiveness of some of the most useful control measures available in agricultural industries. The introduction of transgenic cotton varieties has reduced the dependence on insecticides for control of *H. armigera* and has provided an opportunity to reduce the environmental footprint of cotton production. Nevertheless, chemical insecticides remain the primary method of pest control in sprayed non-transgenic cotton, as an alternative to Bollgard II, or as a refuge for Bollgard II, (as well as other viable and important summer cropping options).

Resistance represents the largest threat to the continued efficacy of conventional chemistries. In addition, selection pressure for Bt resistance is strong, due to the widespread uptake of transgenic cotton, and Bt resistance monitoring has shown higher than expected resistance frequencies in Helicoverpa. Hence, insecticide resistance research, incorporating monitoring and management, is essential to ensure the risk of resistance development in *H. armigera* is minimised, and ensure the viability and sustainability of conventional insecticides in cotton and other crops within a mixed cropping system.

Resistance monitoring and active management has been conducted since pyrethroid resistance was first detected at the field level in 1983. Within the mixed cotton/grains/pulse farming system, resistance monitoring and research continues to be important as part of an integrated approach to pest control, with the system currently dominated by both Bollgard II cotton and increasingly large plantings of pulses and grains. Resistance monitoring and management has ensured viability of conventional pest control options and has provided information for formulation and implementation of insecticide resistance management strategies (IRMS) followed by the cotton industry, with benefits for insect control in grains and pulse crops. Helicoverpa monitoring and management schedules between 2008 and 2011 included twelve chemicals in ten groups. The schedule included older broad-spectrum chemistries as well as the newer more selective insecticides which are highly compatible with integrated pest management approaches.

This project was concerned with the incidence and cause of conventional insecticide resistance in *H. armigera* and, to lesser extent, in *H. punctigera*. Research was focussed on monitoring resistance across all cotton growing regions in the eastern Australian cotton belt in order to detect emerging resistance problems. Information generated from the monitoring program was also used to design and implement management responses, incorporating pests and chemical use within a mixed grains/pulse/cotton farming system. Research also focussed on selecting resistance genes from the field, and on the underlying mechanisms of resistance in *H. armigera*, particularly in relation to understanding of the role of esterases in resistance.

The project operated at base level from March 2010 to April 2011, focussing predominantly on insecticide resistance monitoring. Staff vacancies existed at the research scientist and technical officer level for much of the duration of this project. Dr Louise Rossiter relocated to Orange in March 2010, while technical officer Fiona McKenzie resumed duties two days a week in January 2011 following a period of maternity leave. These staff absences precluded further research and support associated with several objectives in this project, relating particularly to characterisation of resistance mechanisms in *H. armigera*.

2. Objectives and achievements

1. Co-ordinate egg collections for annual resistance monitoring

Local collection teams were assembled and sampling commenced in mid-spring predominantly in chickpea crops and from uncultivated hosts such as roadside weeds. Areas sampled included the upper and lower Namoi valleys extending out to Walgett. Local sampling for early season populations extended to the Gwydir valley from November onward. Host crops sampled included cotton (transgenic and conventional), maize, pigeon pea, sorghum, sunflower, mungbean and soybean.

Dedicated collectors for areas remote to Narrabri were organised in collaboration with Dr Sharon Downes, CSIRO, involving either liaising with the RCEO in a particular area, or directly with consultants involved with collecting activities. Sampling from remote locations commenced from mid October / early November (Emerald and Darling Downs) to mid November (all other areas). The host crop sampled were predominantly cotton (transgenic and conventional), with smaller proportions of collections sourced from pigeon pea, maize, sunflower, chickpea, sorghum and mungbean.

2. Conduct annual insecticide resistance monitoring

Insecticide resistance monitoring for *H. armigera* and *H. punctigera* was successfully conducted on field collected material from most cotton growing regions during the 2008/09, 2009/10 and 2010/11 cotton seasons. Attempts were made to include all chemistries registered for use where practicable, however where limited numbers of *H. armigera* were available, emphasis was placed on the newer softer chemistries due to their widespread use as part of beneficial preservation and IPM.

3. Annually formulate and promote improved resistance management guidelines and strategies

Resistance monitoring results were analysed, interpreted and presented to the Transgenic and Insect Management Strategy (TIMS) Committee as part of assessing the success of the IRMS. Dr Rossiter was involved in formulating improved resistance management guidelines incorporating secondary pests and crops other than cotton in her role as a member of the TIMS Insecticide Resistance Technical Panel and TIMS BT Technical Panel.

The IRMS and its basis and were promoted through a number of oral and written presentations (see Section 7)

4. Establish insecticide resistant Helicoverpa spp. *colonies using field survivors for use in further resistance research.*

Insects found to be resistant to key chemistries through the monitoring program were to be reared to adults and either mated with each other where multiple resistant insects were found (of both sexes), or single pair mated with lab reared susceptible insects, with subsequent selection and outcrossing events to establish a resistant colony. Achievement of this objective was therefore largely dependent upon the detection of resistant individuals to key insecticides of interest in the monitoring program. Only 1-2 survivors at most to these insecticides were detected in each of the sampling seasons. Efforts to establish resistant colonies from these survivors were met with little success, due to the difficulties in rearing these successfully to adults and then achieve success in single pair matings.

Upon the appointment of the replacement researcher, selection experiments were conducted for indoxacarb resistance in *H. armigera* by mating indoxacarb survivors from F_0 screens together, or by outcrossing F_0 survivors to a susceptible strain and reselecting in the F_2. F_2 screening for indoxacarb resistance was undertaken to investigate the presence of recessive genes that may confer resistance to this insecticide. This involved the use of a discriminating dose to screen F_2 progeny from individuals collected from the field and single-pair mated with moths from a laboratory susceptible strain of *H. armigera*. Selection for indoxacarb resistance also involved low-level selection using sub-lethal doses of insecticide. The aim of this process is to establish resistant and susceptible strains of *H. armigera* to be used in resistance characterisation and cross-resistance studies.

5. *Investigate the features of resistance to key insecticides using resistant colonies established from field survivors*

Due to unsuccessful attempts to establish resistant colonies from field survivors, as well as the absence of a research scientist in this project from March 2010 to April 2011, investigations into inheritance of resistance to insecticides were limited. With the appointment of a replacement scientist in April 2011, research to select for resistance to newer chemistries has recommenced with the aim of subsequent work in the future to characterise the resistance mechanism and its mode of inheritance

6. *Investigate potential cross resistance between insecticides*

Cross-resistance can significantly influence the rate at which resistance will develop in a population. Following the appointment of a replacement researcher in April 2011, studies were initiated to establish patterns of cross-resistance in *H. armigera* between Bt toxins and conventional insecticides utilising Bt resistant strains established by CSIRO, and the newly established pyrethroid resistant strain, KOS. The dose-responses of insecticide resistant strains and a susceptible strain were to be tested against seven conventional insecticides and two Bt toxins.

7. *Using appropriate bioassay techniques, accumulate baseline dose-response data for new Helicoverpa insecticides.*

The dose-response to a number of new chemistries with potential for registration against Helicoverpa were investigated between June 2008 and March 2010. These included pyridalyl, and cyazypyr. Dose response assays for pyridalyl were discontinued in 2008/09 when it was confirmed Sumitomo would not seek registration of this chemical. Preliminary assays in 2009/10 also commenced for cyazypyr (DuPont).

8. *Characterise esterase protein produced by gene isolated from H. armigera and assess role in resistance*

While confirmation of the full length esterase sequence isolated from *H. armigera*, allowed for initiation of the characterisation of this gene and its protein product, the absence of a research scientist in this position from March 2010 resulted in the discontinuation of this research.

9 *Conduct further molecular investigations of esterase properties in H. armigera*

The full length esterase cDNA gene sequence from pyrethroid susceptible (S) and resistant (R) larvae were determined using a high fidelity polymerase on. This allowed for final confirmation of the esterase gene sequence previously identified in DAN 193. The gene sequence between resistant and susceptible larvae was also found to be different at a number of sites, resulting in coding for alternative proteins on 5 occasions. The implications for resistance of these point mutations are discussed.

10. Support other Helicoverpa related research projects with field collected material and filed collected laboratory reared insect colonies

Field collected laboratory-reared insect colonies were made available for use by several other Helicoverpa related projects.

3. Methods

1. Insect collection and laboratory rearing

The *Helicoverpa* resistance monitoring program relies on the collection of insect eggs for lab rearing and resistance testing. Eggs are preferable as they can be collected and transported with relative ease. Unlike larval populations, eggs are abundant in both sprayed conventional and transgenic cotton, and the risk of losing material due to disease and parasitism is reduced. The disadvantage of egg collections is the time delay in rearing larvae to the appropriate size for bioassay (30-40 mg 3rd or 4th instars). In addition, *H. armigera* eggs cannot be distinguished from *H. punctigera* eggs. Moreover, Trichogramma parasitised white eggs cannot be identified. These factors can have significant affects on numbers of *H. armigera* available for testing. *H. armigera* has a demonstrated ability to develop field resistance to insecticides. Therefore, monitoring efforts are concentrated on *H. armigera*, with *H. punctigera* included to a lesser extent and screened against only the most relevant chemistries used specifically to target this species.

Despite the disadvantages associated with collecting eggs for resistance monitoring purposes, they remain the preferred collection material because of the high incidence of parasitism and disease which contribute to high levels of mortality in larval field collections

Helicoverpa eggs were collected across all key cotton growing districts across the Australian cotton belt by a central team of collectors working out of ACRI Narrabri, as well as CRDC-supported collection teams co-ordinated by CCA representatives in remote locations. Regular sampling was conducted in the upper and lower Namoi valleys and Gwydir valley by local collectors. Sampling by dedicated collectors in NSW was undertaken in the Macquarie and Lachlan Valleys as well as in the Mungindi district. The Mungindi area was of particular interest as this area had maintained a reasonable proportion of conventionally sprayed cotton, relative to most other cotton growing regions where Bollgard II cotton dominated plantings. Sampling in southern Queensland covered the Macintyre valley, the Darling Downs, and St George. Sampling in central Queensland was concentrated around the cotton growing area of Emerald.

Specific collecting protocols and guidelines were distributed to all collectors together with all equipment required for collecting and sending eggs to ensure collections were conducted to our requirements and arrived at ACRI in optimum condition.

Sampling regimes had little spatial or temporal structure. This is because egg pressure can be highly variable in terms of where and when eggs are laid. Collections were nevertheless, co-ordinated as much as practicable to be representative of a particular region and to include as many time points from within a season as was logistically achievable. Sampling operations were also aimed at incorporating all Helicoverpa hosts from within a mixed cropping system, including conventional and transgenic cotton, pigeon pea, maize, sorghum and other attractive crops, as well as uncultivated hosts such as weed species.

Egg collections were sent to the central testing laboratory at ACRI and transferred to artificial soy flour based diet (modified from Teakle and Jensen, 1985), contained in 12-well Linbro tissue culture trays. Larvae were reared at 25 ºC with a photoperiod of 14h light : 10h dark until they reached the appropriate size for testing (30-40 mg 3rd or 4th instar larvae). Prior to testing, larvae were speciated using established visual identification techniques. Larvae that were not tested directly in the F_0 generation were reared to pupae. Pupae were washed in 0.1 % bleach solution and transferred to 5 L round containers with honey solution as a food source for emerging adult moths. Adults were maintained at 25 °C and 70 % relative humidity with a photoperiod of 14 hr light : 10hr dark. Eggs were laid on cloth liners which were replaced three times per week. Harvested eggs were placed in plastic bags and allowed to hatch at 25 °C. Neonates were transferred to artificial diet in order to establish an F_1 strains for resistance testing.

2. Insecticide Resistance Monitoring

Resistance monitoring was conducted using established techniques described by Forrester *et al.* (1993), covering all chemistries used against Helicoverpa. Where adequate numbers permitted, individual collections were split in order to screen the full compliment of insecticidal classes. Where insect material was limited the newer, more selective chemistries with a key role in IPM systems were prioritised for testing. For *H. armigera*, which has historically presented the greatest resistance threat, these included indoxacarb (Steward®), spinosad (Tracer®) and emamectin benzoate (Affirm®), with the former two registered for use in a number of grains and pulse crops. Other insecticides tested included endosulfan, methomyl (carbamate), chlorpyrifos (organophosphate), bifenthrin and fenvalerate (pyrethroids). Chemistries included in the resistance monitoring schedule for *H. punctigera* were abamectin, endosulfan and pyrethroid (fenvalerate) which are the only chemistries with baseline data and discriminating doses established, and which are highly relevant for this species.

Larvae were tested for resistance at the 3rd or 4th instar within a weight range of 30-40 mg by a topical discriminating dose bioassay, using a discriminating dose ($LD_{99.9}$) and time to assessment as determined by previous research projects. Bioassays were performed by topical application of 1 μL of acetone dissolved technical grade insecticide to the dorsal surface of insects. Larvae were held at 25 ºC for 2-4 days, with mortality assessed at a time dependent on the individual insecticide. Insects were considered dead if they could not demonstrate co-ordinated movement when prodded from behind. Insects that survived a discriminating dose of insecticide were reared through to adults and used to establish a colony to confirm resistance status and for use in mechanism studies.

3. Resistance Management Strategy Formulation and Promotion

IRMS formulation and promotion involved the Transgenic and Insect Management Strategy (TIMS) committee. The formulation of resistance guidelines incorporated the resistance monitoring data and accounted for other pests of cotton and associated resistance issues. Before her departure, Dr Rossiter was involved in formulating improved resistance management guidelines incorporating secondary pests and crops other than cotton in her role as a member of the TIMS Insecticide Resistance Technical Panel and TIMS Bt Technical Panel. The promotion of resistance management involved various written and oral presentations (see Section 7).

4. Establishment of resistant H. armigera colonies

Field derived insects that survived discriminating dose bioassays used for resistance monitoring were reared to adults and either mated with other survivors (where both sexes were available), or single pair mated with laboratory-reared susceptible insects, with subsequent selection and outcrossing to establish a resistant strain. Of particular interest were the newer chemistries including spinosad, indoxacarb and emamectin benzoate, about which little is known.

In April 2011, a different approach to selection was taken by utilising F_2 families established by CSIRO. F_2 screening for indoxacarb resistance was undertaken to investigate the presence of recessive resistance genes to this insecticide. This involved the use of a discriminating dose to screen F_2 progeny from *H. armigera* individuals that has been collected from the field and single-pair mated with moths from a laboratory susceptible strain of *H. armigera*.

Colonies resistant to other insecticides, which have been studied more extensively, were established for use in investigating cross-resistance to Bt toxins. In particular, a pyrethroid resistant strain of *H. armigera* was established by selecting a laboratory strain (KOS) that had previously demonstrated a measure of resistance to fenvalerate.

5. Investigation of features of resistance to key insecticides using resistant colonies established from field survivors

The mode of inheritance of resistance is one of the most important factors influencing the rate at which resistance will develop in a population. Due to unsuccessful attempts to establish resistant colonies from field survivors, as well as the absence of a research scientist from March 2010 to April 2011, studies on inheritance of resistance to insecticides was limited. Nevertheless, with the appointment of a replacement researcher in this project, research to select for resistance to newer conventional chemistries will be undertaken as well as subsequent work to determine the mode of inheritance of such resistance genes.

6. Investigate potential cross-resistance between insecticides

Cross-resistance can significantly influence the rate at which resistance will develop in a population. Following the appointment of a replacement researcher in April 2011, studies were initiated to establish patterns of cross-resistance in *H. armigera* between Bt and conventional insecticides utilising a Bt (Cry2Ab) resistant strain established by CSIRO, the newly established pyrethroid resistant strain KOS, and a an isogenic laboratory susceptible strain of *H. armigera*, known as GR. The dose-responses of these strains were tested against eight conventional insecticides and two Bt toxins.

Bioassays of conventional chemistries were conducted using 20 larvae per treatment (each treatment consisted of an insecticidal concentration produced by a series of two-fold dilutions from a stock solution). Dose-responses vary between chemical groups. Therefore, the number of doses required to produce the full range of response depends on the chemical being tested (usually between four and seven doses).

Larvae were tested using established bioassay techniques described by Forrester *et al*. (1993) where larvae were chosen within a specific age-structure and weight range (30-40 mg 3rd or 4th instar) and each larva dosed dorsally on the thorax with 1 µl of insecticide dissolved in acetone. Doses were expressed as ng or µg of insecticide (depending on the efficacy of the insecticide tested). Larvae were tested in 45-well disposable assay trays and contained within using heat-sealed Mylar® film perforated for aeration and kept at 25 ºC until assessment.

Assessment was based on physiological criteria. Larvae are alive if they were able to demonstrate coordinated movement when prodded. Each bioassay was performed in triplicate with acetone used as the control, with control mortality adjusted by the formula of Abbott (1925). A dose-response regression was used to determine LD$_{50}$, 95 % fiducial limits, slope and χ^2 values determined by Probit analysis (POLO-PC, LeOra Software, Berkeley, CA).

Bioassays of Bt toxins (Cry1Ac and Cry2Ab) were performed on unfed 1st instar *H. armigera* larvae using a diet surface contamination method where artificial diet was dispensed into 45-well disposable assay trays, approximately 2 mL of diet per well. Aliquots of 50 µL of serially diluted Bt toxin were pipetted onto the surface of diet with residual surface liquid allowed to evaporate in a cool airflow. Doses were expressed as ng of toxin per cm^2 of diet surface with a minimum of six doses required to cover the full range of response. Larvae were transferred to trays, one larva per well and contained within using heat-sealed and perforated Mylar® film. Larvae were maintained at 25 °C with a photoperiod of 14 h light : 10 h dark. Larvae were assessed at seven days using physiological criteria. Larvae were assessed as alive if they demonstrated normal movement or dead if moribund and demonstrating uncoordinated movement. Each bioassay was performed in triplicate with untreated diet as the control, with control mortality adjusted by the formula of Abbott (1925). The dose responses of larvae to Cry toxins were analysed with LC$_{50}$, 95 % fiducial limits, slope, and χ^2 values determined by Probit analysis (POLO-PC, LeOra Software, Berkeley, CA).

Given the unexpected high frequency of Bt resistance in Australian populations of *H. punctigera* further cross-resistance studies have been initiated to investigate the dose-response of Bt-resistant and -susceptible *H. punctigera* strains.

7. *Using appropriate bioassay techniques accumulate baseline dose-response data for new Helicoverpa insecticides*

The establishment of a realistic range of susceptibility (including low level, polygenic tolerance) is imperative in resistance monitoring because variation in susceptibility impacts on the criteria for resistance. Appropriate bioassay techniques and accurate determination of discriminating doses is, therefore of critical importance in order to detect the presence of resistance genes in populations before they reach high frequency.

Two chemicals with potential for registration against Helicoverpa were the subject of dose response assays. Assays were conducted on pyridalyl over 2008/09 however these were discontinued due to confirmation this chemical would not be registered in Australia. These results shall remain the property of NSW DPI in the event registration is sought in the future, however they shall not be further reported here.

Pilot studies were initiated in 2009/10 to determine the appropriate range of concentrations that covered the full range of responses for the anthranilic diamide, cyazypyr using a feeding bioassay. These insecticides activate the ryanodine receptor and cause the unrestricted movement of calcium into the muscle cells of insects (Cordova *et al*. 2006). The new insecticide cyazypyr has the added advantage of being active against whitefly. This class of insecticide is already represented in the insecticide resistance monitoring schedule by rynaxypyr, the baseline data for which was reported in a previous project (DAN 193).

Feeding bioassays utilised the commercial formulation of cyazypyr, supplied by DuPont, to determine the dose response when incorporated into artificial diet. A stock solution and a series of dilutions were prepared in de-ionised water. The bioassay consisted of nine treatments (pesticide concentrations), with 40 larvae tested at each concentration. Artificial diet was prepared according to a standard protocol, with an appropriate amount of water omitted corresponding to the total volume of insecticide solution to be added. Insecticide was added as a 1 in 10 dilution to the diet (i.e. 10 mL insecticide: 190 mL diet) followed by vigorous shaking. Diet was dispensed into 12-well nLinbo tissue culture trays. When the diet had set larvae were transferred to the trays, one larva/well. Bioassays were conducted on F_1 and F_2 generations of field derived strains and kept in an incubator at 25 ^{o}C, 12:12 hr light: dark until assessment which occurred at 96 hours. Concentrations were expressed as ppm and analysed using probit analysis (PROBIT5, NSW Agriculture).

8. Characterise an esterase protein produced by a gene isolated from H. armigera and assess its role in resistance

While confirmation of the full length esterase sequence isolated from *H. armigera*, allowed for initiation of the characterisation of this gene and its protein product, the absence of a research scientist in this position from March 2010 resulted in the discontinuation of this research.

9 Conduct further molecular investigations of esterase properties in H. armigera

Previous investigations (DAN 193) had resulted in a preliminary full esterase sequence from *H. armigera*. Further investigations within this project involved confirmation of this sequence using high fidelity polymerase to minimise error during PCR. Both pyrethroid resistant (R) and susceptible (S) larvae were used as part of identifying sequence differences which may contribute to resistance.

Standard molecular techniques were used in RNA extraction, cDNA synthesis, PCR and sequencing. RNA extraction from a single neonate larva was carried out using a Nucleospin® RNA II Extraction Kit (Machery Nagel). Two resistant and 2 susceptible larvae were used in the RNA extraction. cDNA synthesis involved all four RNA samples using a Superscript® III First strand Synthesis system for RT_PCR. (Invitrogen). Several RT-PCR's were conducted using Primestar® HS DNA Polymerase and a number of primers designed from the preliminary esterase sequence. These primers were both internal as well as flanking the esterase gene. Ligation of PCR products into plasmids for transformation and cloning into One Shot® TOP10 Chemically Competent cells used a TOPO® Zero Blunt® TOPO® Cloning Kit for Sequencing (Invitrogen). Plasmid preps containing esterase inserts were prepared and sent to The Australian Genome Research Facility, St Lucia, QLD, for sequencing using both gene specific primers as well as M13 primers.

Sequencing data was analysed using the suite of tools within DNAstar® software (Lasergene).

Further molecular investigations, including sequencing of genomic DNA (gDNA) to investigate the presence of exons within the gene were initiated, however with the vacation of the research scientist, these were not progressed.

10. Support other Helicoverpa related research projects with field collected material and field collected laboratory reared insect colonies

Field and laboratory reared insects were reared in accordance with established protocols and made available to Helicoverpa related projects on request.

4. Results

1. Insect collection and laboratory rearing

Annual collection of field material (predominantly Helicoverpa eggs with some supplementary larval sampling) was successfully conducted for the 2008/09, 2009/10 and 2010/11 seasons. Total data collected from field samples for monitoring purposes includes information on overall species complex at the time of sampling (Tables 1a - c). This information is used in the monitoring program and also has implications for resistance management, with *H. punctigera* effectively controlled due to its susceptible status.

Species composition in resistance monitoring collections

Source crops represented in species composition data are those that attract both Helicoverpa species including cotton, pigeon pea, chickpea, sunflower and mungbean, and excludes maize and sorghum. Tables 1a to 1c describe the percent *H. armigera* in samples collected from all regions. Total insect numbers speciated are given in brackets. Blanks indicate no collections were received or collections were exclusively from sorghum, maize or other non *H. punctigera* hosts.

Region	**Sept-Oct**	**Nov**	**Dec**	**Jan**	**Feb**	**March**	**April**
Emerald	56 (89)		60 (156)	97 (89)	100 (26)		
Darling Downs				82 (105)			
St George			52 (419)	59 (243)	62 (34)		
Mungindi							
Macintyre			51 (53)	77 (465)	76 (532)		
Gwydir		5 (422)	56 (660)	85 (327)			
Lower Namoi	0 (27)	11 (298)	79 (431)	57 (1089)	98 (56)	63 (265)	
Upper Namoi		59 (56)	47 (260)	53 (159)			
Macquarie			5 (118)	4 (616)			
Lachlan							

Table 1a: Percentage *H. armigera* in samples collected during 2008/09 (numbers in brackets = n).

Region	**Sept-Oct**	**Nov**	**Dec**	**Jan**	**Feb**	**March**	**April**
Emerald		67 (104)	44 (75)	47 (300)	61 (70)		
Darling Downs			6 (81)	20 (193)			
St George		2 (106)	5 (283)	7 (307)	81 (43)		
Mungindi		0 (130)	0 (22)	49 (41)		80 (20)	
Macintyre			9 (559)	86 (343)	59 (396)		
Gwydir		0 (67)		81 (118)		91 (143)	
Lower Namoi	36 (335)	19 (100)	1 (438)	47 (677)	55 (978)	47 (70)	94 (133)
Upper Namoi			3 (355)	6 (374)	41 (487)	74 (653)	98 (121)
Macquarie			0 (53)				
Lachlan			1 (74)				

Table 1b:. Percentage *H. armigera* in samples collected during 2009/10 (numbers in brackets = n).

Region	Sept-Oct	Nov	Dec	Jan	Feb	March	April
Emerald		89 (123)	83 (119)	93 (28)	97 (36)		
Darling Downs		89 (225)	93 (69)		95 (79)		
St George		76 (365)	87 (143)	79 (121)			
Mungindi		67 (100)	94 (198)	95 (194)			
Macintyre		88 (217)	95 (236)	97 (33)	100 (16)		
Gwydir	74 (295)		79 (378)	97 (68)	100 (31)		
Lower Namoi	40 (43)	52 (348)	88 (279)	92 (2926)	90 (729)	99 (759)	
Upper Namoi				96 (296)	93 (375)	98 (62)	
Macquarie							
Lachlan							

Table 1c:. Percentage *H. armigera* in samples collected during 2010/11(numbers in brackets = n).

In the 2008/09 and 2009/10 seasons insect pressure was moderate in most regions particularly throughout December and January as reflected in the total number of insects speciated. The exception to this was in the southern districts of the Lachlan and Macquarie where pressure was generally low during all three seasons. The Namoi and Gwydir valleys are generally over-represented. This is because samples from these regions are collected and processed by the dedicated local collection team based at ACRI, Narrabri. Here there were fewer logistical issues associated with collecting operations, particularly in terms of the availability of timely and efficient transport links to the central testing laboratory at ACRI which is often presents a challenge in isolated areas.

Throughout the three seasons populations of Helicoverpa occurring in cotton, grain and pulse growing areas of eastern Australia were generally mixed, with dominance by *H. punctigera* early season and late season composed predominantly of *H. armigera*. Interestingly, species composition was dominated by *H. punctigera* during December 2009/10, with less than 10 % *H. armigera* contributing to the Helicoverpa population in all areas, with the exception Emerald (Table 1b). This trend continued to some extent during January, particularly in the southern Queensland districts and in the upper Namoi valley. By February and March *H. armigera* was becoming more dominant in all regions. However, during the first three months of 2010 *H. armigera* contributed only 50 % to the Helicoverpa population in the lower Namoi valley. This had the potential to impact on the samples size available for resistance testing in this species. However, large collections of *H. armigera* from sorghum and maize helped to offset the lack of material available from cotton, pigeon pea and other crops attractive to both Helicoverpa species.

Species composition was dominated by *H. armigera* early in 2010/11 season, particularly in the border regions and in southern Queensland. High proportions of *H. armigera* persisted in all regions from November to March. It should be acknowledged that collecting efforts in were significantly impacted by high rainfall and flooding, particularly in regions of southern Queensland.

2. Insecticide Resistance Monitoring

Insecticide resistance monitoring was successfully conducted for the 2008/09, 2009/10 and 2010/11 seasons. The results will be presented for each chemical below in terms of how they relate to historical resistance trends and the relative differences between regions sampled.

Analysis of the resistance status of *H. armigera* focussed on the detection of resistant individuals and reporting results in terms of whole numbers tested, with trend observations and statistical analysis between areas, years and within the season almost impossible due to the low numbers collected in some areas and in different seasons, particularly in the Queensland. For this reason data has been pooled for the Gwydir/Mungindi areas (represented on the graphs below as ‘Gwydir’) and for the St George, Macintyre and Darling Downs regions (represented on the graphs below as ‘Southern Queensland’).

Helicoverpa armigera

Spinosad (Tracer®)

During these seasons very few survivors to spinosad were detected. This follows a trend of decreasing resistance since a peak in 2001/02 (Figures 1a). The widespread uptake of Bollgard II® by the Australian cotton industry, combined with management strategies to mitigate an increasing resistance trend, and the availability of alternative soft options, has contributed to the observed low frequencies of resistance to this insecticide which did not exceed 1 % in any given region. In fact, no survivors were found in a number of valleys in each year, particularly regions in Queensland. However these were not generally highly sampled. The more highly sampled areas such as the Gwydir and Namoi valleys yielded the occasional survivor (Figure 1b).

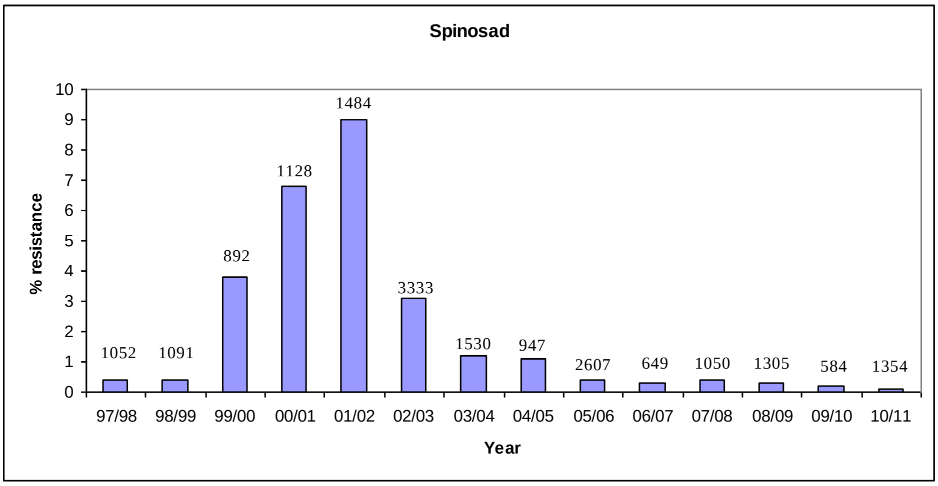


Figure 1a: Spinosad resistance frequencies, average for all valleys for 2008/09, 2009/10 and 2010/11 including historical data from previous monitoring projects from 1997/98 - 2007/08, (R. Gunning & L. Rossiter). Numbers on graph indicate total larvae tested.

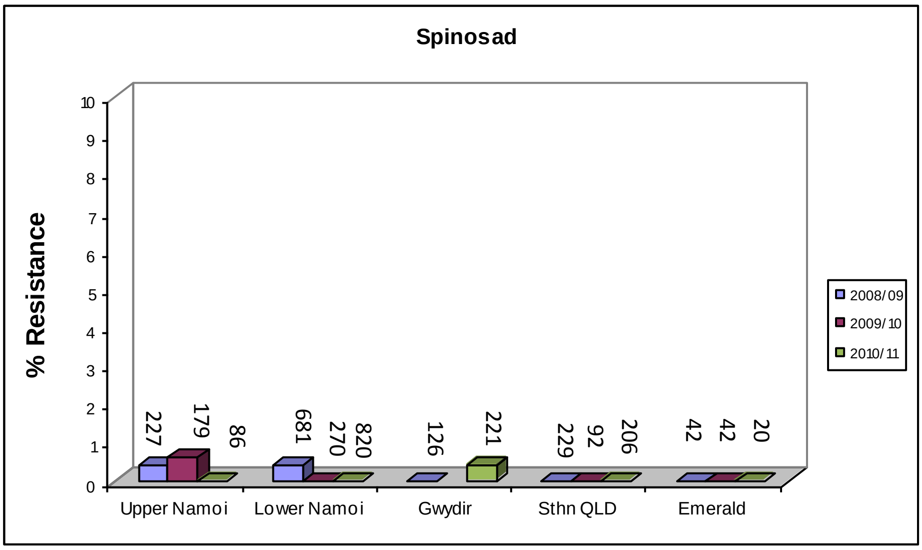


Figure 1b: Spinosad resistance frequencies for each valley tested in 2008/09, 2009/10 and 2010/11. Numbers on the graph indicate total larvae tested.

Indoxacarb (Steward®)

Indoxacarb resistance was detected for the first time in 2002/03 in most valleys and has been in decline ever since (Figure 2a). The threat of compromised efficacy through the development of resistance prompted prioritisation of this insecticide within the monitoring program and hence is well represented by high sample sizes, particularly from the Namoi valley, where no survivors detected. Lack of material from Emerald and southern Queensland in some years, precluded extensive testing of indoxacarb for these areas.

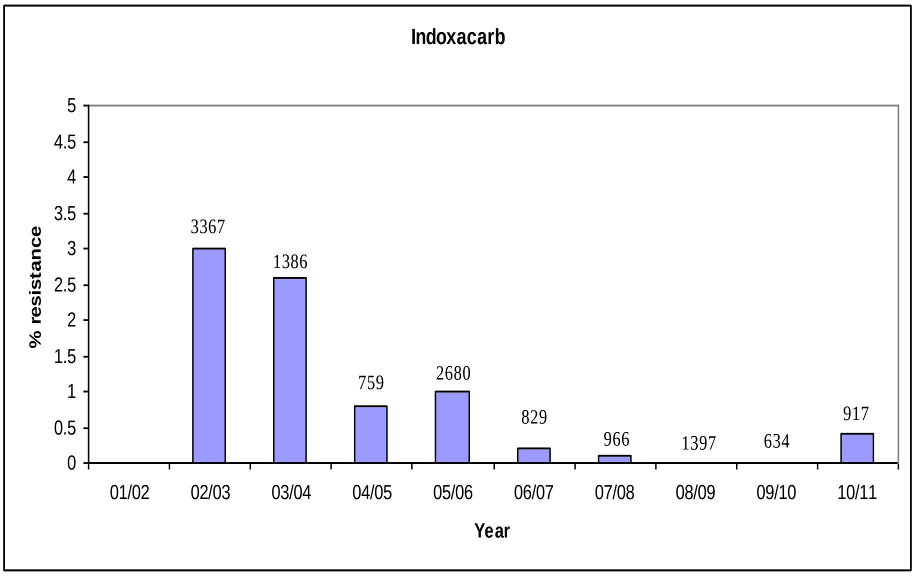


Figure 2a: Indoxacarb resistance frequencies, average for all valleys for 2008/09, 2009/10 and 2010/11 including historical data from previous monitoring projects from 1997/98 - 2007/08, (R. Gunning & L. Rossiter). Numbers on graph indicate total larvae tested.

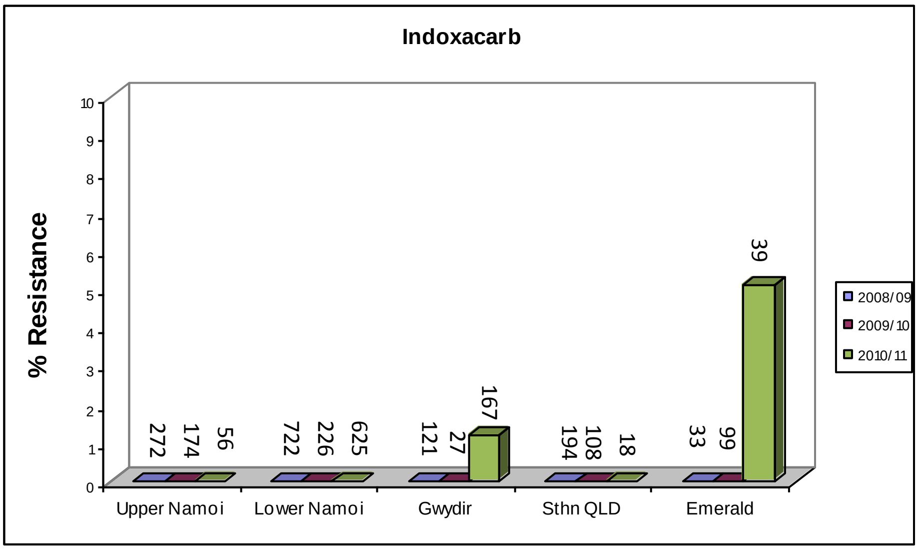


Figure 2b: Indoxacarb resistance frequencies for each valley tested in 2008/09, 2009/10 and 2010/11. Numbers on the graph indicate total larvae tested.

Emamectin Benzoate (Affirm®)

The 2002/03 season was the first season that emamectin benzoate resistance was detected throughout the Australian cotton growing regions (Figure 3a). Resistance frequencies have remained generally low with the exception of the Gwydir valley where resistance was 5.1% in 2009/10 (Figure 3b). Emamectin benzoate is not registered for use against Helicoverpa in broad acre crops other than cotton. The consequence of this is that selection is restricted to populations that establish in cotton and has been further minimised with the introduction of Bollgard II. However, emamectin benzoate is an important component within an IPM system for controlling Helicoverpa and monitoring must continue to ensure that efficacy if not compromised, particularly in areas such as the Gwydir valley where resistance frequencies were significantly higher in 2009/10 than in 2008/09. The result from 2010/11, however offers some reassurance that resistance is not establishing because of the very high sample size tested.

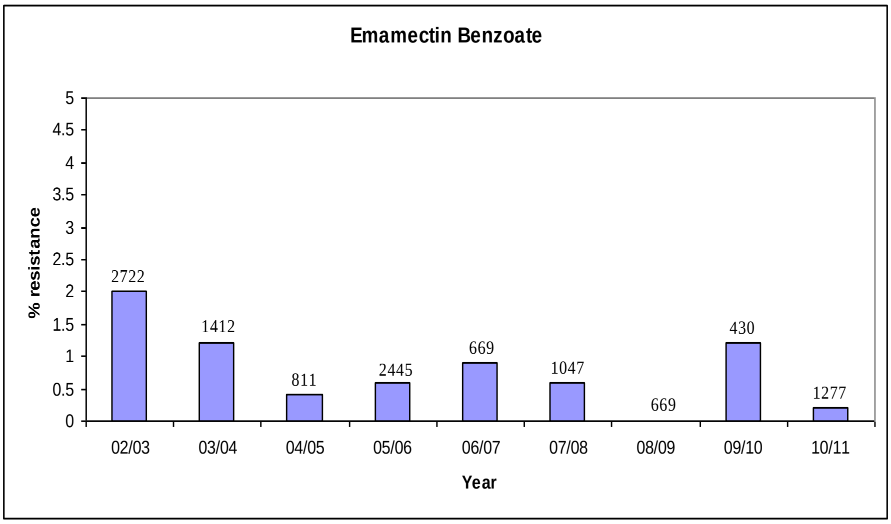


Figure 3a: Emamectin benzoate resistance frequencies, average for all valleys for 2008/09, 2009/10 and 2010/11 including historical data from previous monitoring projects from 1997/98 - 2007/08, (R. Gunning & L. Rossiter). Numbers on graph indicate total larvae tested.

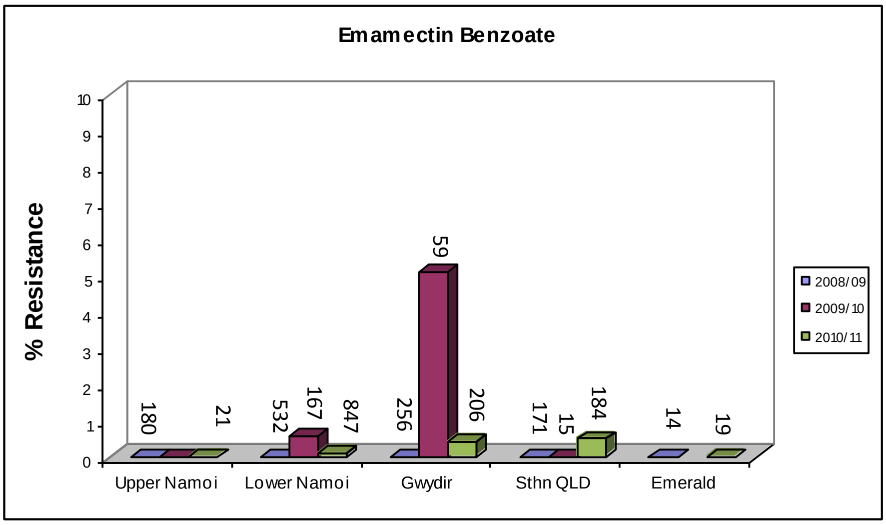


Figure 3b: Emamectin benzoate resistance frequencies for each valley tested in 2008/09, 2009/10 and 2010/11. Numbers on graph indicate total larvae tested.

Organophosphate (Chlorpyrifos)

Resistance to chlorpyrifos remains present in *H. armigera* populations since being detected again in 2001/02 (Figure 4a). However, resistance frequencies have declined to very low levels which have been maintained over the last three seasons. Again this probably reflects a reduction in selection pressure in line with reduced spray applications in a cotton production system that is dominated by Bollgard II cotton and is consistent between regions (Figure 4b).

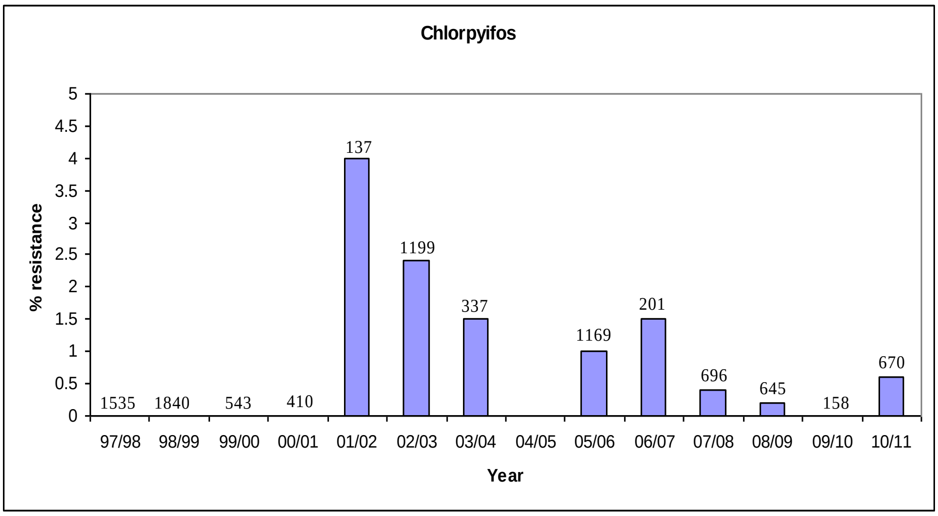


Figure 4a: Chlorpyrifos resistance frequencies, average for all valleys for 2008/09, 2009/10 and 2010/11 including historical data from previous monitoring projects from 1997/98 - 2007/08, (R. Gunning & L. Rossiter). Numbers on graph indicate total larvae tested.

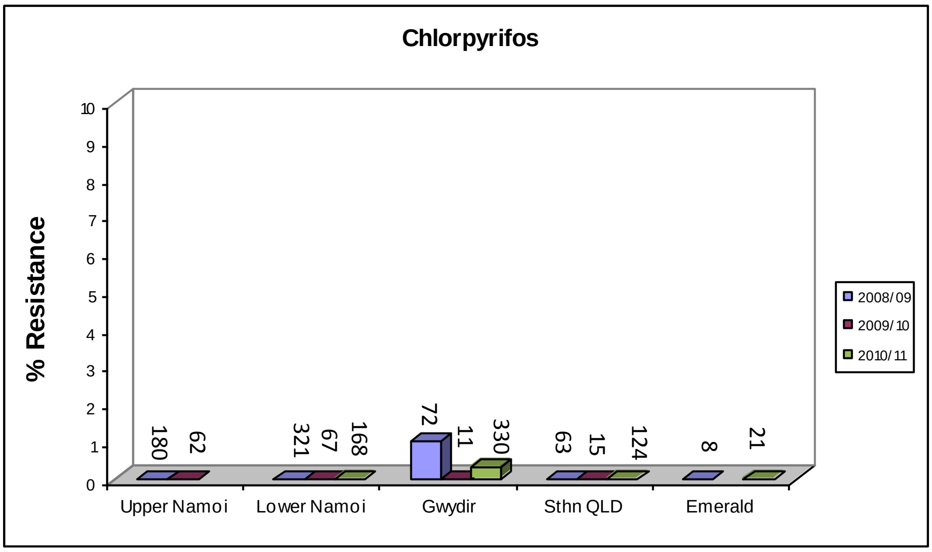


Figure 4b: Chlorpyrifos resistance frequencies for each valley tested in 2008/09, 2009/10 and 2010/11. Numbers on the graph indicate total larvae tested.

Endosulfan

Endosulfan resistance has been present in *H. armigera* since first reported in 1972, and variation in resistance frequency reflects overall use patterns in different areas (Figure 5a). Monitoring over the last three seasons demonstrates significant reductions in the incidence of endosulfan resistance (Figure 5b), with some regional variability (Figure 5c). Given that endosulfan resistance has historically varied in response to its usage patterns, the reduction in resistance over the last three seasons is likely to be a result of the reduction in use due to high uptake of Bollgard II.

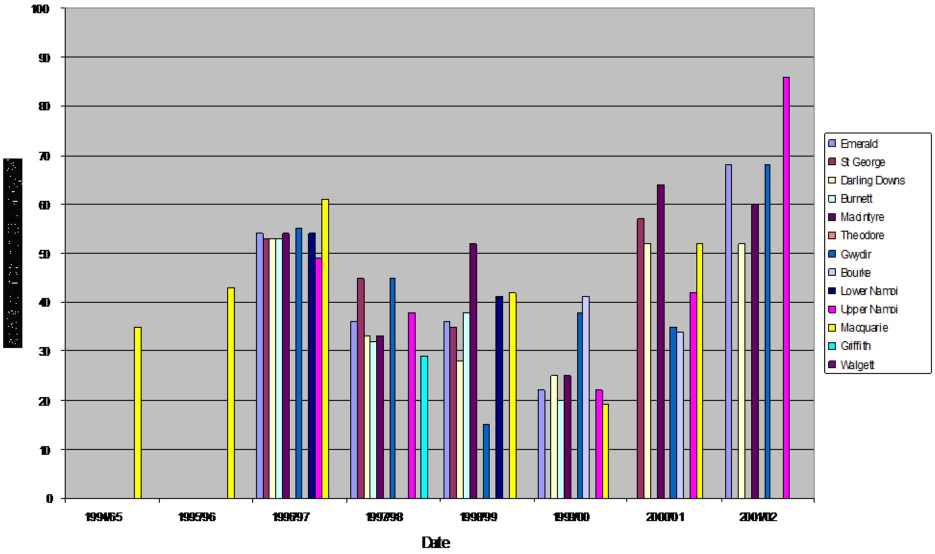


Figure 5a: Historical endosulfan resistance frequencies from individual valleys (R. Gunning, NSW DPI, pers comm.).

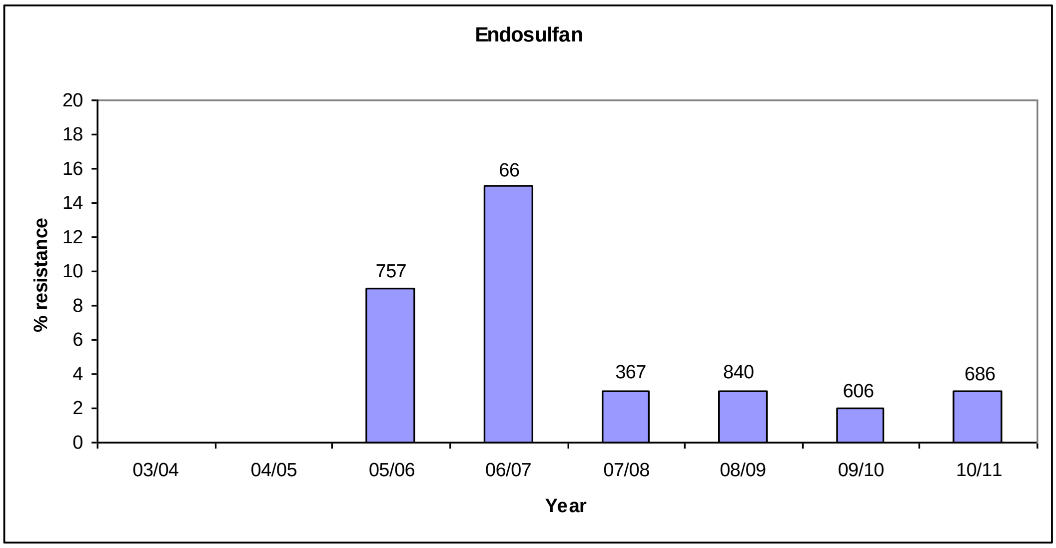


Figure 5b: Endosulfan resistance frequencies, average for all valleys for 2008/09, 2009/10 and 2010/11 including historical data from previous monitoring projects from 1997/98 - 2007/08, (R. Gunning & L. Rossiter). Numbers on graph indicate total larvae tested.

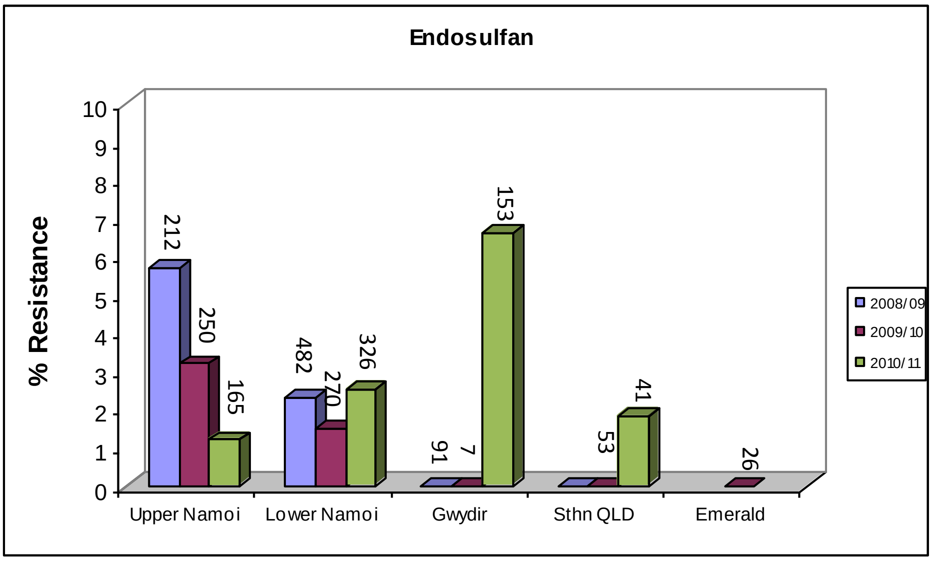


Figure 5c: Endosulfan resistance frequencies for each valley tested in 2008/09, 2009/10 and 2010/11. Numbers on graph indicate total larvae tested.

Pyrethroids

Bifenthrin (Talstar®)

Pyrethroid resistance is well established in *H. armigera* populations in cotton growing regions of Australia, at variable but generally high frequencies. Monitoring has historically involved the pyrethroid fenvalerate. While not registered for use in cotton, fenvalerate provided a good indicator of the level of general pyrethroid resistance.

Between the years 2000 and 2007 pyrethroid resistance was estimated by measuring resistance frequencies to a single pyrethroid bifenthrin (Talstar®), which is registered for use in cotton. Resistance to this pyrethroid decreased significantly during this time. In fact no survivors were detected in the 2007/8 and the 2008/09 seasons. However, in 2009/10 and 2010/11 resistance increased to an average of 7 % across all regions (Figure 6a) with some variability between regions (Figure 6b). It is noteworthy that numbers of insects were relatively low and sampling should continue in subsequent seasons to confirm this increase.

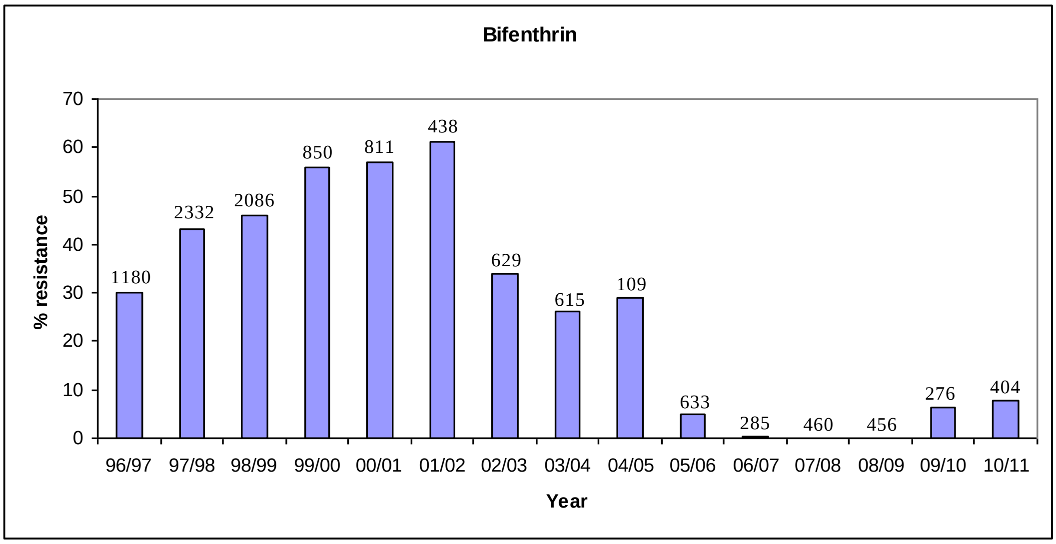


Figure 6a: Bifenthrin resistance frequencies, average for all valleys for 2008/09, 2009/10 and 2010/11 including historical data from previous monitoring projects from 1997/98 - 2007/08, (R. Gunning & L. Rossiter). Numbers on graph indicate total larvae tested.

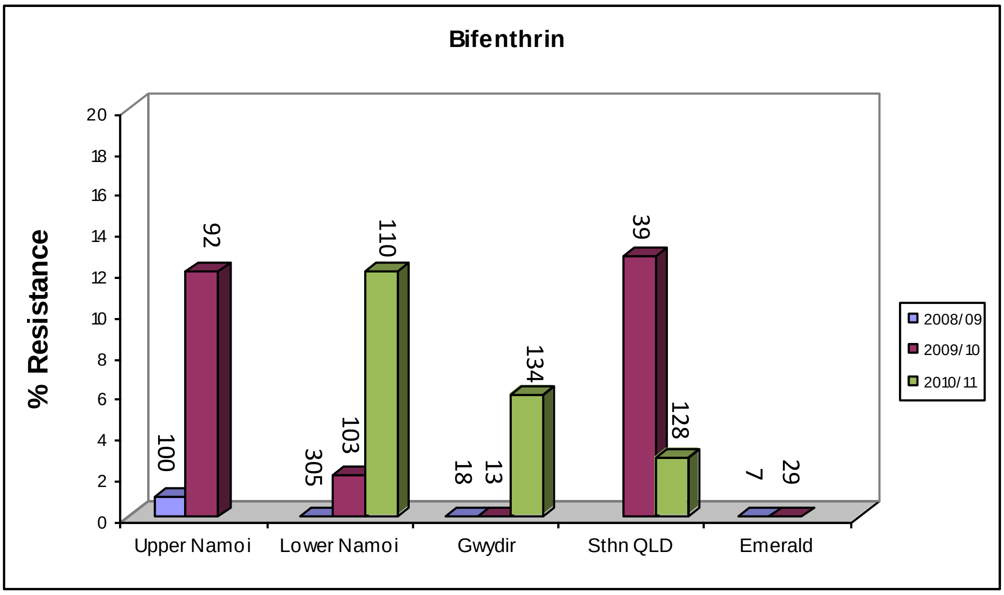


Figure 6b: Bifenthrin resistance frequencies for each valley tested in 2008/09, 2009/10 and 2010/11. Numbers on graph indicate total larvae tested.

To examine the effect of the general decline bifenthrin resistance on other pyrethroids, fenvalerate was reintroduced to the monitoring schedule in 2007/08 for the first time in several years and demonstrated an average resistance frequency of 41 % across all regions. Resistance to fenvalerate continued to be monitored between 2008 and 2011. Results demonstrate the presence of high level fenvalerate resistance which indicates that the genes that confer resistance to this insecticide are stable in Australian populations of *H. armigera*

(Figure 6c). Although frequencies vary slightly between regions (ranging from 53 % in southern Queensland to 85 % in Emerald) results are generally consistent between regions and between seasons (Figure 6d).

Results from a previous project (DAN193) confirmed the reduction in resistance to bifenthrin is specific to that insecticide and does not extend to other pyrethroids, with the fenvalerate results indicating that general pyrethroid resistance remains at a high frequency.

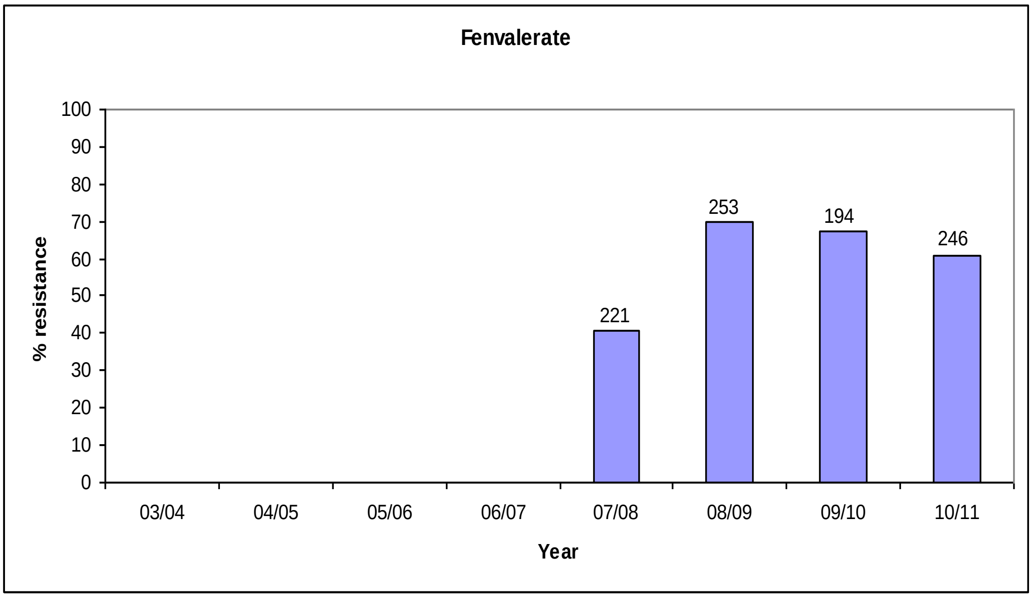


Figure 6c: Fenvalerate resistance frequencies, average for all valleys for 2008/09, 2009/10 and 2010/11 including historical data from previous monitoring projects from 1997/98 - 2007/08, (R. Gunning & L. Rossiter). Numbers on graph indicate total larvae tested.

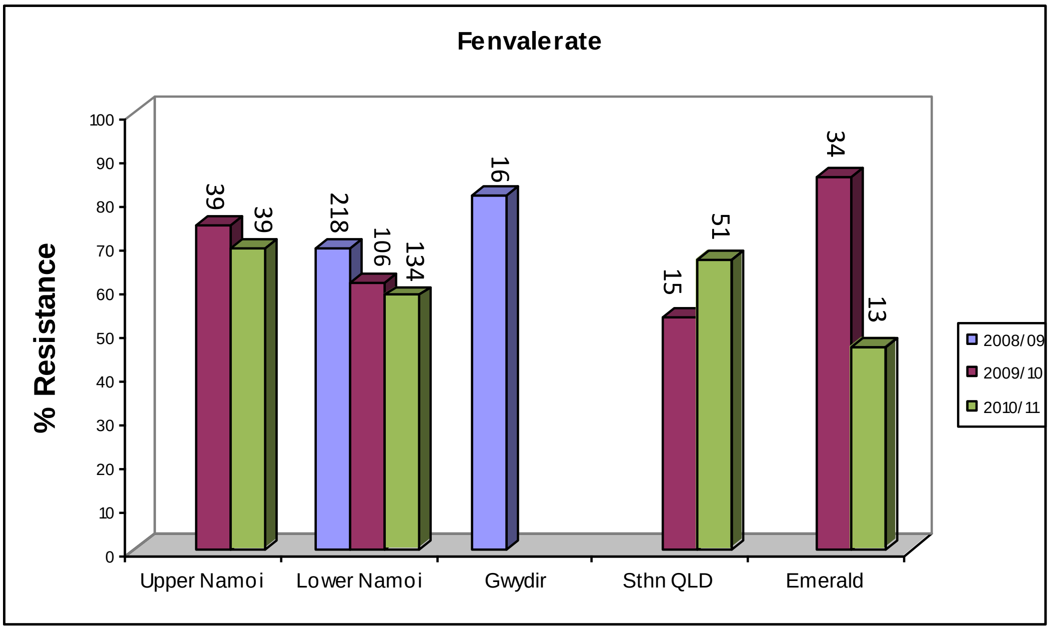


Figure 6d: Fenvalerate resistance frequencies for each valley tested in 2008/09, 2009/10 and 2010/11. Numbers on graph indicate total larvae tested.

Carbamate (Methomyl)

Methomyl (carbamate) resistance has been present at high frequencies throughout all cotton growing regions for over ten years, with typical frequencies of 60 % or more. Over the last three years, monitoring has been conducted on large numbers of insects to determine the response of reduced selection pressure which has occurred due to the deployment of Bollgard II. Results show that resistance frequencies to remain at moderate and stable levels in the population ranging from 17 to 33 % (Figure 7a) and that these frequencies are consistent between regions (Figure 7b).

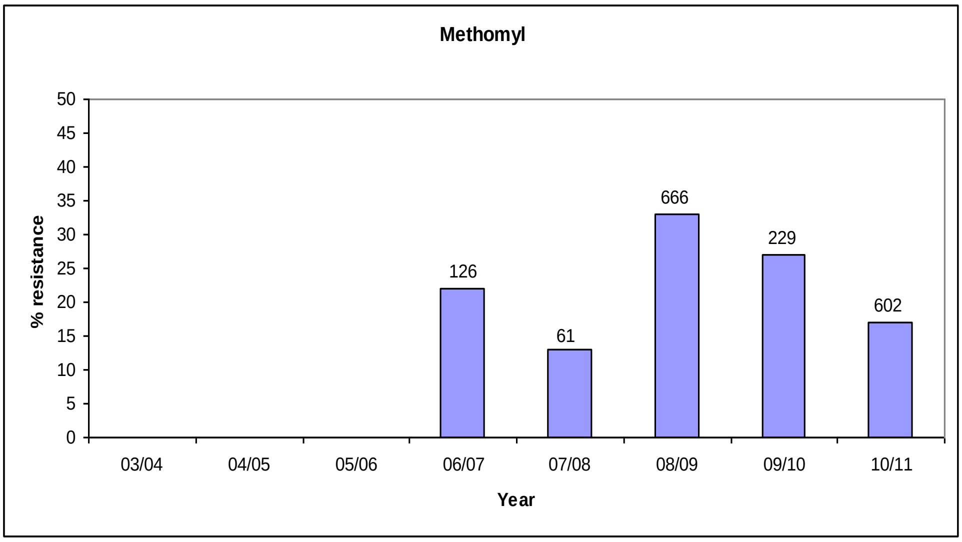


Figure 8a: Methomyl resistance frequencies, average for all valleys for 2008/09, 2009/10 and 2010/11 including historical data from previous monitoring projects from 1997/98 - 2007/08, (R. Gunning & L. Rossiter). Numbers on graph indicate total larvae tested.

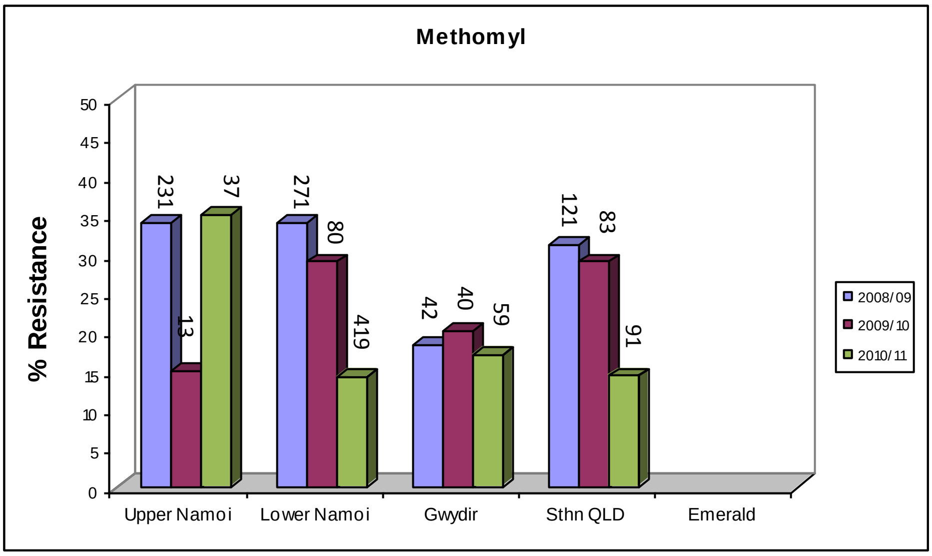


Figure 8b: Methomyl resistance frequencies for each valley tested in 2008/09, 2009/10 and 2010/11. Numbers on graph indicate total larvae tested.

Rynaxypyr (Altacor®)

In 2008/09 the newest of the soft chemistries, rynaxypyr, became available for use in cotton production for the control of Helicoverpa. Rynaxypyr (chloranthraniliprole) is a compound belonging to the anthanilamide class of insecticide. These insecticides act by binding to and activating the ryanodine receptor, causing the release of stored calcium from the sarcoendoplasmic reticulum, resulting in impaired regulation of muscle function and ultimately insect death (Cordova *et al*. 2006). This class of compounds is highly target specific and, therefore highly desirable in pest control because of its suitability for use within an IMP framework. Since its inclusion in the resistance monitoring schedule there have been very few survivors at the discriminating dose of rynaxypyr, with only three survivors out of 2497 insects tested over the past three seasons.

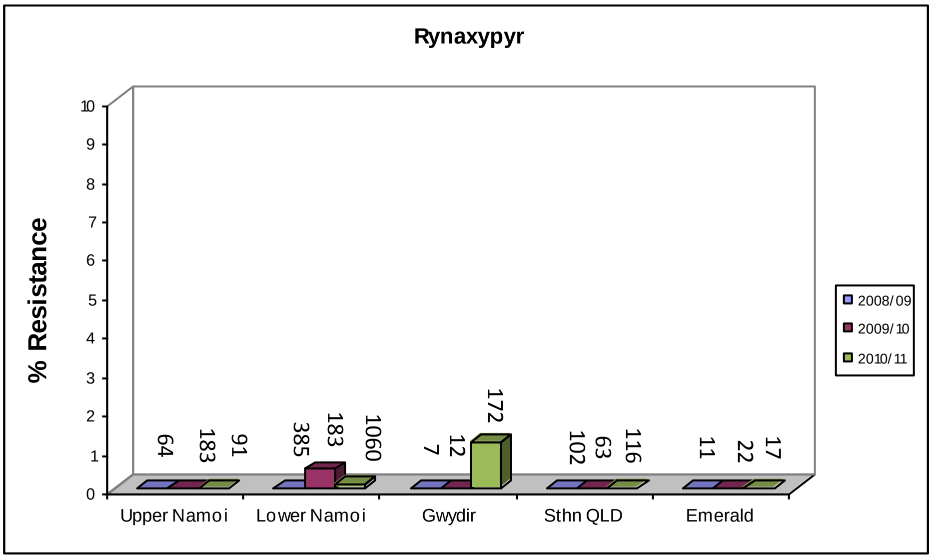


Figure 9:: Rynaxypyr resistance frequencies for each valley tested in 2008/09, 2009/10 and 2010/11. Numbers on graph indicate total larvae tested.

Helicoverpa punctigera

Moderate pressure combined with prolonged dominance of *H. punctigera* in 2009/10 made it possible to test all chemistries in most areas, while in the other two seasons *H. punctigera* pressure was variable and generally lower.

Endosulfan

Endosulfan resistance has been detected in previous years at very low frequencies, with no indication that resistance was increasing. Monitoring in 2008/09 and 2009/10 confirmed that this was the case (Figure 10). However, results from 2010/11 indicate a significant increase in endosulfan resistance frequency in the Gwydir/Mungindi regions and in southern Queensland. Although sample sizes were small (36 and 44 tested from the Gwydir/Mungindi and Southern Queensland respectively) it is intriguing that surviving individuals originated from only two collection sites. Results suggest that monitoring should be increased in these areas to confirm resistance status in local *H. punctigera* populations.

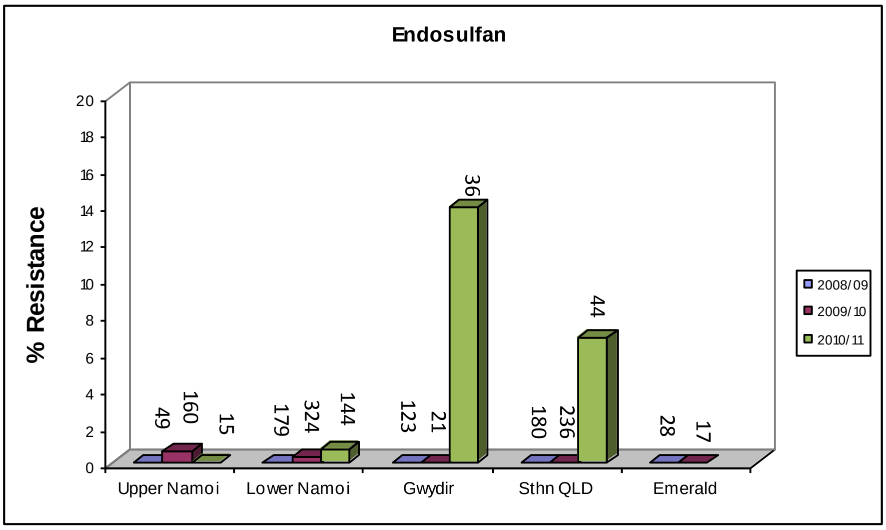


Figure 10: Endosulfan resistance frequencies for each valley tested in 2008/09, 2009/10 and 2010/11. Numbers on graph indicate total larvae tested.

Pyrethroids (Fenvalerate)

Pyrethroid resistance has also been detected in previous years at low frequencies. However, apart from a small number of survivors that were detected in the upper and lower Namoi in 2009/10, there were with no survivors detected in the other areas or in other years (Figure 11).

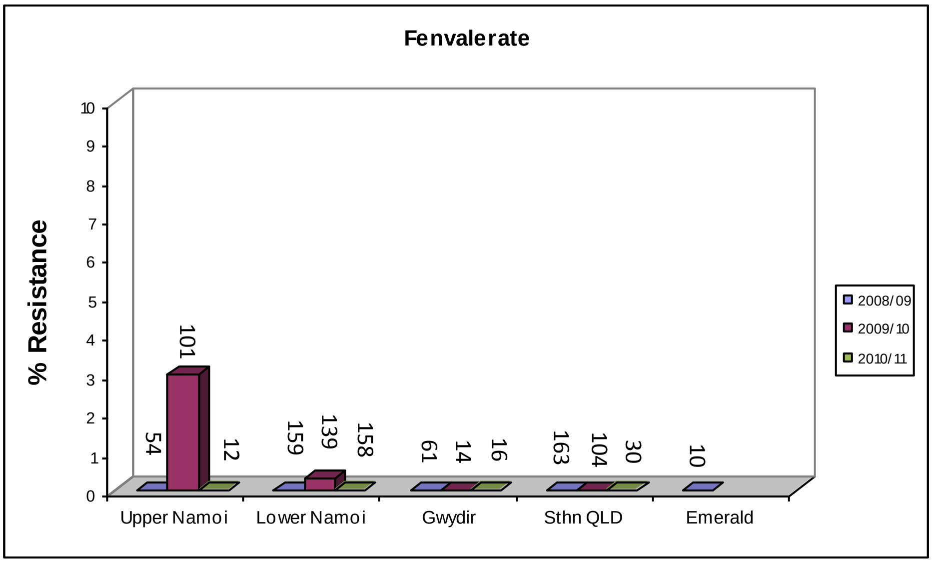


Figure 11: Pyrethroid (fenvalerate) resistance frequencies for each valley in 2008/09, 2009/10 and 2010/11. Numbers on graph indicate total larvae tested.

Abamectin

Low *H. punctigera* pressure in some regions precluded extensive testing of this species against abamectin, particularly in Emerald. Despite the findings of the occasional survivor in the lower Namoi and Gwydir valleys, there was no indication that resistance is developing beyond a very low level (Figure 12).

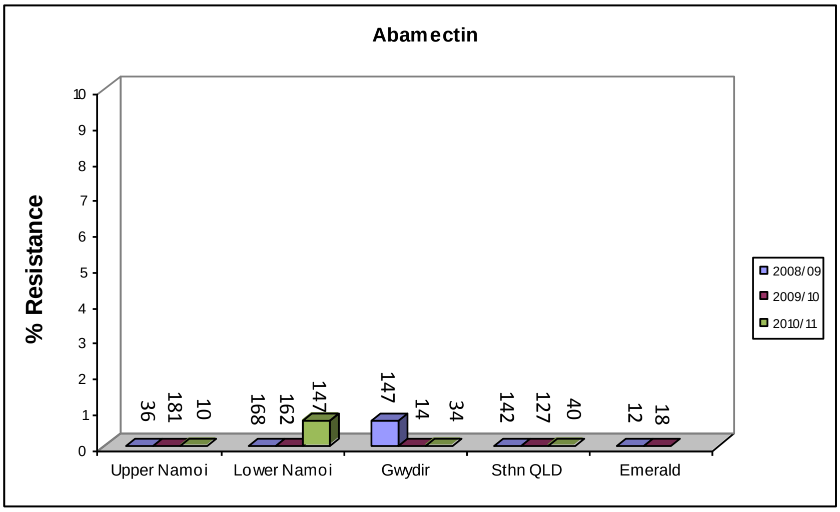


Figure 12: Abamectin resistance frequencies for each valley 2008/09, 2009/10 and 2010/11. Numbers on graph indicate total larvae tested.

3. Resistance Management Strategy Formulation and Promotion

Resistance monitoring data were presented to the TIMS committee as part of assessing the IRMS, used primarily by the cotton industry. The IRMS was disseminated through the DPI publication Cotton Pest Management Guide, and promoted through the TIMS Resistance Roadshow, and is also available on the Cotton Catchment Communities CRC website.

The 2008/09 IRMS had only two minor amendments. The abamectin window was extended by 15 days in all regions except the Darling Downs. This was primarily in response to a need for abamectin later in the season for use against mites. This was not considered necessary in the Darling Downs area, so was not extended. Altacor® (active rynaxypyr) was also added to the IRMS following its registration against Helicoverpa in cotton. This was introduced for season long use to allow industry to determine its best fit during the season.

In 2009/10 a number of changes were made to the IRMS. The Darling Downs IRMS was re-incorporated into the Central Regions IRMS, enabling insecticide use windows to be uniform across northern NSW and southern QLD, thereby minimising the exposure period for selection pressure This incorporation however did result in the southern regions of the Macquarie, Lachlan and Murrumbidgee Valleys returning to a separate strategy which allowed for earlier season pyrethroid use (Dec 15 – end of season) to account for higher *H. punctigera* populations in areas less prone to silverleaf whitefly problems. In contrast the pyrethroid window in the Central Regions IRMS was moved to start a month later (Jan 15 – end of season) because of the risk of flaring silverleaf whitefly outbreaks.

Altacor® was windowed for use after an initial season wide use period the previous year. In the Central and Southern Regions the window extended from Dec 1 – Feb 15, with an earlier window in northern regions of Nov 15 – Feb 1. The Northern Regions IRMS also moved the

Steward window to start 15 days later in the season in response to industry feedback in regard to where its use was most required.

The final amendment aimed to simplify the strategy by separating from the main part of the strategy and reducing the fontic emphasis on those chemicals which remained registered but were commercially unavailable. They were not removed completely from the IRMS to account for left over commercial stocks and on-farm stocks.

4. Establishment of resistant H. armigera colonies

Insects that survived discriminating dose bioassays were to be kept for subsequent use in establishing resistant strains. However, resistance frequencies to the insecticides of most interest (spinosad, indoxacarb and emamectin benzoate) were very low, making establishing colonies resistant to these insecticides difficult.

In April 2011, a different approach to selection was taken by utilising F_2 families established by CSIRO. F_2 screening for indoxacarb resistance was undertaken to investigate the presence of recessive resistance genes to this insecticide. This involved the use of a discriminating dose to screen F_2 progeny from *H. armigera* individuals that has been collected from the field and single-pair mated with moths from a laboratory susceptible strain of *H. armigera*. Of the 72 F_2 families that were screened, three demonstrated the presence of resistant genotypes, indicating that the F_0 parent was a potential carrier of heterozygous resistance of a recessive resistance gene. The survivors from these screens were then single-pair mated with moths from a laboratory susceptible strain and retested in the F_4 generation. However, homozygous resistance was unable to be detected. The possible reason for the loss of this gene may be related to a fitness cost associated with genes that confer resistance to indoxacarb in *H. armigera*.

Selection for indoxacarb resistance using F_2 survivors from field material will be ongoing. In addition, low-level selection using sub-lethal doses of insecticide is currently underway with results pending.

Colonies resistant to other insecticides, which have been studied more extensively, were established for use in investigating cross-resistance to Bt toxins. In particular, a pyrethroid resistant strain of *H. armigera* was established by selecting a laboratory strain (KO) that had previously demonstrated a measure of resistance to fenvalerate. This strain was selected at 2.5 µg/µL (> 10 x the discriminating dose of fenvalerate), resulting in a strain that was 24-fold more resistant, named KOS (Table 2) than the parental susceptible strain (GR).

In addition, the parental strain of this pyrethroid resistant strain (KO) was deselected to produce a fully pyrethroid and organophosphate susceptible strain of *H. armigera* (New GR). This involved creating multiple single pair families and allowing only those families with full susceptibility to fenvalerate (pyrethroid) and chlorpyrifos (organophosphate) to contribute to the establishment of the susceptible strain.

5. Investigation of features of resistance to key insecticides using resistant colonies established from field survivors

The mode of inheritance of resistance is one of the most important factors influencing the rate at which resistance will develop in a population. However, due to the absence of a research scientist in this project from March 2010 to April 2011, studies of resistance inheritance in *H. armigera* were limited. Nevertheless, with the appointment of a replacement researcher in a subsequent project, research to select for resistance to newer conventional chemistries will be undertaken as well as work to determine the mode of inheritance of such resistance genes.

6. Investigate potential cross-resistance between insecticides

The results from cross-resistance studies are shown in Table 2 and describe the dose-response of eight conventional insecticides and two Bt toxins against Cry2Ab and pyrethroid resistant strains of *H. armigera* (SP15 and KOS respectively) as well as a laboratory susceptible strain of *H. armigera* (GR). The pyrethroid resistant strain (KOS) had a resistance ratio of 24-fold to fenvalerate. The Cry2Ab resistant strain (SP15) sourced from CSIRO had a resistance ratio of > 6000-fold to Cry2Ab (Mahon et al. 2007). The data summarised in Table 2 show that Cry2Ab resistance (conferred by a single fully recessive gene) does not confer cross-resistance to conventional chemistries used in the field to control *H. armigera*. In fact, 5-fold negative cross-resistance was found between Cry2Ab and the organophosphate chlorpyrifos, and the carbamate methomyl. There is some evidence for low-level cross-resistance to Cry1Ac in the pyrethroid resistant strain KOS (4.6-fold) and the Cry2Ab resistant strain SP15 (1.5-fold). However, these results need to be confirmed as bioassays of the susceptible strain GR, were conducted by CSIRO laboratories in 2007. Validation of these bioassays is currently being undertaken at ACRI. Further experiments are planned to investigate cross-resistance between conventional chemistries and Cry1Ac which is the other Bt toxin deployed in Bollgard II.

With the unexpected high frequencies of Bt resistance in Australian populations of *H. punctigera* further cross-resistance studies have been initiated to investigate the dose-response of Bt-resistant and -susceptible *H. punctigera* strains.

Strain	Insecticide	LC50	95% Confidence Intervals		Slope	Resistance Ratio
			Lower	Upper		
GR	Fenvalerate	0.07(µg/larva)	0.049	0.089	2.3	
	Bifenthrin	0.02 (µg/larva)	0.018	0.026	3.6	
	Chlorpyrifos	3.83 (µg/larva)	3.091	4.752	2.5	
	Methomyl	0.91 (µg/larva)	0.757	1.132	1.8	
	Spinosad	0.31 (µg/larva)	0.254	0.361	2.2	
	Indoxacarb	0.03 (µg/larva)	0.024	0.031	3.6	
	Rynaxypyr	4.66 (ng/larva)	3.794	5.755	2.8	
	Emamectin Benzoate	5.51 (ng/larva)	3.425	7.763	1.6	
Mahon et al. 2007	Cry1Ac	0.01 (µg/cm^2)	0.009	0.018	1.2	
Mahon et al. 2007	Cry2Ab	0.09 (µg/cm^2)	0.070	0.110	1.1	
SP15	Fenvalerate	0.08 (µg/larva)	0.054	0.104	2.0	ns
	Bifenthrin	0.02 (µg/larva)	0.015	0.027	3.5	ns
	Chlorpyrifos	0.72 (µg/larva)	0.623	0.835	4.2	- 5.3
	Methomyl	0.17 (µg/larva)	0.112	0.250	1.3	- 5.4
	Spinosad	0.46 (µg/larva)	0.289	0.659	2.2	ns
	Indoxacarb	0.02 (µg/larva)	0.020	0.030	4.3	ns
	Rynaxypyr	4.26 (ng/larva)	3.217	5.703	2.7	ns
	Emamectin Benzoate	5.02 (ng/larva)	3.622	6.492	2.2	ns
Mahon et al. 2007	Cry1Ac	0.02 (µg/cm^2)	0.013	0.024	1.2	1.6
Mahon et al. 2007	Cry2Ab	607.83 (µg/cm^2)	269.8	1369.4	0.8	6830
KOS	Fenvalerate	1.66 (µg/larva)	1.195	2.225	2.4	23.7
	Bifenthrin	0.13 (µg/larva)	0.112	0.151	5.6	6.6
	Chlorpyrifos	2.66 (µg/larva)	2.025	3.432	2.0	ns
	Methomyl	1.32 (µg/larva)	0.982	1.840	1.6	ns
	Spinosad	0.20 (µg/larva)	0.158	0.243	2.4	ns
	Indoxacarb	0.02 (µg/larva)	0.016	0.028	3.9	ns
	Rynaxypyr	3.44 (ng/larva)	2.901	4.071	3.0	ns
	Emamectin Benzoate	6.47 (ng/larva)	3.888	9.535	1.3	ns
	Cry1Ac	0.05 (µg/cm^2)	0.042	0.057	2.6	4.6?
	Cry2Ab	0.07 (µg/cm^2)	0.064	0.086	2.6	ns

Table 2: Cross resistance in *Helicoverpa armigera*

7. *Using appropriate bioassay techniques accumulate baseline dose-response data for new Helicoverpa insecticides*

Preliminary investigations were carried out in 2009/10 relating to the dose-response of *H. armigera* to the anthranilic diamide cyazypyr . These insecticides activate the ryanodine receptor and cause the unrestricted movement of calcium into the muscle cells of insects (Cordova *et al*. 2006). The new insecticide cyazypyr has the added advantage of being active against whitefly.. The results of these assays indicated an appropriate range for assays between 0.005 and 0.5 ppm final concentration of active ingredient in diet. For *H. punctigera*, a slightly lower concentration range between 0.002 and 0.2 ppm appeared appropriate. For the three *H. armigera* strains tested that gave statistically acceptable results (Table 3), LD$_{50}$ values in the range of 0.015-0.070 ppm were obtained. These preliminary assays provide the necessary information to facilitate further accumulation of baseline dose-response data for final determination of a discriminating dose for use in resistance monitoring in the event that cyazypyr is registered for use against *Helicoverpa* spp.

Population (*H. armigera*)	**n**	**Slope**	**LC$_{50}$** (ppm final conc in diet) **(FL$_{95\%}$)**	**χ^2 (df)**
3009N F2	240	3.67	0.053 (0.041-0.070)	11.0 (4)
3016Tw F2	240	3.76	0.064 (0.052-0.080)	1.5 (4)
3126M F1	233	3.42	0.019 (0.015-0.024)	9.6 (4)

Table 3: Dose-response results of three *H. armigera* strains against cyazypyr.

8. *Characterise an esterase protein produced by a gene isolated from H. armigera and assess its role in resistance*

While confirmation of the full length esterase sequence isolated from *H. armigera*, allowed for initiation of the characterisation of this gene and its protein product, the vacation of the research scientist from this position in March 2010 resulted in the discontinuation of this research.

9. *Conduct further molecular investigations of esterase properties in H. armigera*

Two RNA and subsequent cDNA samples were prepared for RT-PCR and sequencing for a 2 single larvae of both a pyrethroid resistant and susceptible strain. Several PCR products from these four larvae encompassing the entire gene sequence were ligated into cloning vectors and transformed, with subsequent plasmid extraction for sequencing. Overall, for the resistant larvae cDNA, good sequencing data was obtained for both larvae, from 4 different PCR samples using different primer combinations. Within these 4 samples, there were also replicate sequences from clones obtained from the same PCR For the susceptible larvae cDNA, good sequencing data was also obtained for both larvae, from 3 different PCR samples using different primer combinations. Within these 3 samples, there were also replicate sequences from clones obtained from the same PCR. Having sequencing data from separate and replicate PCR samples as well as replicate larvae reduces the chance of an error in the esterase consensus sequence caused by a mismatching during the PCR.

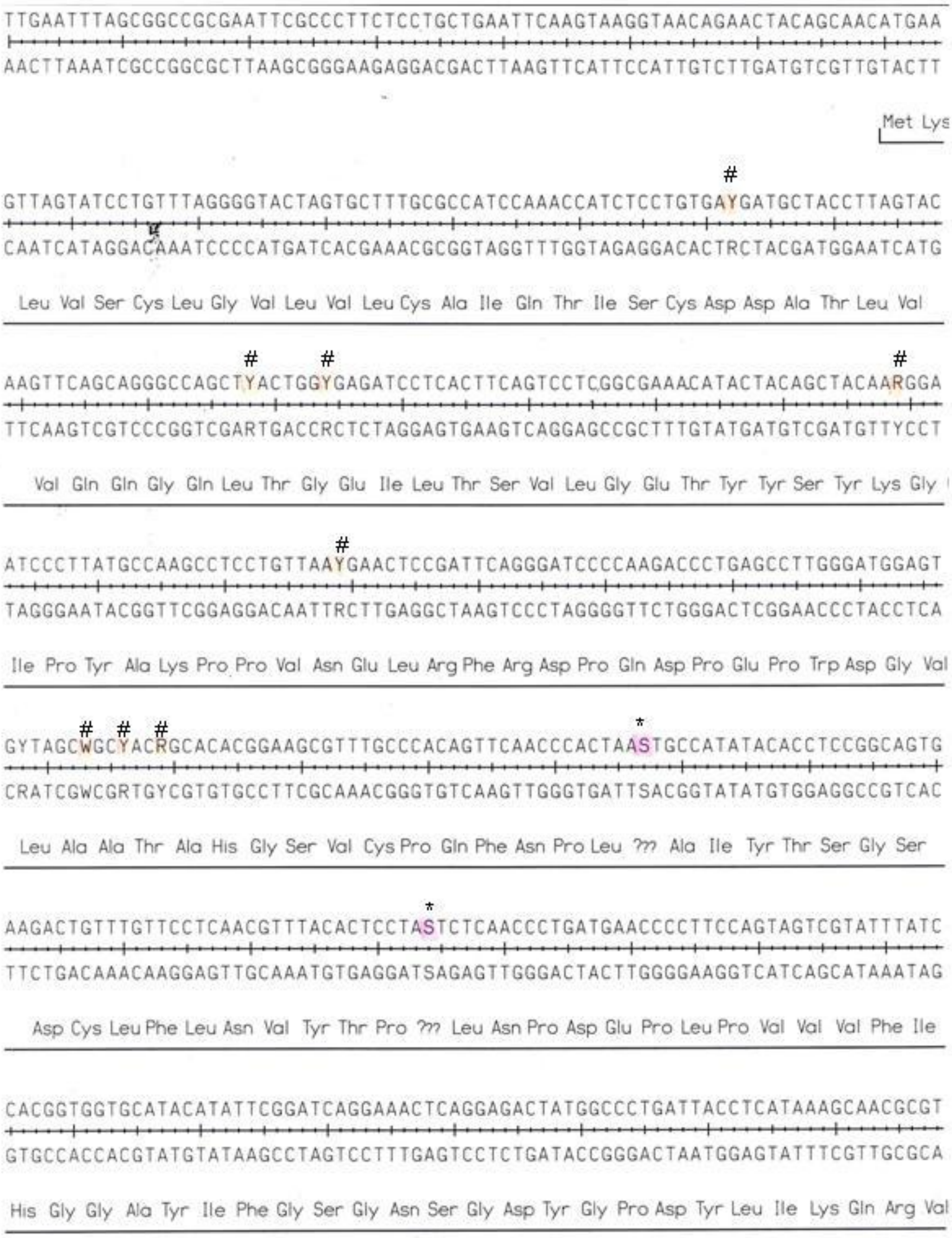
All resistant sequences and all susceptible sequences were aligned to determine a consensus sequence for each. The resistant and susceptible sequences were then aligned to identify any differences which might be related to resistance. A consensus sequence was obtained from both resistant larvae, which were the same as each other. A consensus sequence for both susceptible larvae was likewise determined, however the sequence for each larva was different. Comparison of these sequences with the resistant sequence revealed that one of the susceptible larvae had an esterase sequence that matched the resistant sequence. There was only one cDNA sequence used for this larva. The possible causes of this difference that matched the resistant sequence include that this larva was a heterozygote, or there was contamination or a mix up of samples at either the larval stage, or the RNA/cDNA stage.

Disregarding this susceptible sample, there were at least 19 base differences between the resistant and susceptible cDNA sequences (Figure 13), with possibly a number of further differences which could not be confirmed due to insufficient sequence data from replicate samples. Of the 19 differences that were consistent and found in replicate cDNA sequences, five resulted in coding for different amino acids, the other point mutations resulted in coding for the same amino acid between the R and S sequence.

10. Support other Helicoverpa related research projects with field collected material and field collected laboratory reared insect colonies

This project maintains an insecticide susceptible and field derived *H. armigera* and *H. punctigera* colonies for use within the project, which are available to support other related projects. Regular support has been provided to a number of NSW DPI projects investigating *Helicoverpa* semiochemicals (supervised by Dr Robert Mensah). The colonies have also been used by various other NSW DPI and CSIRO researchers at ACRI. *H. armigera* have also been supplied to Department of Rural Science and Environment, University of New England, for teaching purposes on a number of occasions.

Figure 13: Final esterase consensus sequence with point mutations highlighted. #: mutation that codes for the same amino acid, *: mutation that results in a different amino acid in the R and S sequences. Y: C/T, R: A/G , W: A/T, S: C/G , M: C/A



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                                                                   Met Lys

                                                         #
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 Leu Val Ser Cys Leu Gly Val Leu Val Leu Cys Ala Ile Gln Thr Ile Ser Cys Asp Asp Ala Thr Leu Val

                   #     #                                        #
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 Val Gln Gln Gly Gln Leu Thr Gly Glu Ile Leu Thr Ser Val Leu Gly Glu Thr Tyr Tyr Ser Tyr Lys Gly

                          #
ATCCCTTATGCCAAGCCTCCTGTTAAYGAACTCCGATTCAGGGATCCCCAAGACCCTGAGCCTTGGGATGGAGT
TAGGGAATACGGTTCGGAGGACAATTRCTTGAGGCTAAGTCCCTAGGGGTTCTGGGACTCGGAACCCTACCTCA

 Ile Pro Tyr Ala Lys Pro Pro Val Asn Glu Leu Arg Phe Arg Asp Pro Gln Asp Pro Glu Pro Trp Asp Gly Val

      #  #  #                                     *
GYTAGCWGCYACRGCACACGGAAGCGTTTGCCCACAGTTCAACCCACTAASTGCCATATACACCTCCGGCAGTG
CRATCGWCGRTGYCGTGTGCCTTCGCAAACGGGTGTCAAGTTGGGTGATTSACGGTATATGTGGAGGCCGTCAC

 Leu Ala Ala Thr Ala His Gly Ser Val Cys Pro Gln Phe Asn Pro Leu ??? Ala Ile Tyr Thr Ser Gly Ser

                                 *
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 Asp Cys Leu Phe Leu Asn Val Tyr Thr Pro ??? Leu Asn Pro Asp Glu Pro Leu Pro Val Val Val Phe Ile

CACGGTGGTGCATACATATTCGGATCAGGAAACTCAGGAGACTATGGCCCTGATTACCTCATAAAGCAACGCGT
GTGCCACCACGTATGTATAAGCCTAGTCCTTTGAGTCCTCTGATACCGGGACTAATGGAGTATTTCGTTGCGCA

 His Gly Gly Ala Tyr Ile Phe Gly Ser Gly Asn Ser Gly Asp Tyr Gly Pro Asp Tyr Leu Ile Lys Gln Arg Val
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                                         *
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                                #                                      #
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TTGTCGCAATGATAAATGCCTCTGTGACGACCRCCTCGAAGCCAGTGAGAAGTATACGACAGTGGCTACAGWTT
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                                                            #         *
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 Gly Leu Phe His Lys Ala Ile Ala Met Ser Gly Ser Pro Thr Cys Asp Tyr Gly Ile Thr Tyr Lys Pro ???

                         #
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TCTTCCGTTTCTAAAAGCCTTTTAAYGAGCCGCGAGGTCTATGACTTTTGTGATGTCGTGAGGAACTTAAGGAA
  Lys Ala Lys Ile Phe Gly Lys Leu Leu Gly Ala Pro Asp Thr Glu Asn Thr Thr Ala Leu Leu Glu Phe Leu

                          #      #       #
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GTCTCACGACTGATACTATGAAAGATRGGATAGYGAGGTTGRCACGACCGAAGTCTTCTCTAATGGCTACAAAA
 Gln Ser Ala Asp Tyr Asp Thr Phe Tyr Pro Ile ??? Pro Thr Val Leu Ala Ser Glu Glu Ile Thr Asp Val Leu

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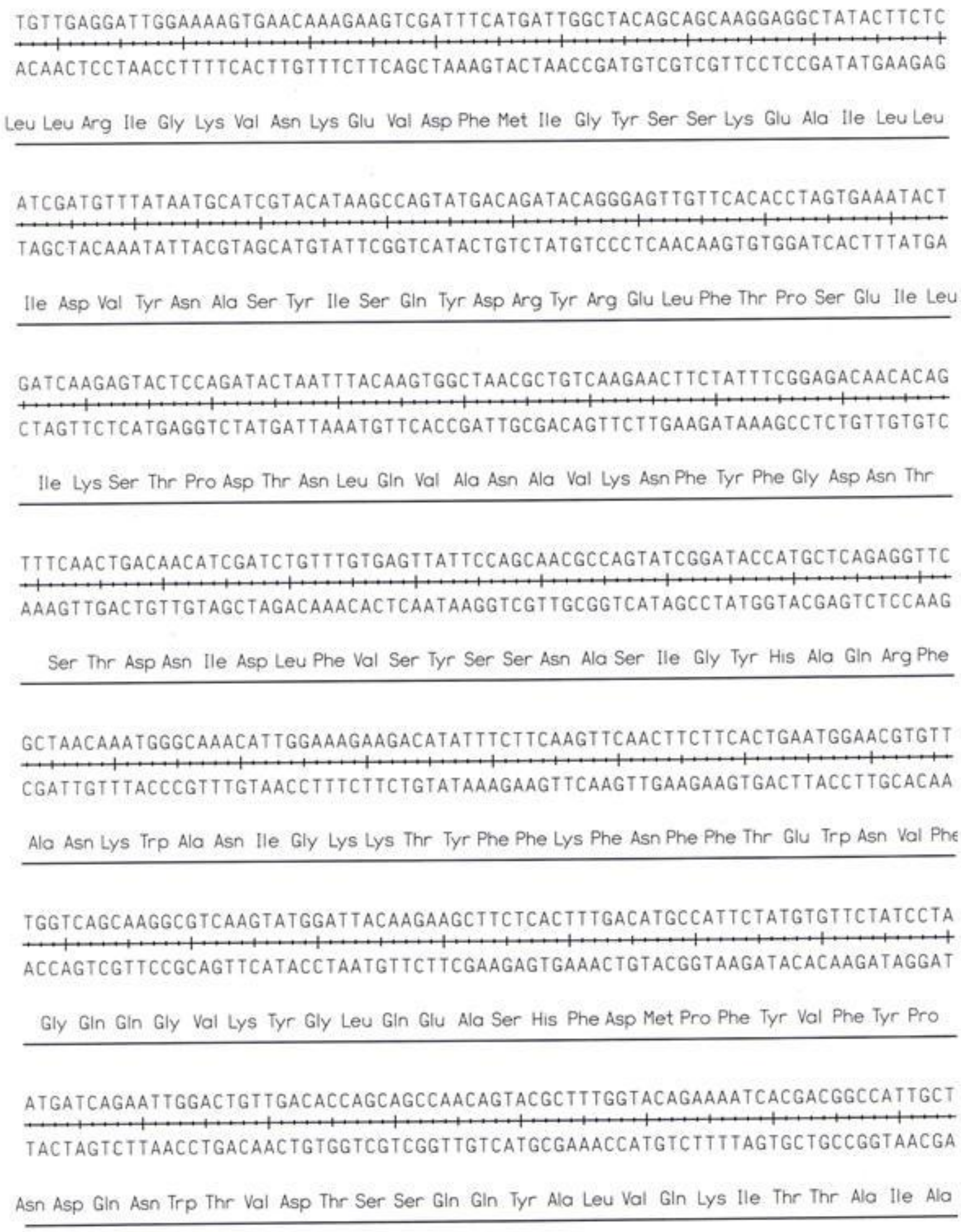

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Ile Asp Val Tyr Asn Ala Ser Tyr Ile Ser Gln Tyr Asp Arg Tyr Arg Glu Leu Phe Thr Pro Ser Glu Ile Leu

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Ser Thr Asp Asn Ile Asp Leu Phe Val Ser Tyr Ser Ser Asn Ala Ser Ile Gly Tyr His Ala Gln Arg Phe

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Ala Asn Lys Trp Ala Asn Ile Gly Lys Lys Thr Tyr Phe Phe Lys Phe Asn Phe Phe Thr Glu Trp Asn Val Phe

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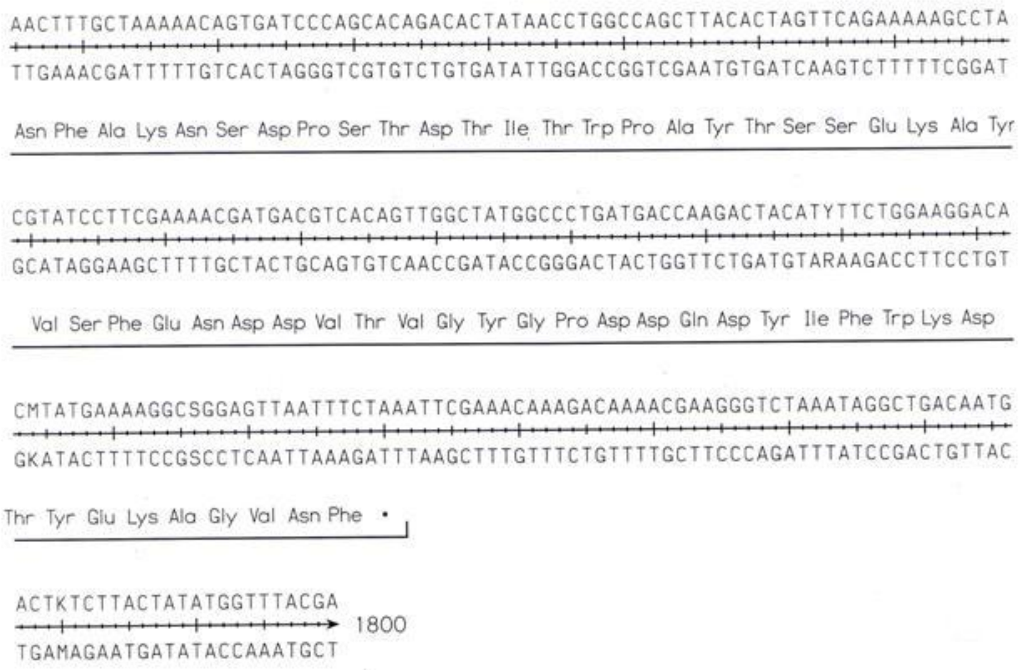

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Val Ser Phe Glu Asn Asp Asp Val Thr Val Gly Tyr Gly Pro Asp Asp Gln Asp Tyr Ile Phe Trp Lys Asp

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5. Outcomes

Outcome 1: Throughout this project data was accumulated to describe the incidence of insecticide resistance in *Helicoverpa* species in mixed farming systems incorporating cotton in Australia over a three year period from the spring of 2008 to the autumn of 2011. This data was available to be used by the TIMS committee to assess the success of management strategies and to support amendments to the IRMS in response to emerging resistance issues. Without this information the committee could not confidently endorse amendments to the IRMS, nor could it measure benefits of the strategy. Moreover, promotion of the strategy would be difficult, with little incentive for cotton growers to utilise conventional insecticides in compliance with recommendations of the IRMS.

Outcome 2: Results from the insecticide resistance monitoring component of this project is a direct measure of the success of the IRMS because the data describes spatial and temporal variation in resistance frequencies in response to patterns of insecticide use. The trends observed by analysis of this data facilitates a greater understanding of effective insecticide resistance management in cotton production and enables formulation of improved strategies incorporating all insect pests and associated resistance issues within an IPM framework. The monitoring data provided herein indicates that strategies in place (supported by other factors) have contributed to the observed stabilisation of resistance frequencies at low levels.

In 2008 an amendment was made to the length of the abamectin window in response to a request for use on mites. This amendment was supported by monitoring data for the target pest *H. punctigera*. Data from this project suggests that this change has not impacted on the resistance risk for abamectin in this species.

Outcome 3: Accumulation of dose-response data from geographically diverse populations contributes to knowledge of the resistance risk for different insecticidal classes. The establishment of a realistic range of susceptibility (including low level, polygenic tolerance) is imperative in resistance monitoring because variation in susceptibility impacts on the criteria for resistance. Appropriate bioassay techniques and accurate determination of discriminating doses is, therefore of critical importance in order to detect the presence of resistance genes in populations before they reach high frequency.

The development of appropriate topical and feeding bioassays for the anthanilic diamide cyazypyr, directly contributes to this outcome. Although progress in establishing baseline data for this new insecticide was limited (due to staff absences), pilot studies were initiated including range-finding experiments to determine the dose-response on a limited number of strains.

Outcome 4: Genetic/biochemical mechanisms to key IMP compatible chemistries. This outcome was also largely dependent on colonies resistant to key IPM compatible chemistries.

Outcome 5: The patterns of cross-resistance were studied in isogenic strains of pyrethroid resistant and Cry2Ab resistant strains of *H. armigera*. Studies showed no cross-resistance when pyrethroid (fenvalerate) resistant insects were exposed to Cry2Ab toxin or the IPM compatible chemistries utilised for control of *H. armigera* in cotton production, indicating that elevated resistance to older chemistries does not compromise efficacy of Cry2Ab or the newer chemical classes. Results also suggest 5-fold negative cross-resistance between Cry2Ab and carbamate/organophosphate insecticides. Although requiring verification preliminary results suggest low level cross-resistance between pyrethroid and Cry1Ac.

Outcome 6: Comparison of the one esterase sequence from resistant and susceptible larvae yielded an interesting outcome in terms of differences that resulted in coding for different amino acids. Such a difference could be a cause of resistance. Studies such as these are part of building an overall picture of resistance and the mechanisms behind it, and provide the basis for further investigation.

6. Conclusions

Insecticide resistance monitoring was successfully conducted in 2008/09, 2009/10 and 2010/11. The objective was to detect resistance to chemistries used against *Helicoverpa* and monitor trends and shifts in resistance allele frequency. These results were utilised by TIMS as part of assessing the success of the IRMS and formulating changes in response to possible resistance events.

Conclusions from this monitoring were:

- Very low but detectable levels of resistance to IPM compatible chemistries including indoxacarb, spinosad, emamectin benzoate and rynaxypyr.
- Resistance remains present to those chemistries that *H. armigera* is known to have developed resistance to, including endosulfan and methomyl. However frequencies remain stable following significant declines in recent years.
- Widespread general pyrethroid resistance remains present in *H. armigera* populations as indicated by high resistance frequencies to fenvalerate. However resistance to bifenthrin is low and relatively stable.
- Very low frequency of resistance to endosulfan, pyrethroids and abamectin in *H. punctigera*.
- Spatial and temporal data recorded for species composition within cotton growing regions, with implications for the monitoring project and also for resistance management.

These results have direct implications for insecticide use within the cotton, grains and pulse industries. They demonstrate that the IPM compatible insecticides continue to be effective for control of *Helicoverpa* species with older insecticides providing useful alternatives for early season control of *H. punctigera*. However the reduction and stabilisation of resistance to conventional chemistries can largely attributed to a reduction in insecticide use since the introduction of Bollgard II. Nevertheless conventional insecticides continue to be used within the IRMS to control Helicoverpa in conventional cotton where it is planted as an alternative to Bollgard II and when used as a sprayed refuge. Therefore, it is imperative to retain efficacy of these chemistries, particularly if usage increases, and the cotton, grains and pulses industries are currently well placed to resume the use of those insecticides, if required, within an IPM system.

Experiments were conducted to investigate resistance development within a *H. armigera* population. However, attempts to select for resistance to the newer chemistries which are of key importance in IMP systems were problematic, and had limited success. This can be attributed to the fact that there was very low survival to discriminating doses of insecticides being investigated. Where surviving individuals were established in single-pair families, mating success was low, or resulted in progeny that could not be established as long-term resistant strains. Although this hindered investigations, the demonstration of a possible fitness cost associated with the genes that confer resistance to these chemistries, in particular indoxacarb, is of interest in terms possible impacts on delaying resistance development in the future, and this will be pursued in ensuing seasons. Strains resistance to older insecticides, particularly the strain selected for pyrethroid resistance which is conferred by a putative metabolic resistance mechanism, was utilized in studies to investigate patterns of cross-resistance in *H. armigera*.

Studies of cross-resistance indicated that the efficacy of newer chemical classes is not compromised by high frequencies of resistance to either pyrethroid (fenvalerate) or Cry2Ab. Results also suggest 5-fold negative cross-resistance to carbamate/organophosphate insecticides, and Cry2Ab. Preliminary results suggest low level cross-resistance between pyrethroid and Cry2Ab.

Cross-resistance studies will continue into the future and focus on resistance to new chemical classes of insecticide as well as investigations into mechanisms that confer low level resistance and the possibility that these genes confer cross-resistance between different chemical classes and insecticidal toxins.

Comparison of the sequence of one esterase from pyrethroid resistant and susceptible larvae suggested a possible role in resistance for this esterase, with several point mutations between the S and R strain coding for different amino acids. Investigation of the molecular aspect of resistance in *H. armigera* has now ceased, however the results shall remain the property of NSW and shall be available for future reference if this research is recommenced.

7. Extension

The results of this project have been disseminated using various methods as outlined below. In addition the TIMS committee has been annually updated on the status of insecticide resistance by *H. armigera* as part of assessing and formulating management strategies. The methods of extension outlined shall continue to be utilised. Aspects of the project shall be further disseminated to the scientific community through the publication of peer reviewed scientific articles (in preparation).

Publications

Extension articles

Rossiter, L. (2009). Data for management strategies. In Boehm R. (Ed.) Spotlight on Cotton R&D, Autumn 2009 pg 21.

Contributions to extension publications and manuals

Rossiter, L., Larsen, D., Downes, S., Wilson, L. Murray, D. & Miles, M (2010). Insecticide Resistance Management Strategy (IRMS) for 2010/11. In Cotton Pest Management Guide 2010/11, pages 61-65. Industry and Investment NSW

Rossiter, L., Larsen, D., Downes, S., Wilson, L. Murray, D. & Miles, M (2009). Insecticide Resistance Management Strategy (IRMS) for 2009/10. In Cotton Pest Management Guide 2009/10, pages 47-51. Industry and Investment NSW.

Rossiter, L., Farrell, T., Larsen, D., Downes, S., and Wilson, L. (2008). Insecticide resistance management strategy (IRMS) for 2008/2009. In Farrell, T. (Ed) Cotton Pest Management Guide, 2008-09. NSW Dept. of Primary Industries.

Oral presentations

Rossiter, L. (2009). End of season Bollgard II spray: insecticide choices. ACGRA Research and Extension in Bt resistance and management meeting, 19th Feb 2009, oral presentation.

Rossiter, L. Chemical control in cotton pest management. Presentation to UNE Cotton Production Course students. August 2008 and 2009.

TIMS Resistance Roadshow 2009. Oral presentations to growers and consultants at five locations in NSW and QLD: ‘Conventional Insecticide Resistance Monitoring, 2008/09’: *Helicoverpa* spp. results.

Rossiter, L. Insecticide Resistance – overview and management within the cotton industry. Presentation to students at Tocal Agricultural College, 2009

Media presentations

25 years of insecticide resistance management. CSD Web on Wednesday Website: 15th October 2008.

8. Executive Summary

Insecticide resistance represents one of the most significant limitations to successful pest control and economic production in Australian cotton industry. This project has continued a long-term resistance monitoring program for *Helicoverpa armigera* and *H. punctigera* for three seasons from 2008 to 2011. Other aspects of the project include cross-resistance and resistance mechanism research, accumulation of dose response data for new insecticides and resistance management formulation and promotion.

Insecticide resistance monitoring was conducted in 2008/09, 2009/10 and 2010/11. The objective of the monitoring program was to detect resistance to conventional insecticidal chemistries used for control of *Helicoverpa* species and monitor trends and changes in resistance frequency. Conclusions from monitoring were:

- Very low resistance frequencies detected to the IPM compatible chemistries of indoxacarb, spinosad, emamectin benzoate and rynaxypyr.
- Resistance remains present but stable in field populations of *H. armigera* to chemistries that this species has developed resistance to in the past, including endosulfan and methomyl
- Widespread general pyrethroid resistance remains present in *H. armigera* populations as indicated by high resistance frequencies to fenvalerate. However resistance to bifenthrin is low and relatively stable.
- Very low level resistance to endosulfan, pyrethroids and abamectin in *H. punctigera*.

These results have direct implications for insecticide use within the cotton, grain and pulse industries. They indicate that effective control of Helicoverpa will continue to be provided by the IPM compatible insecticides utilised by these industries. Studies of cross-resistance conducted in this project demonstrate a lack of cross-resistance between these chemistries and Bt toxins which also gives confidence that efficacy will not be compromised by the high uptake of Bollgard II by the Australian cotton industry. Nevertheless, detectable resistance to these compounds, albeit at very levels, highlights the importance that insecticides continue to be used within the IRMS and that they continued to be monitored to ensure that their effectiveness is retained, particularly if their overall use increases.

The observed stabilisation of resistance to older insecticides is consistent a reduction in selection pressure associated with declines in insecticide use since the introduction of Bollgard II.

Comparison of the sequence of one esterase from pyrethroid resistant and susceptible larvae suggested a possible role in resistance for this esterase, with several point mutations between the S and R strain coding for different amino acids. Studies such as this are part of building an overall picture of resistance and the mechanisms behind it, and provide the basis for further investigation.

In addition to resistance monitoring and mechanism research for chemicals currently registered for use on cotton, it is essential that baseline responses be established for new chemistries prior to registration. Following on from similar work in establishing discriminating dose bioassays for rynaxypyr, a pilot study for determining the dose-response of a chemically similar anthranilic diamide, cyazypyr was undertaken. This enables early detection of resistance development in populations before it is evident in the field and allows appropriate management responses to be activated in order to minimise future resistance risk.

The final aspect of this research is the formulation and promotion of resistance management strategies and principles. The assessment and formulation of the IRMS by TIMS has utilised resistance monitoring data which has enabled changes to be made to the IRMS at the request of the industry.

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Forrester, N.W., M. Cahill, L.J. Bird and K. Layland. 1993. Management of pyrethroid and endosulfan resistance in *Helicoverpa armigera*. *Bulletin of Entomological Research, Supplement Series* **Supp. 1**, 1-132.

Teakle, R.E. and J.M. Jensen. 1985. Rearing *Heliothis punctiger*. pp. 313-322 *in* Singh, P. and Moore, R.F. (*Eds*) *Handbook of insect rearing Vol II*. Amsterdam, Elsevier.

10. Acknowledgements

This final report was compiled by Dr Lisa Bird, with significant contribution from Dr Louise Rossiter. While the research scientist position was vacated in March 2010, Dr Rossiter continued to informally manage the project until the position was filled by Dr Bird in April 2011. This ensured the project continued to operate at a base level, including analysis of the 2009/10 resistance monitoring results and their presentation to the TIMS committee, submission of progress reports, and the completion of the resistance monitoring in 2010/11. The successful completion of this project could not have been achieved without the contribution and efforts of:

Fiona McKenzie (Technical Officer), Debbie Richardson (Technical Assistant) and Cynthia Wilson (Technical Assistant), NSW DPI, who provided technical expertise in the insectary and laboratory through the three years of the project, particularly the extra efforts of Debbie that allowed for the project to continue after the research scientist vacated the position during which time Fiona was on maternity leave.

Dawn Smith, Barbara Bowning, Stacey Dorahy and Katie Browning, egg collectors for the Gwydir and Namoi Valleys, NSW DPI.

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Dr Grant Herron and Martin McLoon for their collaborative contribution to the esterase gene identification.

Dr Sharon Downes and Tracey Parker, CSIRO for access to their field derived lab colonies.

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Regional Cotton Extension Officers (RCEOs), particularly in regard to co-ordinating collections in regional areas ensuring their timely arrival in good condition at ACRI. We appreciate the excellent assistance provide by Sally Ceeney, Susan Maas, Dallas King, and James Hill in co-ordinating the collections as part of their RCEO role.

Numerous growers and consultants throughout all the valleys, but particularly in the Gwydir and Namoi Valleys for access to and information regarding cotton fields for sampling purposes.

DuPont for supporting the preliminary baseline dose response research and provision of Cyazypyr samples to complete the work.

The GRDC provided additional financial support to this project.