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Conventional insecticide resistance in Helicoverpa – monitoring, management and novel mitigation strategies in Bollgard III

FINAL REPORT: DAN1506 & DAN00204

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Part 1 - Summary Details

CRDC Project Number: DAN1506

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Project Title: Conventional insecticide resistance in Helicoverpa – monitoring, management and novel mitigation strategies in Bollgard III

Project Commencement Date: 1.7.2014 **Project Completion Date:** 30.6.2019

CRDC Program: Farm

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Part 3 – Final Report

Background

Species from the Helicoverpa genus are among the most damaging pests in agriculture. The cotton bollworm, *Helicoverpa armigera* is particularly problematic because of a capacity to develop resistance to insecticides. Field resistance in this species results in reduced efficacy of insecticides and represents a major cost to agricultural production in terms economic, environmental and social consequences. While the introduction of transgenic cotton has reduced reliance on insecticides for control of *Helicoverpa* spp., chemical insecticides remain the primary method of pest control in sprayed non-transgenic cotton and in other broad-acre production systems which are important rotational options for cotton growers. In particular, the use of some Helicoverpa-selective products with broad registration across different summer and winter pulses represents a significant challenge for resistance management in mixed production systems. Therefore, stewardship of key Helicoverpa insecticides to support cross-sector agricultural sustainability is an issue shared by both the cotton and grains industries.

Resistance monitoring and associated research delivers an evidence-based evaluation of resistance risk associated with the use of insecticides in Helicoverpa management as well as being critical for informing a broader process of formulating insecticide resistance management strategies (IRMS in cotton and RMS in grains). These industry-endorsed strategies underpin strategic responses to emerging resistance issues, and provide a platform to promote adoption of key recommendations that support product stewardship.

An understanding of the genetic and molecular basis of resistance provides a powerful predictive tool in resistance management. To achieve this, research is focused on resistance isolated from the field to 1) ensure the frequency distribution selected is representative of wild populations 2) reduce the unintended consequences of polygenic selection 3) progress development of molecular diagnostics in resistance surveillance. Serial backcrossing of resistant strains followed by quantitative genetic analysis and molecular characterisation provides an evidence-based assessment of the resistance risk associated with presence of resistant genotypes in the *H. armigera* population and provides valuable insight for improvement of resistance management strategies.

Selection pressure for Bt resistance is intense due to the widespread uptake of transgenic cotton. A higher than expected resistance frequency of Bt resistance genes in *Helicoverpa* spp. increases risk for resistance Bollgard cotton. A key priority of the project has been to maintain collaborative links with other research agencies for investigating interactions between Bt toxins and insecticidal chemistries as this is important for identifying potential risks to both the efficacy of sprays and the sustainability of transgenic technologies.

A key imperative in resistance surveillance is that the most scientifically rigorous methods available are utilized to generate data. A priority throughout the DAN1506/00204 project was to capitalise on improvements in monitoring techniques implemented during the previous project (DAN1204/00164) by investigating innovative methods for improving field sampling. Both approaches have significantly enhanced the efficiency for detecting resistance, firstly because of higher sensitivity of genic screening compared with phenotypic screening, and secondly because of improved efficiency through the adoption of moth trapping which has replaced traditional labour intensive methods that rely on visual sampling of immature life stages. As a result of these substantive improvements, NSW DPI is well positioned to deliver a scientifically robust and cost-effective program to meet the needs of the cotton and grains industries.

List of objectives (and milestones) and extent of achievement

Objective 1. Conduct insecticide resistance monitoring annually including delivery of outcomes across the cotton and grains industries

Milestone 1.1. Pheromone trapping networks established in the Namoi/Gwydir and Macintyre valleys and monitored fortnightly from December to April. Achieved.
Milestone 1.2. Pheromone traps co-ordinated in other cotton growing areas through liaison with industry collaborators. Achieved.
Milestone 1.3. Pheromone traps co-ordinated in pulse growing areas through liaison with industry collaborators. Achieved.
Milestone 1.4. Resistance monitoring completed annually for *H. armigera* using F_2 screening methods for detection of resistance to selective chemistries. Achieved.
Milestone 1.5. Insecticide Resistance Management Strategy (IRMS) formulated through involvement with TIMS committee and accepted by the Australian cotton industry. Additional resistance management guidelines formulated and promoted through publications and presentation at meetings. Achieved.
Milestone 1.6. Resistance monitoring specifically aimed at determining potential for enrichment of resistance genes (resistance risk) in preferred host crops for *H. armigera* with monitoring focused in mixed cropping areas of the Namoi valley. Achieved.
Milestone 1.7. Support other Helicoverpa related research projects with field collected material and field collected laboratory reared insect colonies. Achieved.

Objective 2. Isolation and characterisation of mechanisms that confer resistance to selective chemistries in *H. armigera* if detected from the field

Milestone 2.1. Using survivors from F_2 screens of *H. armigera*, establish strains that confer resistance to selective chemistries by multiple backcrossing. Achieved.
Milestone 2.2. Characterise the genetic basis of resistance for novel mechanisms isolated from survivors of F_2 screens using specific crosses and bioassays. Achieved.
Milestone 2.3. Investigate patterns of cross-resistance in strains with novel resistance to selective chemistries. Achieved.

Objective 3. Conduct studies to determine patterns of cross-resistance between Bt-resistant Helicoverpa strains and conventional chemistries

Milestone 3.1. In collaboration with CSIRO test the dose response of VIP3A resistant strains of *H. armigera* and *H. punctigera* against conventional chemistries registered for use against Helicoverpa. Achieved.

Objective 4. Preparing for new product options

Milestone 4.1. Using appropriate bioassay techniques, accumulate baseline data for new Helicoverpa insecticides pending registration. Achieved.

Objective 5. Develop a collaboration with Monsanto for investigating cross-resistance in recently isolated Bt resistant strains of *H. punctigera*

Milestone 5.1. Test dose response of Bt resistant strains of *H. punctigera* against conventional chemistries registered for Helicoverpa. Pending, carried over to new project.

Objective 6. Undertake project review and gather industry feedback regarding work completed and establish whether there are new industry research priorities Achieved.

Objective 1. Conduct insecticide resistance monitoring annually including delivery of outcomes across the cotton and grains industries

Milestone 1.1 Pheromone trapping networks established in the Namoi/Gwydir and Macintyre valleys and monitored fortnightly from December to April

In the first year of this project an alternative method for sourcing insect samples for resistance testing was trialled in a pilot study conducted between October 2014 and March 2015. This involved the use of pheromone traps for live capture of male moths. Samples from the Liverpool Plains (collected by Dr Peter Gregg UNE, and surplus to the needs of the Bt-monitoring project) were utilized for establishing isofemale families for F_2 screening of selective Helicoverpa insecticides.

Following the success of this pilot study the pheromone trapping network was expanded throughout the Namoi valley in 2015-2016 using specialised Heliothis net traps (Scentry Biologicals Inc., Billings, MT) baited with *H. armigera* female pheromone lures (InSense Pty. Ltd. Cobram, VIC) (Text Box 1.1.1). This provided further evidence that pheromone trapping was an effective alternative to traditional visual egg and larval Helicoverpa sampling (see *Supplementary Milestone 1.9* for supporting data). By 2016-17 the project had fully transitioned to a pheromone-based method for sourcing insects for resistance testing.

From 2015-16 to 2018-19 pheromone traps were deployed every two to three weeks in suitable host crops within the Namoi, Gwydir, Macintyre valleys from September/October to March (except that in 2018-19 sampling concluded in late February). The Macquarie valley was sampled in all years except 2018-19. Host crops included cotton, chickpeas, faba beans, soya beans, mung beans, pigeon pea, canola, maize, sorghum and wheat (2018-19 only). The full data set for annual moth collections by local collectors is shown in *Appendix 1*, Table A.1.2.

The adoption of live pheromone trapping increased outputs from the monitoring program for several reasons:

1. Increased capacity for generating test insects due to elimination of egg and larval parasitism and disease which cause high insect mortality.

2. Increased efficiency for generating isofemale families due to increased mating success from pheromone caught samples compared with samples collected as immature life stages (see *Supplementary Milestone 1.9* for comparison of methods).

3. Effective for targeting *H. armigera* in Helicoverpa populations with mixed species composition.

4. Increased time efficiency associated with field and laboratory processes ie. visual scouting for eggs/larvae and rearing of immature life stages were eliminated.

5. More efficient use of labour and resources.

6. Faster rates of accumulating resistance data.

In summary, the development and implementation of the pheromone trapping method enabled uninterrupted delivery of the insecticide resistance surveillance program following the cessation of the Crop Consultants Australia (CCA) Helicoverpa collection project in 2016, and led to substantive improvements in the quality and rigour of resistance monitoring outcomes.

Milestone 1.2 Pheromone traps co-ordinated in other cotton growing areas through liaison with industry collaborators

The full data set for annual moth collections by external collaborators is shown in *Appendix 1*, Table A.1.2. Collaborators from QDAF commenced pheromone sampling in August in the Emerald irrigation area. Since pheromone trapping was implemented at Emerald in 2016 the sampling effort by QDAF staff has been outstanding and has resulted in a marked increase in the volume of material available for testing which has subsequently improved the statistical power for interpretation of regional differences in resistance frequency (see Figure 1.4.1).

For example, in the years before implementation of pheromone trapping in Emerald (prior to and including 2014-15) it was not possible to analyse results for this region because of the low numbers of F_2 isofemale families available for testing from egg and larval samples (see *Supplementary Milestone 1.9*). Of particular concern was the indoxacarb frequency recorded in 2015-16 when no positive tests were recorded for this insecticide. In this instance there were only four F_2 isofemale families recovered from a possible sample size of 226 *H. armigera* collected in that year (Table A.1.3). In comparison, indoxacarb frequency was recorded as 8.0% in 2014-15 (n = 50) and 9.2% in 2016-17 (n = 426) (*Appendix 2*). This demonstrates that regional resistance frequencies can be dangerously underestimated without sufficient numbers in the testing cohort.

Text Box 1.1.1 Instructions for using the Scentry™ Heliothis net trap

Scentry Heliothis Traps are reusable insect traps for use with pheromone lures.

HOW TO USE:

The trap comes in two parts. Reshape the mesh upon removal from the package so that the inner cone (A) is clear and open at the top. Assemble by fitting the smaller top (1) cone over the bottom (2) cone and secure with velcro material (F).

Trap should be mounted on a pole or stake of sufficient length to be driven two feet into the ground and have enough height to position trap above crop.

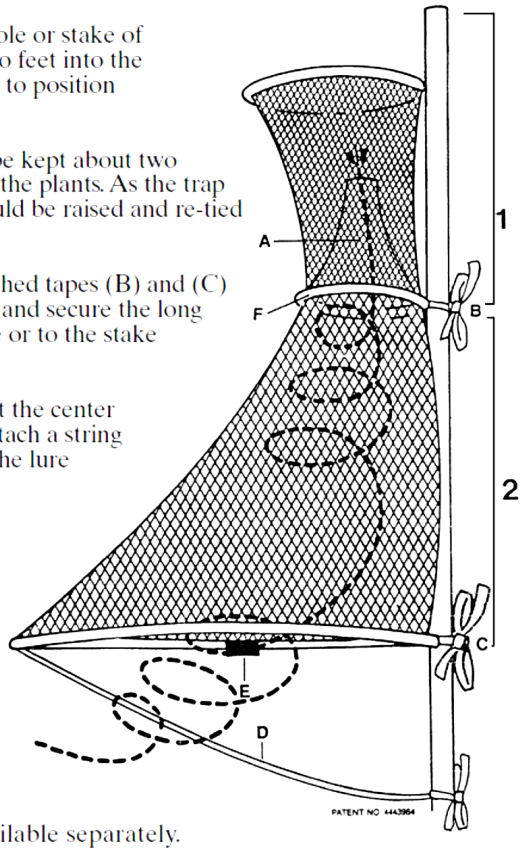
The bottom of the trap should be kept about two inches (5 cm) above the tops of the plants. As the trap is inspected and serviced, it should be raised and re-tied to maintain this distance.

Tie tightly to the pole with attached tapes (B) and (C) to prevent it from sliding down, and secure the long front tape (D) either to the pole or to the stake provided for wind protection.

The pheromone lure is placed at the center of the large bottom opening. Attach a string across the opening, and secure the lure with a paper clip (E).

DO NOT CROSS-CONTAMINATE. Each trap should be used for monitoring only one species.

The top portion (1) of the trap can be removed by pulling the velcro fastening loose. Remove insects, replace top and refasten in place.



NOTE: Top portion (1) is available separately.
Item No. R08300

Milestone 1.3 Pheromone traps co-ordinated in pulse growing areas through liaison with industry collaborators

In May 2018, with support from GRDC, this project was expanded to include grains production areas of central Qld. The project expansion was in response to industry concerns that *H. armigera* in grains regions of central and northern Qld were at increased risk of developing resistance due to the intensive use of insecticides to manage *H. armigera* in pulse crops in these locations. The risk was elevated due to ecological factors unique to northern populations which will also favour resistance selection (see *Milestone 2.5*).

The objective of targeted surveillance, particularly in pulse growing regions where insecticide use is high, was to detect regionally specific shifts in resistance frequency in discrete populations of *H. armigera* and increase industry understanding of resistance risk in key grains production areas of Qld and NSW. Importantly, detection of early-stage resistance in northern populations will improve the level of preparedness in grains and other industries that play host to this pest.

Collaborators from QDAF Emerald (Ms Gail Spargo and Ms Sharna Holman), with assistance from a local consultant (Mr Jamie Iker), commenced pheromone sampling of chickpea crops during June 2018 in the northern highlands region (Clermont district) of central Qld. Private consultants from Prospect Ag, Bowen (Mr Chris Monsour) and AgnVet, Theodore (Mr Stuart Olsson) provided the project with moth samples collected in the Burdekin and Dawson/Callide regions, respectively. Crops sampled in these central and northern districts of Qld were primarily pulse crops including soya beans, mungbeans and chickpeas with smaller collections from cotton (Dawson/Callide only).

Regionally specific resistance data will be important for ratification of the *H. armigera* Resistance Management Strategy (RMS) for Australian grains which was developed by the National Insecticide Resistance Management (NIRM) working group and released by GRDC in April 2018. Resistance monitoring is a key component in evaluating the effectiveness of the RMS and an ongoing program of surveillance is necessary to provide evidence-based reasoning for any proposed changes to the strategy. In short, there is little value in industry adoption of an RMS without an ongoing monitoring program to support and validate its existence.

Text Box 1.3.1 Investigations of alternative trapping systems in Helicoverpa resistance surveillance.

In addition to the deployment of net traps, canister traps were evaluated as a more economical trap design for catching live male *H. armigera* for the pheromone trapping network. Canister traps are considerably cheaper (~$15 each) compared with net traps (~$180 each), less cumbersome to operate and more efficient for catching male *H. armigera*. However, canister traps have a solid plastic base which may compromise the quality of moth samples due to heat stress and mechanical damage, thus reducing fitness compared with those caught in net traps which have a more ventilated mesh trapping receptacle. Our objective was to develop a cost-effective system of trapping that could be used in central and northern Qld that would not compromise sample quality. Therefore, NSW DPI trialled a hybrid trap design comprising the top of a canister trap with a mesh trapping receptacle for greater ventilation. These trials were conducted on the Liverpool Plains and in the Macquarie valley during the 2017-2018 season. Results from comparisons of trapping methods are described in

Milestone 1.4 Resistance monitoring completed annually for *H. armigera* using F_2 screening methods for detection of resistance to selective chemistries

Introduction and aims

Monitoring for resistance to selective chemistries (indoxacarb, chlorantraniliprole and emamectin benzoate) involved use of the F_2 screen adapted for a diet-incorporation bioassay of Helicoverpa larvae developed in the DAN1204/00164 project (Bird 2015). Implementation of these methods has increased capacity for accurate and robust determination of selective insecticide resistance allele frequencies in Australian populations of *H. armigera* (Bird et al. 2017). Isofemale lines for F_2 screening were established from egg, larval and moth collections from all cotton regions in 2014-15. Thereafter the project transitioned to a system that utilized only pheromone-caught moths which further enhanced efficiencies for delivery of resistance monitoring outcomes (data shown in *Supplementary Milestone 1.9*).

The aim of resistance surveillance activities was to determine annual resistance frequencies over the five years of the project in major cotton and grains production areas of eastern Australia and provide an evidence-based assessment of the effectiveness of the Insecticide Resistance Management Strategy (IRMS) primarily used by the cotton industry.

Materials and methods

Resistance screening. Resistance was tested by using an F_2 screening procedure which generates isofemale lines that produce a proportion of individuals homozygous for haplotypes present in their field derived parents (Andow and Alstad 1998). This method comprised a stepwise process for generating isofemale lines: (1) collecting the parental (F_0) generation from the field; (2) rearing F_1 offspring for each line; (3) sib-mating F_1 adults. The method used herein was modified from Stodola and Andow (2004) where field derived moths were individually mated to moths from a laboratory susceptible strain to generate isofemale lines tested for resistance in the F_2 generation.

Step 1. Establishment of F_0 generation from field collected material. Two methods were used to source *H. armigera* from the field for resistance monitoring. The first method involved visual searches for *Helicoverpa* spp. eggs and larvae conducted in a range of cultivated hosts (primarily cotton, sorghum, maize and pulses) in the major cropping areas of northern NSW and central/southern Qld between September and March, during the first field season of the project (2014-15). Eggs and larvae collected from the field were reared individually to adults in the laboratory on artificial diet. Pupae were washed in a 1% bleach solution, sorted by gender and transferred to 500ml round, plastic containers (WF Plastics Australia Pty. Ltd., Warwick Farm, NSW, Australia) open at the top and covered with cloth liners. Following emergence moths were single-pair mated with an individual of opposite sex from the laboratory susceptible strain.

The second method commenced in the 2014-15 field season and involved a live pheromone trapping system (*Text Box 1.1.1*) as a method for sourcing adult male *H. armigera* for mating directly with laboratory-reared female moths. Trapping was conducted initially in the upper Namoi valley and was expanded throughout the industry in subsequent seasons from 2015-16 to 2018-19. Moths were removed daily from the traps, transported to the laboratory and placed into single-pair mating containers along with a recently emerged female moth from the susceptible strain.

Step 2. Establishment of F_1 generation. Single-pair mating chambers containing a pheromone-caught male and female from the susceptible laboratory strain (New GR) were checked daily for the presence of eggs. Cloth liners with fertile eggs were collected and stored at 25° C. A cohort comprising 135 neonates from each pair was used to establish individual isofemale lines.

Step 3. Establishment of F_2 generation. Larvae were reared to adults and bulk-mated with siblings. A minimum of 40 moths (20 males and 20 females) constituted each isofemale line. Eggs from each

line were harvested daily for up to 4 days to ensure that at least 300 eggs had accumulated for testing.

Bioassay method. All bioassay screening procedures were performed on artificial diet into which formulated insecticide was incorporated. The ratio of diet to toxin determined the concentration calculated as micrograms of insecticide per millilitre of diet.

The discriminating concentration for emamectin benzoate, chlorantraniliprole, and indoxacarb was 0.2, 1 and 12µg of insecticide/ml of diet, respectively (Bird 2015). Commercial insecticide formulations were used in all bioassays: indoxacarb (Steward® [15% active ingredient], Du Pont Australia Ltd., Macquarie Park, Australia), emamectin benzoate (Affirm® [1.9% active ingredient], Syngenta Crop Protection) and chlorantraniliprole (Altacor® [35% active ingredient], Du Pont Australia Ltd., Macquarie Park, Australia).

Diluted insecticide was added to 1L of diet and incorporated by using a stick blender to produce a homogenous mixture. Insecticide-incorporated diet was then dispensed into 45-well bioassay trays. We aimed to expose 90 larvae (two 45-well trays) from each isofemale line to each of the three insecticides. A minimum of 500 lines were screened for each insecticide in each season.

For complementation tests for allelism, stock suspension of insecticide was diluted with distilled water to produce six or seven two-fold dilutions which were added to 200ml of diet and incorporated by vigorous shaking by hand for 30 seconds to produce a homogenous mixture then dispensed into 45-well bioassay trays, as above. Bioassays were performed on three cohorts of insects with individual treatments (insecticide concentrations) of each cohort consisting of a minimum of 20 individuals; untreated diet was used as the control.

Insects used in all bioassays were reared to the late second or early third larval instar on untreated diet. To ensure the fitness of test insects, F_2 progeny of isofemale families that did not reach the required stage of development within four to five days were discarded. Test larvae were transferred to trays containing bioassay diet (one larva per well) and covered with heat-sealed, perforated lids.

Bioassays were maintained for seven days under general rearing conditions. Larvae were considered dead if one or more of the following criteria were demonstrated: larvae unable to perform coordinated movement when prodded; paralysis of prolegs; larvae very slow to right themselves (time exceeding three seconds).

Isolation of resistance. If resistance is recessive, 6.25% of F_2 progeny will be homozygous (*rr*) for resistance. However, if resistance is inherited as a non-recessive trait the heterozygote is more likely to survive a discriminating dose of insecticide in the F_1 and the proportion of survival in the F_2 will also be increased depending on the degree of dominance of resistance. All surplus larvae were retained as a "back-up" cohort of each isofemale family and stored at 15 °C pending the result of bioassays and discarded only if screens of emamectin benzoate, chlorantraniliprole and indoxacarb produced no survivors.

If, on the other hand, one or more survivors were detected, a process of validation was undertaken which comprised two steps. Firstly, a minimum of 90 additional larvae from the back-up cohort of isofemale lines that tested positive were retested on the discriminating concentration of insecticide. Secondly, if there was more than one survivor in the retested cohort, 90 unselected larvae along with survivors of the F_2 screens were reared to adult. Progeny of surviving larvae were mated among themselves or crossed to the untreated cohort. The subsequent F_3 generation was retested and the line was retained for further characterization if it demonstrated survival at the discriminating concentration. In some cases, where there were insufficient numbers of F_2 larvae for retesting, validation of resistance was determined from the results of the F_3 bioassays.

Seven lines that tested positive for indoxacarb resistance (three from 2013-14 and four from 2014-15) were retained in laboratory culture for further analysis. In the F_4, each strain was outcrossed (except EM14-1) to the laboratory susceptible New GR strain to reduce the effects of inbreeding depression which can influence the outcome of bioassays. Each strain was then reselected with the discriminating dose of indoxacarb for four to six generations. Complementation tests for allelism

were performed by backcrossing to the reference strain GY7-39, which was the first indoxacarb strain isolated from the field in 2013 (Bird 2016). Male and female reciprocal crosses within each test were pooled because there was no difference in the median lethal concentration when bioassayed by diet incorporation.

Data analysis. A χ^2 goodness-of-fit test (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) was used to compare the proportion of survivors within isofemale lines that scored positive for resistance relative to the proportion of survivors that would be expected under a hypothesis that resistance was recessive. If resistance is completely recessive, 6.25% of larvae would be expected to survive a discriminating concentration of insecticide.

The 95% confidence intervals for proportional data of resistance frequency were calculated according to methods described by Newcombe (1998) and derived from a procedure by Wilson (1927) http://vassarstats.net. Pooled binomial standard errors were calculated using the formula of Forrester et al. (1993). Control mortality in bioassays was corrected by the formula of Abbott (1925). Estimates of LC_{50} values and their 95% fiducial limits (FL) were calculated using stand-alone software developed by Barchia (2001) where source codes were developed by implementing previously described procedures (Finney 1971, Robertson et al. 2007).

Results

Progression to F_2 generation

The full testing data set is shown in *Appendix 2* (Tables A.2.1 to A.2.6) with annual results summarised, below. Details of the annual number of F_0 lines established as single-pairs, and the number of isofemale lines that resulted from these pairs and passed through a defined stage in the five seasons from 2014-15 to 2018-9 are shown in Table 1.4.1. Of the 10153 F_0 lines established field derived eggs, larvae or moths 46% were lost through failure to mate or where mating had resulted in either non-viable eggs or too few viable eggs to establish an F_1 strain. Approximately 44% of isofemale lines established were bioassayed with one or more insecticides. Of the F_1 larvae produced, 99% successfully emerged as F_1 moths and produced fertile progeny.

Table 1.4.1 Establishment of isofemale lines of *H. armigera* and progression through an F_2 screen.

Year	Total F_0 isolines	Total F_1 isolines	Total F_1 isolines that produced sufficient viable F_2 progeny	Total emamectin benzoate tests	Total chlorantraniliprole tests	Total indoxacarb tests
2014-15	1733	874	674	652	656	666
2015-16	2702	1045	696	628	636	650
2016-17	2314	1287	1026	933	988	1026
2017-18	1745	1143	990	945	976	988
2018-19	1659	1182	1070	1004	1051	1070
Total	10153	5531	4459	4162	4307	4400

Detection of resistance

The combined testing data from all years and pooled data from all collecting sites is shown in Table 1.4.2. None of the 4142 lines bioassayed against emamectin benzoate scored positive for resistance to this insecticide. From the five year combined total of 4287 lines tested with chlorantraniliprole, 22 scored positive. This resulted in an average resistance frequency of 0.005 (95% CI 0.0034 - 0.0077). Of 4380 lines tested with indoxacarb 256 lines scored positive resulting in an average resistance frequency of 0.058 (95% CI 0.0518-0.0657).

F_2 screens from 2014-15 (sourced from egg, larval and moth samples). Progression of isofemale lines through to the F_2 screen in 2014-15 is summarized in Table 1.4.1. Insect sampling conducted by the use of pheromone traps was introduced to supplement egg and larval collection in the 2014-15 season. Of the 1733 single-pair crosses, 50% (874 pairs) produced sufficient numbers of viable eggs for establishment of an F_1 generation. The majority (77%) of F_1 larvae went on to provide viable F_2 larvae for testing of one or more insecticides (n = 674).

No isofemale lines scored positive for emamectin benzoate resistance (n = 652 tests) while a single line scored positive for chlorantraniliprole resistance (n = 656 tests) resulting in a resistance frequency of 0.002 (95% CI 0.0003 - 0.0085) (Table 1.4.2). A total of 666 lines were tested with indoxacarb. Of these, 25 lines scored positive for indoxacarb resistance after being rescreened in the F_3 generation resulting in a resistance frequency of 0.038 (95% CI 0.0255 - 0.0548) (Table 1.4.2).

F_2 screens from 2015-16 (sourced from egg, larval and moth samples). Of the 2702 single-pair crosses established from F_0 insects 38.7% produced viable F_1 larvae (Table 1.4.1). Of these 1045 lines, 655 (62.7%) then progressed to the moth stage and went on to produce sufficient larvae for testing of one or more insecticides. No lines scored positive for emamectin benzoate resistance (n = 628) while four lines scored positive for chlorantraniliprole resistance (n = 636), resulting in a resistance frequency of 0.006 (95% CI 0.0025 - 0.0161) (Table 1.4.2). A total of 650 lines were tested with indoxacarb and, of these, 16 lines scored positive after being rescreened in the F_3 generation resulting in a resistance frequency of 0.025 (95% CI 0.0152 - 0.0396) (Table 1.4.2).

F_2 screens from 2016-17 (sourced from a small larval sample collected at Emerald and moth samples). The majority of F_0 isofemale lines tested in the 2016-17 season were established from males captured in pheromone traps. The transition to a system of pheromone trapping is reflected in a 35% increase in volume of testing in 2016-17 compared with the previous season. Of the 2314 lines set up 55.6% progressed to the F_1 generation and 80.0% of the F_1 strains went on to produce viable F_2 material for testing (Table 1.4.1).

As in previous seasons no lines scored positive for resistance to emamectin benzoate (n = 933) while four lines scored positive for chlorantraniliprole resistance (n = 988) resulting in a resistance frequency of 0.004 (95% CI 0.0015 - 0.0103) (Table 1.4.2). Indoxacarb resistance was tested in 1026 lines of which 62 tested positive after being rescreened in the F_3 generation resulting in a resistance frequency of 0.060 (95% CI 0.0767 - 0.0772) (Table 1.4.2).

F_2 screens from 2017-18 (sourced from moth samples). Of the 1745 lines set up using pheromone trapped males mated with laboratory susceptible females 65.5% progressed to the F_1 generation and 86.6% of the F_1 strains went on to produce viable F_2 material for testing (Table 1.4.1). Again, no lines scored positive for resistance to emamectin benzoate (n = 945) while eight lines scored positive for chlorantraniliprole resistance (n = 976) resulting in a resistance frequency of 0.008 (95% CI 0.0042 - 0.0161) (Table 1.4.2). Indoxacarb resistance was tested in 988 lines of which 64 tested positive after being rescreened in the F_3 generation resulting in a resistance frequency of 0.065 (95% CI 0.0511-0.0819) (Table 1.4.2).

F_2 screens from 2018-19 (sourced from moth samples). Of the 1659 lines set up 71.2% progressed to the F_1 generation and 90.5% of the F_1 strains went on to produce viable F_2 material for testing (Table 1.4.1). As in all previous seasons no lines scored positive for resistance to emamectin benzoate (n = 1004) while five lines scored positive for chlorantraniliprole resistance (n = 1051) resulting in a resistance frequency of 0.005 (95% CI 0.0021 - 0.0111) (Table 1.4.2). Indoxacarb resistance was tested in 1070 lines of which 93 tested positive after being rescreened in the F_3 generation resulting in a resistance frequency of 0.087 (95% CI 0.0715-0.1053) (Table 1.4.2).

Regional resistance frequency

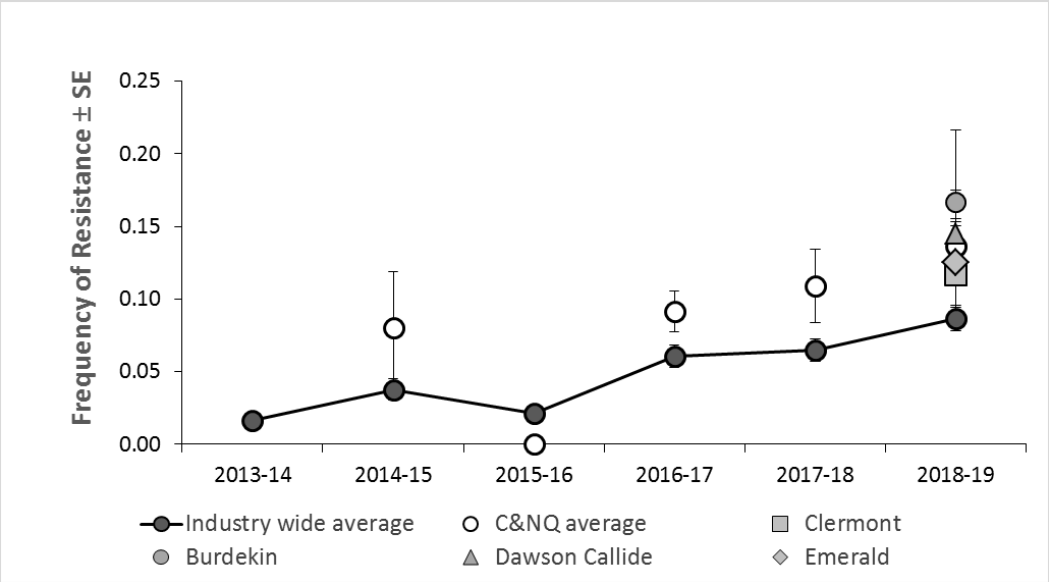
Indoxacarb. Testing results for individual regions are provided in *Appendix 2* with annual frequencies summarised in the Figure 1.4.1. In the years from 2014-15 to 2017-18 resistance monitoring in central Qld was conducted in the Emerald irrigation area (EIA). In years when high

numbers of samples were tested (2016-2019) resistance frequency was significantly higher in the EIA compared with the industry-wide average, based on non-overlap of binomial standard errors.

Table 2.4.2 Emamectin benzoate, chlorantraniliprole and indoxacarb resistance in *H. armigera* determined from F_2 bioassays conducted on insecticide-incorporated diet (annual average all regions). Data from the 2013-14 season has been included for comparison.

Insecticide	Year	Total tests	Total positives	Frequency of *r*	95% CI
Emamectin Benzoate	2013-14	500	0	0.000	-
	2014-15	652	0	0.000	-
	2015-16	628	0	0.000	-
	2016-17	933	0	0.000	-
	2017-18	945	0	0.000	-
	2018-19	1004	0	0.000	-
Pooled five year frequency		4162	0	0.000	-
Chlorantraniliprole	2013-14	525	0	0.000	-
	2014-15	656	1	0.002	0.0003-0.0085
	2015-16	636	4	0.006	0.0025-0.0161
	2016-17	988	4	0.004	0.0015-0.0103
	2017-18	976	8	0.008	0.0042-0.0161
	2018-19	1051	5	0.005	0.0021-0.0111
Pooled five year frequency		4307	22	0.005	0.0034-0.0077
Indoxacarb	2013-14	548	9	0.016	0.0086-0.0309
	2014-15	666	25	0.038	0.0255-0.0548
	2015-16	650	16	0.025	0.0152-0.0396
	2016-17	1026	62	0.060	0.0474-0.0767
	2017-18	988	64	0.065	0.0511-0.0819
	2018-19	1070	93	0.087	0.0715-0.1053
TOTAL frequency over life of project		4400	260	0.059	0.0525-0.0665

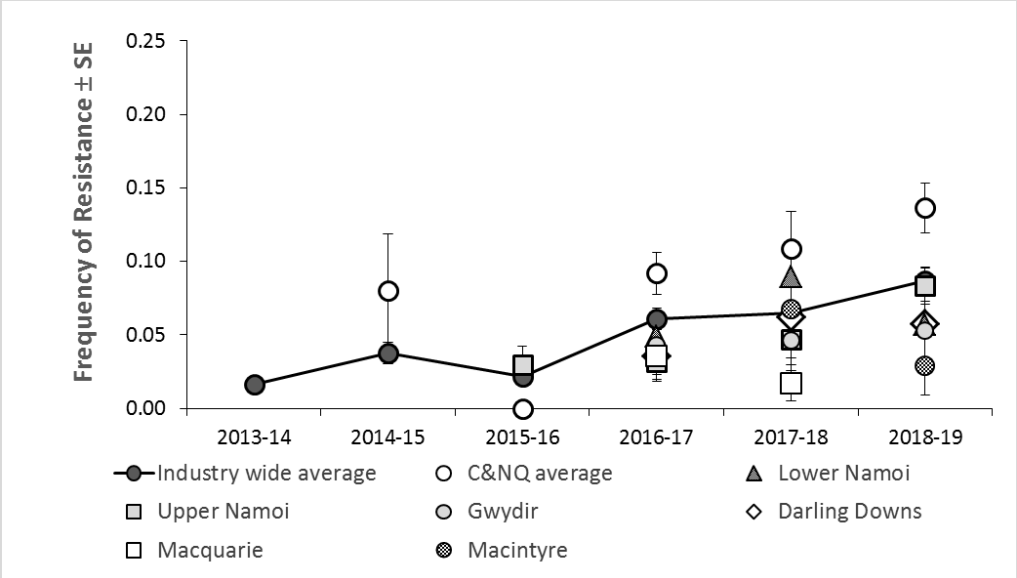
Figure 3.4.1 Annual regional frequency of indoxacarb resistance in central/northern Qld compared with the eastern Australian average ± binomial standard error (SE).



In 2018-19 surveillance was expanded in central Qld to include areas of high pulse production as a means of targeting areas of high insecticide use. These regions included large-scale chickpea-dominant landscapes of the northern highlands, pulse growing landscapes of the Burdekin region and the mixed farming landscape (pulses and cotton) of the Dawson/Callide region. Indoxacarb resistance in samples collected from these four regions during 2018-19 were higher than the industry average (Figure 1.4.1).

Throughout this study frequencies of indoxacarb resistance in southern Qld and NSW generally remained at levels similar to or at levels below the industry-wide average (Figure 1.4.2). Resistance in the upper and lower Namoi regions were slightly higher than the industry average in 2015-16 and 2017-18, respectively. However, indoxacarb resistance in these regions decreased in 2018-19 to below the industry average. Levels recorded in southern Qld and NSW during the final year of the project were significantly lower than the central and northern Qld average, based on non-overlap of binomial standard errors.

Figure 4.4.2 Annual regional frequency of indoxacarb resistance in southern Qld and NSW compared with the central/northern Qld and eastern Australian average ± binomial standard error (SE).



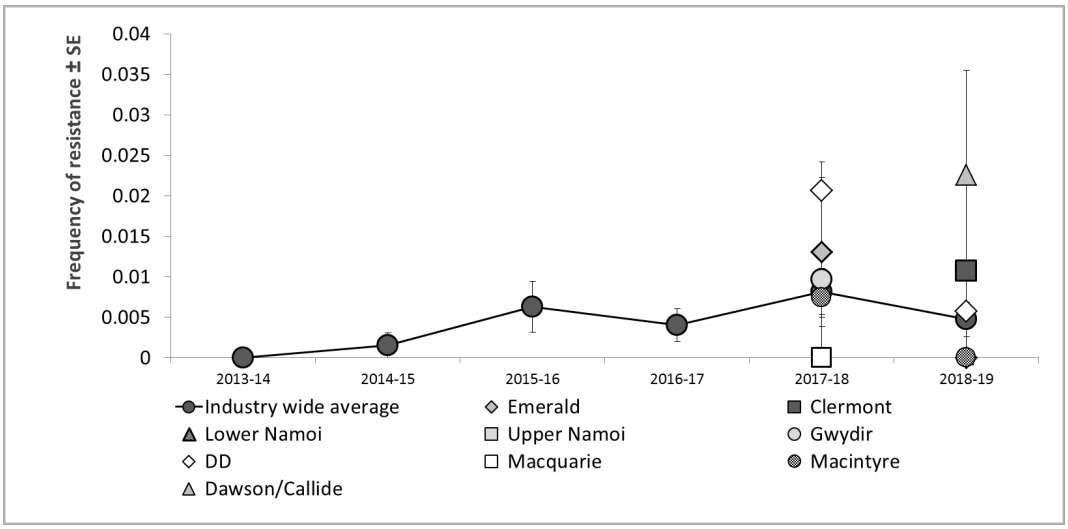
Although levels of indoxacarb resistance in Emerald were 2.6-fold higher than those in southern Qld and NSW in the final year of the project, it is unlikely that this is a result of substantially different management practices and is more likely associated with ecological differences unique to northern regions. These factors relate to a longer growing season which supports a higher number of insect generations per year compared with southern regions resulting in more insecticide applications and increased selection pressure in northern populations of *H. armigera*. In addition, the lack of an overwintering phase in the lifecycle of *H. armigera* may also contribute to elevated resistance frequencies in northern populations of *H. armigera*. Investigations of the impact of diapause on indoxacarb resistance are discussed in *Supplementary Milestone 2.5*.

Chlorantraniliprole. Resistance to chlorantraniliprole is shown in Figure 1.4.3. Regional frequencies were generally consistent with the industry average in years prior to 2016-17 (data not shown, refer to *Appendix 2,* Tables A.2.3 and A.2.4). Elevated levels in some regions and in some years is reflective of small sample sizes (eg. Gwydir 2015-16, Table A.1.3). In 2017-18 elevated chlorantraniliprole resistance was found in the Darling Downs (2.1%, $n = 97$) (Figure 1.4.3) and in 2018-19 the Dawson/Callide region had the highest number of positive with a frequency of 0.023 ($n = 133$) (*Appendix 2*).

Survival in isofemale lines that carried resistance alleles

Indoxacarb. A total of 260 isofemale lines tested positive in indoxacarb screens over the five years of this study from 2014-15 to 2018-19 (Table 1.4.2). The first strain that tested positive during 2013 was the

Figure 5.4.3 Annual regional frequency of chlorantraniliprole resistance in all regions compared with the eastern Australian average ± binomial standard error (SE).



GY7-39 strain (DAN1204/00164). In this case, the proportion of larvae that survived the discriminating concentration of indoxacarb was 0.1 on the initial F_2 screen. The genetic factor that confers indoxacarb resistance in the GY7-39 strain was non-recessive (Bird 2016). Therefore, if the proportion surviving the initial F_2 screen is >0.1, we assume this was indicative of non-recessive resistance. This was found to be the case for a total of 213 of the 260 lines that tested positive for indoxacarb resistance (proportion observed = 0.100 – 0.578, $\chi^2 > 3.63$, $P < 0.0568$). On the other hand, in another 47 lines survival was not significantly different from the proportion expected to survive (0.0625) if resistance was completely recessive (proportion observed = 0.011 – 0.089, $\chi^2 < 1.07$, $P > 0.0568$). However, the possibility that this may indicate a level of polygenic resistance or enhanced tolerance to indoxacarb in the *H. armigera* population cannot be excluded.

The proportion of the UN1U3-10 isofemale line that survived the initial F_2 screen (0.278) was also indicative of non-recessive inheritance. Although this was not the highest level of survival observed

in an F_2 screen, the rate of larval development in this strain was markedly increased compared with all other surviving F_2 progeny on the discriminating concentration of indoxacarb.

Chlorantraniliprole. Twenty two isofemale lines scored positive for chlorantraniliprole resistance from 2014-15 to 2018-19 (Table 1.4.2). In the majority of lines (19/22) the proportion of larvae that survived a discriminating concentration of chlorantraniliprole was not significantly different from that expected if resistance was recessive (proportion observed = 0.011 – 0.111, $\chi^2 < 3.63$, $P > 0.0568$). In three cases, the proportion of larvae that survived was significantly greater than the proportion expected for recessive resistance (proportion observed = 0.133 – 0.344, $\chi^2 > 10.314$, $P < 0.0013$), suggesting that more than one mechanism of resistance may be involved. Complementation tests were not performed on any lines that demonstrated reduced susceptibility to chlorantraniliprole as it was difficult to maintain these strains in laboratory culture beyond one or two generations.

Complementation tests for allelism in strains positive for indoxacarb resistance. Strains selected for resistance and then backcrossed to an indoxacarb resistant strain GY7-39, were bioassayed with indoxacarb. Results from inter-strain complementation tests are presented in Table 1.4.3. All selected strains exhibited resistance ratios of >100-fold compared with the laboratory strain, New GR. The F_1 progeny from crosses between five of the six strains (UN29-4, LN12-1, MQ6-4 and EM14-1) and the GY7-39 strain were not significantly more susceptible to indoxacarb compared with the parental strain of GY7-39, suggesting that a major resistance locus may be common to these strains. In one strain, LN16-40, the F_1 progeny from crosses of this and the GY7-39 strain were significantly more susceptible to indoxacarb than the parental GY7-39 strain which may have been the result of inbreeding depression following six generations of selection in the LN16-40 strain.

Table 6.4.3 Indoxacarb bioassays (complementation tests) performed on strains isolated for indoxacarb resistance by F_2 screening between 2013 and 2015 and then backcrossed to an indoxacarb resistant strain GY7-39.

Strain	Date collected	Number of generations selected	LC_{50} [µgAI/ml diet] (95% FL)	Fit of probit line			Toxicity Ratio (95% CI)	RR
				Slope	X^2 (df)	*P*		
GY7-39	Nov. 2013		21.0 (18.0, 24.6)	2.6 ± 0.22	3.622 (4)	0.4596	-	117
UN29-4	Feb. 2014	5	20.8 (15.7, 28.0)	2.9 ± 0.26	7.658 (4)	0.1049	1.01 (0.82, 1.25)	116
UN29-4 x GY7-39			20.5 (18.6, 22.7)	2.8 ± 0.17	1.573 (4)	0.8136	1.03 (0.86, 1.24)	114
GY7-39			18.8 (10.1, 40.1)	2.2 ± 0.22	15.36 (4)	0.0040	-	104
LN49-9	Mar. 2014	4	27.5 (20.2, 37.6)	2.8 ± 0.27	4.482 (3)	0.2139	0.76 (0.62, 0.93)	153
LN49-9 x GY7-39			29.2 (23.1, 37.4)	2.7 ± 0.27	9.834 (4)	0.0433	0.72 (0.60, 0.86)	162
GY7-39			37.1 (31.3, 44.9)	2.2 ± 0.21	2.647 (4)	0.6185	-	206
LN12-1	Sept. 2014	4	34.1 (30.1, 38.5)	3.9 ± 0.40	0.520 (2)	0.7711	1.09 (0.87, 1.35)	189
LN12-1 x GY7-39			29.0 (25.8, 32.9)	2.3 ± 0.14	4.486 (5)	0.4818	1.28 (1.00, 1.59)	161
GY7-39			37.1 (31.3, 44.9)	2.2 ± 0.21	2.647 (4)	0.6185	-	206
LN16-40	Nov. 2014	6	18.2 (9.7, 40.8)	1.6 ± 0.14	26.22 (5)	0.0001	2.04 (1.54, 2.69)	101
LN16-40 x GY7-39			22.3 (19.7, 25.4)	2.0 ± 0.13	2.442 (4)	0.6551	1.66 (1.33, 2.08)	124
GY7-39			29.8 (26.4, 33.5)	4.3 ± 0.44	2.221 (3)	0.5278	-	166
MQ6-4	Dec. 2014	4	33.3 (25.3, 45.0)	3.2 ± 0.30	7.668 (4)	0.1045	0.89 (0.74, 1.07)	185
MQ6-4 x GY7-39			28.8 (26.4, 31.4)	3.8 ± 0.26	2.320 (3)	0.5087	1.03 (0.89, 1.20)	160
GY7-39			24.8 (19.0, 33.2)	2.4 ± 0.20	8.085 (5)	0.1516	-	138
EM14-1	April 2015	5	27.8 (18.6, 45.1)	2.1 ± 0.24	6.923 (5)	0.2265	0.89 (0.68, 1.17)	154
EM14-1 x GY7-39			34.4 (28.1, 42.8)	2.7 ± 0.18	7.197 (4)	0.1258	0.72 (0.59, 0.88)	191
New GR (laboratory susceptible strain)			0.18 (0.11, 0.28)	4.8 ± 0.54	4.176 (2)	0.1239	-	-

Discussion and conclusions

The time frame to respond to insecticide resistance before field failures occur largely depends on the precision with which resistance alleles can be detected in the population and the dominance of resistance alleles (Andow and Ives 2002). Prior to the present study there were no documented cases of genetic resistance to emamectin benzoate, chlorantraniliprole or indoxacarb isolated from Australian populations of *H. armigera*, even after commercial use of these products over many years. It was therefore presumed that alleles that confer resistance to these insecticides were rare in Australian *H. armigera*, an assumption supported by baseline studies which show high susceptibility in geographically diverse populations (Bird 2015). We made no presumption about the dominance of resistance to these insecticides and adopted the F_2 approach as a means of increasing the limits of detectability if recessive resistance alleles were present in the population.

The F_2 screen was originally designed to use mated field-caught females (Andow and Alsad 1998). However, as noted by Stodola and Andow (2004) the F_2 screen is sufficiently flexible as to allow modification of the breeding steps without compromising the effectiveness of the technique. A notable methodological variation on the theoretical basis of the F_2 screen used herein involved a combination of wild-caught males and moths derived from natural populations as field collected eggs and larvae, which were then individually mated to a laboratory susceptible strain.

Although this modification reduces by half the number of genes screened, it nevertheless enhances the likelihood of obtaining offspring from heterozygous moths, as we had previously experienced very poor mating efficiency where both parental moths were derived from field collected eggs and larvae (data provided in *Supplementary Milestone 1.9*). The capability for detecting resistance using this method was demonstrated by the isolation of genetic indoxacarb resistance from F_2 screens (*Milestone 2.2*, Bird 2016).

The F_2 screen was designed to estimate monogenic inheritance (Andow and Alstad 1998). Genetic characterization indicates monogenic inheritance of indoxacarb resistance in *H. armigera* (*Milestone 2.2*, Bird 2016). In contrast, analysis of chlorantraniliprole resistance in *Plutella xylostella* suggests that resistance may be conferred by a combination of multiple metabolic factors and/or target site resistance due to reduced binding affinity at the ryanodine receptor (Guo et al. 2014, Liu et al. 2015). Because the F_2 screen is equally efficient for recovery of all forms of recessive and non-recessive resistance we expect this method will be highly sensitive for detection of chlorantraniliprole resistance in *H. armigera*.

The lack of recessive inheritance of indoxacarb resistance implied from this study is consistent with previous findings (Bird 2016). However, deviations from a non-recessive model of indoxacarb resistance in *H. armigera* were evident in 47 of 260 lines that tested positive for resistance to this insecticide. It is unclear if this represents a genetic variant of the first indoxacarb resistant strain isolated. However, complementation tests for allelism were performed on six other strains isolated between 2013 and 2016 and, in five of these cases, results support the conclusion that resistance was due to indoxacarb resistance alleles with high levels of dominance. However, the possibility that survival could be increased by enhanced tolerance or low level polygenic resistance mechanisms cannot be discounted.

Strains that tested positive for chlorantraniliprole resistance were difficult to maintain in laboratory culture which may indicate the presence of a fitness cost associated with enhanced survival to this insecticide in *H. armigera*. Reduced fitness has also been observed in strains of *P. xylostella* resistant to chlorantraniliprole (Ribeiro et al. 2014) and cyantraniliprole (Liu et al. 2015).

Results from the present study demonstrate that the *H. armigera* population remains fully susceptible to emamectin benzoate with low but detectable levels of resistance to chlorantraniliprole, highlighting the need for continued use of methods with a high level of sensitivity for resistance detection. This will be particularly important for monitoring the development of resistance conferred by non-recessive alleles, such as the case with indoxacarb resistance, which favor resistance evolution

through selection of heterozygotes and which, therefore, pose more of a challenge for implementing management and mitigation tactics (Gould 1998).

The detection of differential levels of indoxacarb frequencies in northern populations compared with more southern cohorts is likely the result of ecological factors unique in *H. armigera* in more northerly regions of Australia. These factors may be related to an increased number of generations exposed annually to insecticides and a lack of a winter diapause in northern cohorts (*Supplementary Milestone 2.5*). At the levels currently detected from this surveillance program resistance may not be conspicuous at the field level. In other words, resistance phenotypes may not currently be at a high enough frequency to cause field failures of indoxacarb. Nevertheless, the information generated from this study provides a powerful predictive tool in resistance management because it identified a differential level of genotypic resistance in populations residing in central and northern regions of Australia. Importantly, this finding highlights the imperative for pre-emptive adoption of management strategies to mitigate future risk in those particular regions.

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Milestone 1.5 Insecticide Resistance Management Strategy (IRMS) formulated through involvement with TIMS committee and accepted by the Australian cotton industry. Additional resistance management guidelines formulated and promoted through publications and presentations

A key outcome from this project was the delivery of insecticide resistance data to peak industry bodies of the cotton and grains industries. Results were provided for updating resistance status tables in the Cotton Pest Management Guide each year from 2015-16 to 2019-20 and have been featured in a number of Spotlight and Groundcover Articles as well as in media releases by GRDC and the Australian Mungbean Association.

List of presentations given by project leader at cotton and grains industry meetings:

Helicoverpa Insecticide Resistance: 2014-15 Results Summary. TIMS Insecticide Panel meeting, Brisbane April 28th 2015

Characteristics of indoxacarb resistance in Australian populations of *Helicoverpa armigera.* Australian Cotton Research Conference, Toowoomba, QLD, 8th-10th September 2015

Helicoverpa Insecticide Resistance: 2015-16 Results Summary. TIMS Insecticide Panel meeting, Toowoomba May 18th 2016

Insecticide Resistance in Helicoverpa: Monitoring, mechanisms and Management. CRDC Cotton Production Course, Narrabri 1st September 2016

Conventional Insecticide Resistance in Helicoverpa: Monitoring and Research Update. 4th Australian Agrichemical Resistance Meeting, Adelaide, 3rd November 2016

Insecticide Resistance in Helicoverpa: Monitoring, Mechanisms and Management. Centre for Crop and Disease Management / AHRI 2nd Crop Protection Forum, Wagga Wagga 23rd November, 2016

Helicoverpa Insecticide Resistance: 2016-17 Results Update. TIMS Insecticide Panel meeting, Brisbane 10th May 2017

Insecticide Resistance in *H. armigera*. Central Queensland Research Update and Workshop, Emerald 11th July 2017

Insect Resistance Management in Cotton. Cotton Pest Management Short Course, Narrabri 29th August 2017

Insecticide Resistance: Mechanisms, Monitoring and Management. Cotton Production Course, Narrabri 14th September 2017

Resistance Management Strategy for Helicoverpa in Grains. Centre for Crop and Disease Management / AHRI 3rd Crop Protection Forum, Dalby 6th December 2017

Helicoverpa Insecticide Resistance: 2017-18 Monitoring and Research Update. TIMS Insecticide Panel meeting, Brisbane 10th May 2018

Conventional insecticide resistance in Helicoverpa – monitoring, management and mitigation strategies in Bollgard III – A Project Update. Northern Cropping Systems Unit Meeting, 6th August 2018, Tamworth Agricultural Institute

Helicoverpa Insecticide Resistance: 2017-18 Monitoring Update. TIMS Insecticide Panel meeting, Toowoomba 5th June 2019

The development of a resistance management strategy for *H. armigera* in the grains sector was coordinated by the project leader through membership of the National Insecticide Resistance Management (NIRM) working group and involved direct engagement with industry stakeholders including representatives from the agrochemical industry and grains research community.

A key aspect of this process was to undertake industry-wide consultation with leading growers and advisors of cotton and grains. The project leader utilised feedback from industry networks to inform the development of a draft strategy which was completed in October 2017 and endorsed by CropLife Australia in November 2017. The strategy was subsequently approved for public release by GRDC in April 2018 (*Appendix 3*) and was formally launched in May 2018. The strategy was accompanied by a technical report outlining the science behind the grains RMS (*Appendix 4*).

To further raise awareness of *H. armigera* resistance management in grains, the project leader contributed to media communications released by the Australian Mungbean Association and an article published in Groundcover. The project leader was also an invited speaker at the AHRI/CCDM Crop Protection Forum, Dalby December 2017 specifically to outline the rationale for the strategy ahead of its official launch.

The project indirectly contributed to communication channels within the grains sector because of numerous requests by grains industry researchers for the project leader to provide Helicoverpa resistance data for use in industry presentations. A summary of resistance results was regularly provided to researchers from QDAF for inclusion in GRDC Updates and other GRDC supported workshops in the northern grains region. Following the release of the *H. armigera* RMS in Australian grains resistance research featured regularly on the program of northern and southern GRDC Updates in 2018 and 2019.

A summary of results was also provided to QDAF researchers for inclusion in the Northern AgriServices/GRDC Grower Solution Group presentation at Kingaroy in June 2018 aimed at providing support to growers and advisers in the inland Burnett region in grain crops management and to promote the grains RMS for *H. armigera*. A similar series of presentations was also part of Australian Mungbean Association Grower/Advisor Updates from September to November 2018.

Data is also regularly provided to cotton and grains industry researchers for inclusion in their presentations in the Crop Consultants Australia (CCA) annual Cropping Solutions Seminar series.

On each occasion data was provided with approval from the CRDC Program Manager with CRDC and GRDC duly acknowledged in all presentations.

A number of scientific manuscripts and technical reports relating to milestones in this project were prepared by the project leader and accepted for publication in the international scientific literature (see full *Publications List* on p.112).

Milestone 1.6 Resistance monitoring specifically aimed at determining potential for enrichment of resistance genes (resistance risk) in preferred host crops for *H. armigera* with monitoring focused in mixed cropping areas of the Namoi valley

Introduction and aims

In 2014-15 an alternative strategy for identifying management practices that could impact on in-season resistance risk in *H. armigera* was deployed. This work was aimed at determining temporal changes in resistance frequency in locations where successive generations of *H. armigera* were managed across multiple commodities. Specifically, insects collected from sequential *H. armigera* hosts may be enriched for resistance alleles if a previous host crop was treated with insecticide.

Within season comparisons of multiple generations of *H. armigera* with possible exposure to selection pressure was aimed at providing information relating to the magnitude of risk associated with the use of conventional chemistries in mixed farming systems, particularly the use of chlorantraniliprole and indoxacarb in pulse production which could have flow-on effects for the cotton industry.

Materials and methods

Insect sampling. This study utilised insect samples collected as part of the routine resistance surveillance activities. Initially, the insect life stages for inclusion in this study were eggs, larvae, pupae and moths. However, attempts to sample pupae were unsuccessful largely because it was a highly inefficient method compared with other methods of sampling. It was therefore decided that only immature stages and moths would be sampled for resistance testing.

Site selection. Sampling was conducted annually throughout the Namoi and Gwydir regions and farm locations where coarse grains were utilised as a summer cropping option following harvest of winter pulses were selected as sites for this study. Fields of winter pulse and summer grains selected for sampling were generally within a distance of five to ten kilometres of each other.

The Namoi and Gwydir regions were selected because, in general, there was a high degree of diversity in the cropping landscape within and between seasons, and also because these locations were logistically convenient for the local collecting team based at ACRI. At least one growing region was represented in each year, with the exception of 2015-16 when plantings of winter pulses were constrained by a lack of rainfall.

Spring sampling of winter pulses was aimed at targeting the flowering and podding stages of plant development, and also coincided with peak spray periods. After initial sampling of pulse crops the farm locations with plantings of summer cereals were re-sampled once, or twice in the case of Gwydir in 2016-17 (as there were staggered plantings of maize at this location).

Resistance testing and data analysis. Resistance was tested using an F_2 screening procedure described in *Milestone 1.4*. Proportional data of survival were analysed using χ^2 distribution. All analyses were performed using SPSS statistical software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp., 2013).

Results

2014-15 sampling. A total of 14 farm locations with plantings of winter pulses were sampled for eggs and larvae, resulting in a total of ten F_2 isofemale families for resistance testing (Table 1.6.1). At this

point in time, the program had not implemented pheromone trapping and the poor F_2 recovery observed in 2014-15 is consistent with our broader experience with egg and larval sampling (see *Supplementary Milestone 1.9*, below). Approximately six weeks later a follow-up sample was collected from maize fields from a similar location.

2016-17 sampling. During this season both the Upper Namoi and Gwydir regions were sampled. In the Upper Namoi a total of 127 isofemale families were tested from chickpea collections. Approximately seven weeks later a follow-up sample was collected from maize and sorghum from the same location resulting in 27 isofemale families tested for resistance (Table 1.6.1). In the Gwydir 44 isofemale families were tested from samples sourced in chickpea crops while a follow-up sample from maize at the same location yielded 19 isofemale families for testing. However, the majority of these samples were collected only approximately 4.5 weeks after the first collection.

2017-18 sampling. As in the previous season, the Upper Namoi and Gwydir regions were sampled. In the Upper Namoi a total of 83 samples were tested from chickpeas and 68 samples were tested from maize. In the Gwydir 50 samples were tested from chickpeas and 20 samples were tested from maize. There five to six weeks between the first and second samples in both locations (Table 1.6.2).

2018-19 sampling. Samples from the Lower Namoi and Gwydir were tested. In the Lower Namoi a total of 58 samples were tested from chickpeas and 25 samples were tested from sorghum. In the Gwydir 83 samples were tested from chickpea crops and 74 samples were tested from sorghum and maize crops. There were eight and 12 weeks between the first and second samples collected in the Lower Namoi and Gwydir regions, respectively (Table 1.6.2).

Indoxacarb resistance. Comparisons of *H. armigera* populations that had potentially been selected with insecticide in winter pulses and then tested for resistance in a subsequent generation suggest there were generally no increases in temporal indoxacarb resistance frequency within regions. There was however, one instance when indoxacarb resistance increased. In this case, there were no positives recorded in samples collected from chickpea fields in the Gwydir region during November and December of 2016. In subsequent samples collected from maize fields in the same location during January of 2017 resistance had increased significantly to 5.3% ($P = 0.0213$) (Table 1.6.3).

In contrast, samples collected from chickpeas and canola in the Gwydir region during August of 2018 had a significantly higher resistance level than subsequent samples collected from maize field in the same location during November 2018 ($P = 0.0073$).

Chlorantraniliprole resistance. Resistance to chlorantraniliprole was extremely low in all comparisons in all years (Table 1.6.1 and 1.6.2). A single positive was detected in a sample collected in chickpeas in the Liverpool Plains during 2016-17 which was not significantly different ($P = 0.374$) from the result recorded from samples collected in subsequent plantings of maize.

Discussion and conclusions

Species of the *Helicoverpa* genus are highly mobile and although long-range migratory activity is lower in *H. armigera* compared with *H. punctigera*, local within crop movement and flights to nearby crops and wild hosts are important in the seasonal dynamics of this pest which enhances exploitation of the agroecosystem as adults are able to search out more favourable feeding and oviposition sites.

H. armigera is generally more abundant in regions where host plant availability is more predictable such as agricultural landscapes (Fitt 1989) and any insecticide resistance that develops in *H. armigera* populations is more likely to persist locally if selection pressure remains high. Resistance risk could be further increased if cropping landscapes provide a succession of preferred hosts for *H. armigera* which could result in local populations becoming enriched for resistance (Forrester et al. 1993).

This study utilized resistance surveillance data collected over four seasons in three mixed cropping

Table 7.6.1 Emamectin benzoate, chlorantraniliprole and indoxacarb resistance in *H. armigera* determined from F_2 bioassays conducted on insecticide-incorporated diet. Collections from winter pulse crops and subsequent plantings of summer grains in the Namoi and Gwydir regions during 2014-15 and 2016-17.

Regional reference	Date Collected	Crop	F_0 lines	F_2 lines	Number of positive tests		
					Indoxacarb	Chloran-traniliprole	Emamectin benzoate
LOWER NAMOI 2014-15							
LN 1	06/08/2014	Faba beans	2	1	0	0	0
LN 2	06/08/2014	Chickpea	0	0	-	-	-
LN 3	13/08/2014	Faba beans	0	0	-	-	-
LN 4	14/08/2014	Faba beans	2	0	-	-	-
LN 5	14/08/2014	Chickpeas	5	1	0	0	0
LN 6	14/08/2014	Chickpeas	5	2	0	0	0
LN 7	14/08/2014	Faba beans	1	0	-	-	-
LN 8	14/08/2014	Faba beans	3	2	0	0	0
LN 9	14/08/2014	Faba beans	0	0	-	-	-
LN 10	03/09/2014	Chickpeas	4	2	0	0	0
LN 11	03/09/2014	Chickpeas	2	0	-	-	--
LN 12	03/09/2014	Chickpeas	2	2	1	0	0
LN 13	03/09/2014	Canola	0	0	-	-	-
LN 14	20/09/2014	Chickpea	3	0	-	-	-
LN 15	24/09/2014	Chickpeas	1	0	-	-	-
LOWER NAMOI TOTAL - WINTER PULSES			30	10	1	0	0

landscapes to assess the potential for enrichment of insecticide resistance genes in populations of *H. armigera* where farming systems comprised winter pulse crops (typically chickpeas) followed by summer

LN 16	05/11/2014	Maize	53	23	1	0	0
LOWER NAMOI TOTAL - SUMMER CEREALS			53	23	1	0	0
UPPER NAMOI 2016-17							
B1	27/10/2016	Chickpeas	35	20	0	0	0
C1	27/10/2016	Chickpeas	35	26	0	0	0
D1	27/10/2016	Chickpeas	35	25	0	1	0
B2	30/11/2016	Chickpeas	62	37	2	0	0
C2	30/11/2016	Chickpeas	2	2	1	0	0
D2	30/11/2016	Chickpeas	33	17	1	0	0
UPPER NAMOI TOTAL - WINTER PULSES			202	127	4	1	0
B3	25/01/2017	Maize	5	2	1	0	0
DPI RS1	25/01/2017	Sorghum	50	25	0	0	0
UPPER NAMOI TOTAL - SUMMER CEREALS			55	27	1	0	0
GWYDIR 2016-17							
GY1	09/11/2016	Chickpeas	100	44	0	0	0
GWYDIR TOTAL - WINTER PULSES			100	44	0	0	0
GY2	12/12/2016	Maize	100	14	1	0	0
GY3	17/01/2017	Maize	33	5	0	0	0
GWYDIR TOTAL - SUMMER CEREALS			133	19	1	0	0

cereals (typically maize and sorghum). There was no consistent increase in resistance frequency between sequentially planted hosts of *H. armigera.* The only exception was in 2016-17 in the Gwydir region when the frequency of indoxacarb resistance was significantly higher in moths collected from maize compared with moths sampled from chickpeas. In contrast, samples collected from chickpeas in the Gwydir region 2018-19 had significantly higher indoxacarb resistance compared with cohorts from sequential plantings of sorghum and maize.

Table 8.6.2 Emamectin benzoate, chlorantraniliprole and indoxacarb resistance in *H. armigera* determined from F_2 bioassays conducted on insecticide-incorporated diet. Collections from winter pulse crops and subsequent plantings of summer grains in the Namoi and Gwydir regions during 2017-18 and 2018-19.

Regional reference	Date Collected	Crop	F_0 lines	F_2 lines	Number of positive tests		
					Indoxacarb	Chloran-traniliprole	Emamectin benzoate
UPPER NAMOI 2017-18							
UN1	18/10/2017	Chickpeas	160	83	5	0	0
UPPER NAMOI TOTAL - WINTER PULSES			160	83	5	0	0
UN3	28/11/2017	Maize	90	68	2	0	0
UPPER NAMOI TOTAL - SUMMER CEREALS			90	68	2	0	0
GWYDIR 2017-18							
GY1	19/10/2017	Chickpeas	40	25	0	0	0
GY2	19/10/2017	Chickpeas	40	25	1	0	0
GWYDIR TOTAL - WINTER PULSES			80	50	2	0	0
GY6	27/11/2017	Maize	32	20	2	0	0
GWYDIR TOTAL - SUMMER CEREALS			32	20	2	0	0
LOWER NAMOI 2018-19							
LN3	05/11/2018	Chickpeas	50	31	2	0	0
LN4	05/11/2018	Chickpeas	47	27	2	0	0

LOWER NAMOI TOTAL - WINTER PULSES			97	58	4	0	0
LN5	05/01/2018	Sorghum	37	25	1	0	0
LOWER NAMOI TOTAL - SUMMER CEREALS			37	25	1	0	0
GWYDIR 2018-19							
GY1	15/08/2018	Chickpeas	115	83	6	0	0
GWYDIR TOTAL - WINTER PULSES			115	83	6	0	0
GY2	14/11/2018	Sorghum/maize	70	58	0	0	0
GY3	14/11/2018	Sorghum	26	16	0	0	0
GWYDIR TOTAL - SUMMER CEREALS			96	74	0	0	0

In temperate regions of eastern Australia, *H. armigera* moths are generated from both immigrant origins and emergence from local over-wintering pupae (Fitt and Daly 1990) with establishment of spring cohorts on winter pulses (Murray et al. 2005) and, to a lesser extent, uncultivated hosts such as roadside weeds (Wilson 1983). During droughts, spring abundance of *H. armigera* is likely to be reduced due to resource bottlenecks such as the absence of suitable non-crop hosts and reduced plantings of cultivated hosts (Gregg et al. 1995), particularly dryland crops such as chickpeas and sorghum.

This scenario was likely mitigated throughout the duration of the present study by immigration of populations from other areas which may have experienced greater suitability of rainfall and breeding conditions, ensuring sufficient levels of migration to maintain genetic similarity and widespread distribution of resistance alleles (Daly and Gregg 1985, Gregg et al. 1995).

However, if host availability and diversity are severely limited by widespread and prolonged periods of drought we might also expect immigration rates to be substantially affected. In turn, a limited influx of susceptible alleles into cropping areas could increase resistance risk by enrichment of resistance genes selected for and perpetuated in cropping areas. Therefore it is recommended that a similar study should be undertaken in the future as part of a pre-emptive approach to resistance management that takes into account limited gene flow experienced in drought years.

Table 9.6.3 Summary of indoxacarb resistance frequency in *H. armigera* sourced from sequentially planted pulse and coarse grains crops in the Namoi and Gwydir regions between 2014 and 2018.

Region	Number F_2 Lines established	Number of Positive tests	Frequency of R
LOWER NAMOI 2014-15			
WINTER PULSES	10	1	0.100
SUMMER CEREALS	23	1	0.043
UPPER NAMOI 2016-17			
WINTER PULSES	127	4	0.031
SUMMER CEREALS	27	1	0.037
GWYDIR 2016-17			
WINTER PULSES	44	0	0.000
SUMMER CEREALS	19	1	0.053*
UPPER NAMOI 2017-18			
WINTER PULSES	83	5	0.060
SUMMER CEREALS	68	2	0.029
GWYDIR 2017-18			
WINTER PULSES	50	2	0.040
SUMMER CEREALS	20	2	0.100
LOWER NAMOI 2018-19			
WINTER PULSES	58	4	0.069
SUMMER CEREALS	25	1	0.040
GWYDIR 2018-19			

WINTER PULSES	83	6	0.072
SUMMER CEREALS	74	0	0.000*

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Milestone 1.7 Support other Helicoverpa related research projects with field collected material and field collected laboratory reared insect colonies

Throughout the life of this project long-term laboratory susceptible strains of *H. armigera* and *H. punctigera* were in continuous culture. Insect strains and protocols were regularly provided to PhD candidates in the Dept. of Agronomy University of New England. Various strains were requested for experimental purposes by researchers at the Latrobe Institute for Molecular Science, QDAF Toowoomba, and CSIRO. Field and laboratory strains were also used extensively in Dr Robert Mensah's CRDC DAN1404 project for investigating *Helicoverpa* active bio-pesticides.

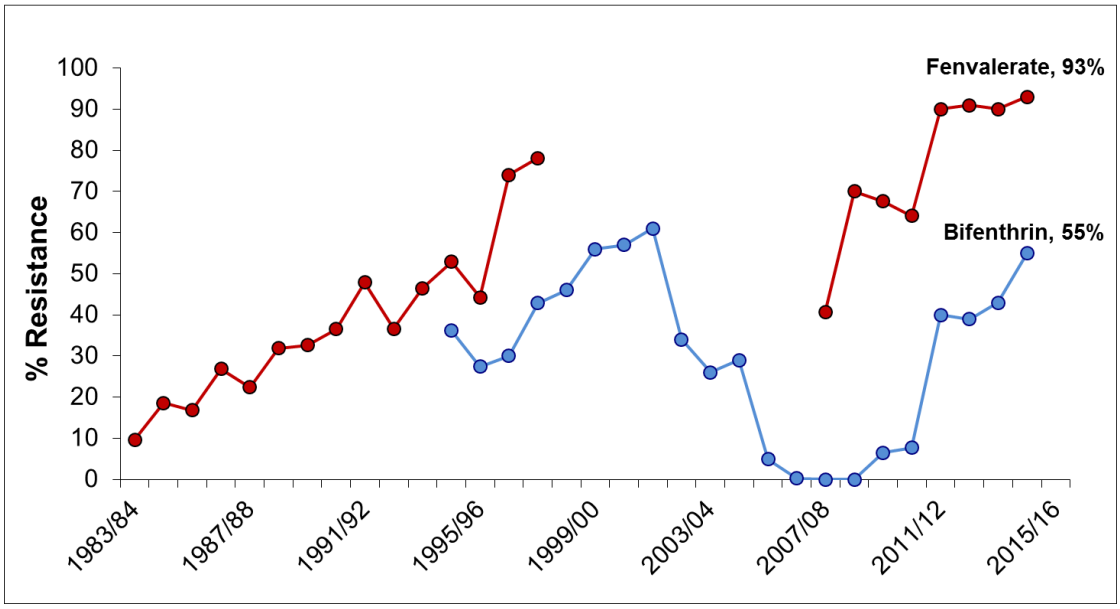
Strains were regularly provided for teaching purposes in the School of Environmental and Rural Sciences, The University of New England, and the Department of Ecology, Environment and Evolution, La Trobe University, and the School of Agriculture at The University of Tasmania.

Supplementary Milestone 1.8 Conduct broad-spectrum insecticide resistance monitoring including delivery of outcomes across the cotton and grains industries

Introduction and aims

This milestone was conducted in Year 1 (2014-15) and involved phenotypic (F_0) testing of broad-spectrum insecticide resistance in *H. armigera* (pyrethroid [fenvalerate and bifenthrin], organophosphate [chlorpyrifos] and carbamate [methomyl]). Resistance was tested to fenvalerate and abamectin in *H. punctigera*. The work described herein completed a 30 year program of continuous broad-spectrum insecticide resistance monitoring in the cotton industry (Figure 1.8.1). The results from this milestone were published along with historical data from the program (Bird 2018, *Appendix 6*). Results were presented at the 2015 TIMS Insecticide Technical Panel meeting held in Brisbane and made available to the grains industry as part of the development of the resistance management strategy for *H. armigera* in Australian grains. https://ipmguidelinesforgrains.com.au/ipm-information/resistance-management-strategies.

Figure 1.8.1 Historical pyrethroid resistance in eastern Australia (average of all regions). Frequency was measured by topically applying a diagnostic dose of insecticide that induces 99.9% mortality in a susceptible strain.



Materials and methods

Sample collection and processing. Visual searches for *Helicoverpa* spp. eggs were conducted in a range of cultivated hosts (primarily cotton, sorghum, maize and pulses) and the scrophulariaceous weed host, *Verbascum virgatum*. Collection sites had not been treated with insecticide at the time of sampling. Eggs (comprising a mixture of both *H. armigera* and *H. punctigera*, in unknown proportions) were collected at random across a wide geographical range in each sampling region and from a range of host crops. The objective of each field collecting trip was to source between 100 and 200 eggs from any one individual farm location. Summary tables of collecting data are presented in *Appendix 2*.

Field collected eggs were transported to the laboratory. They were transferred from plant material using a fine hair paintbrush to 45-well plastic trays and transferred, one egg per well, before being sealed, as above. Eggs were regularly checked for the presence of parasites. The egg parasite, *Trichogramma* spp., occasionally caused high levels of egg mortality in samples. The solitary egg-larval braconid wasp parasite, *Chelonus* spp. was also occasionally present in samples but easily identified by characteristic developmental arrest in *Helicoverpa* spp. larvae and the precocious onset

of metamorphosis in parasitised larvae which were subsequently removed from the test cohort. Hatched larvae were identified to species at the 2nd or 3rd instar as either *H. armigera* or *H. punctigera*. Larvae were then reared, as described above, to the appropriate size for testing.

Insecticides. Insecticide solutions used in all bioassays were prepared from technical material dissolved in analytical grade acetone. Fenvalerate (95.3%) was provided by Sumitomo Chemical (Sydney); bifenthrin (93.3%) was provided by FMC (Brisbane); methomyl (98%) was provided by Bayer CropScience (Melbourne). Discriminating dose bioassays were performed using concentrations previously determined for fenvalerate as 0.125µg/larva (Gunning et al. 1984), bifenthrin as 0.1µg/larva (Forrester et al. 1993) and methomyl as 1µg/larva (Gunning et al. 1992). The insecticide solutions used in control bioassays of the susceptible strain were prepared as twofold serial dilutions corresponding to six or seven insecticide concentrations which were expected to induce 1-99% mortality in 30-40mg *H. armigera* larvae.

Bioassays. Insects were reared on artificial diet (Teakle and Jensen 1985) to the 3rd or 4th instar and bioassayed using the methods of Forrester et al. (1993). Larvae within a weight range of 30-40mg were transferred to fresh diet and allocated randomly to insecticide treatment groups. Each larva was treated by topical administration of 1µL of acetone/insecticide solution applied to the dorsal thorax using a 50µl micro-syringe in a repeating dispenser (Hamilton Company, Reno, NV, USA). Trays containing tested larvae were covered with heat-sealed perforated lids. Bioassays were maintained for 3 days under the conditions described above for larval rearing and assessed for mortality based on the inability to demonstrate coordinated movement when prodded with a blunt probe.

Results

Broad-spectrum resistance in *H. armigera*

Synthetic pyrethroids. In 2011 resistance increased by 30% for both fenvalerate and the resistance-breaking product bifenthrin resulting in very high frequency general pyrethroids resistance (90.5%) and high frequency bifenthrin resistance (39.6%). Resistance remained at these levels for the following two seasons (data shown in Table 1.8.1 and previously reported in DAN1204/00164).

In 2014-15 the average frequency of fenvalerate resistance was 93.3%, with regional frequencies ranging between 76% on the Darling Downs and 98% in the Gwydir valley (Figure 1.8.2 A). In 2014-15 the frequency of resistance to bifenthrin increased to 55.3% (Table 1.8.1) with regional frequencies ranging from 32% on the Darling Downs to 67% in the upper Namoi valley (Figure 1.8.2 B).

Table 1.8.1 Resistance frequencies in *H. armigera* determined in the F_0 generation by topical bioassay (annual average all regions).

Insecticide	2011-12	2012-13	2013-14	2014-15
Fenvalerate - pyrethroid	90.5 (902)	90.6 (2446)	89.5 (581)	93.3 (1053)
Bifenthrin - pyrethroid	39.6 (1197)	38.9 (2215)	42.9 (636)	55.3 (1047)
Chlorpyrifos - OP	1.0 (1051)	1.3 (2037)	1.7 (601)	1.9 (979)
Methomyl - carbamate	34.0 (1049)	23.1 (1965)	27.9 (598)	49.3 (951)

Organophosphates. Resistance to chlorpyrifos declined to very low levels (<2%). Results in 2014-15 indicate a continuing trend of low organophosphate resistance with an annual average frequency of 1.9% (Table 1.8.1). Regional frequencies ranged from 0% at Mungindi to 8% on the Darling Downs. However, the later result was based on a low sample size (n = 25) (Figure1.8.3 A).

Carbamates. Resistance to carbamates (methomyl) has been present at moderate frequencies for over ten years, and typically ranges between 20 and 35%. In 2014-15 average resistance to methomyl increased to 49% (*n* = 951) (Table 1.8.1). Regional frequencies ranged from 26% in the Darling Downs to 56% in the upper Namoi valley (Figure1.8.3 B).

Insecticide resistance in *H. punctigera*

Products tested against *Helicoverpa punctigera* include pyrethroid and abamectin. This species was screened in the F_0 generation using a topical bioassay. While *H. armigera* has repeatedly demonstrated the ability to develop resistance to conventional insecticidal chemistries, *H. punctigera* has maintained a susceptible status to conventional insecticides.

From 2011 to 2015 there was ample opportunity to test resistance in *H. punctigera* because of the strong dominance of this species in all regions, particularly in 2013-14 where approximately 4000 individuals were tested against both chemistries (Table 1.8.3). Survivorship in all years was extremely low indicating a high level of susceptibility to fenvalerate and abamectin.

Table 1.8.2 Fenvalerate and abamectin resistance frequencies in *H. punctigera* determined in the F_0 generation by topical bioassay (annual average all regions).

Insecticide	2011-12	2012-13	2013-14	2014-15
Fenvalerate - pyrethroid	0.07 (1477)	0.2 (2472)	0.2 (3976)	1.1 (1346)
Abamectin	0 (1757)	0.1 (2402)	0.02 (4051)	0.2 (1219)

Discussion and conclusions

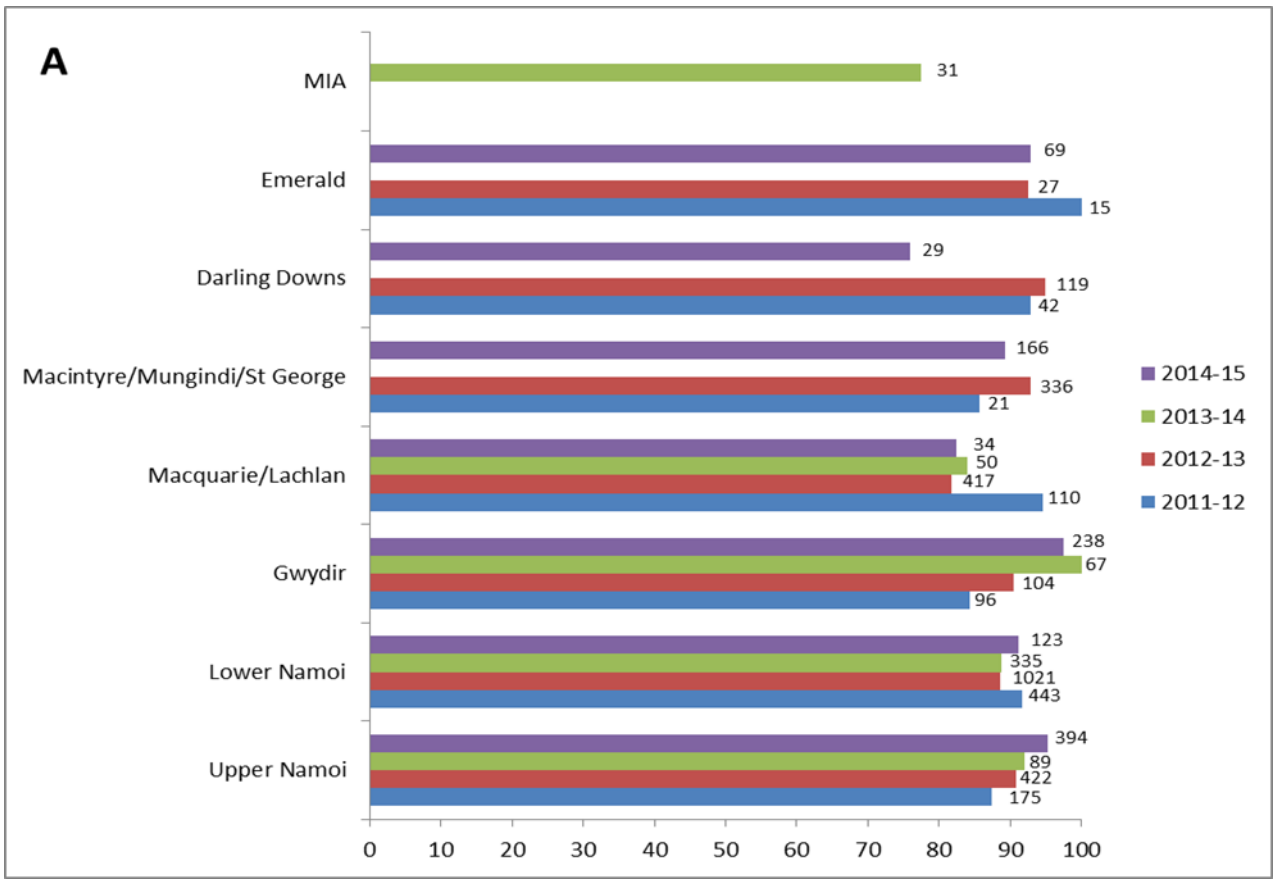
There is little doubt that the introduction of transgenic cotton has reduced the overall need for insecticides that target *Helicoverpa* spp. The commensurate decline in insecticide resistance levels in Australian *H. armigera* was presumed to be associated with reduced selection for resistance in an industry dominated by transgenic cotton (Fitt, 2008, Wilson et al. 2013). Although *H. armigera* was still fully susceptible to bifenthrin in 2008-09, incipient resistance was detected in the following season at a level of 6%, increasing to 36% in 2011-12 and was accompanied by a similar increase in resistance to fenvalerate which exceeded 90% (Bird 2018).

Results from the present study show that high level general pyrethroid resistance was widespread and sustained over the ensuing three seasons. Bifenthrin resistance was also widespread throughout the *H. armigera* population. The frequency of bifenthrin resistance increased by 15.7% between 2011-2012 and 2014-15. These levels of resistance have been shown to result in field failures of ester bonded phenoxybenzyl alcohol (α-cyano) pyrethroids (Forrester et al. 1993). Moderate resistance to methomyl is also consistent with unreliable levels of control observed under field conditions (Gunning et al. 1992). Therefore the implications for management are that applications of pyrethroid on *H. armigera* dominant populations will provide little or no control unless applied in combination with a synergist product such as piperonyl butoxide. In contrast, *H. punctigera* remains fully susceptible to α-cyano pyrethroids and abamectin.

The marked increase in bifenthrin and fenvalerate resistance occurred despite relatively low levels of pyrethroid use to target *Helicoverpa* spp. in cotton and was unlikely to have provided sufficient selection pressure to account for the magnitude of increase in resistance frequency. Likewise, but to a lesser extent, resistance to methomyl had increased significantly in 2014-15 despite very low levels of carbamate use in cotton (Bird 2018).

Although use of these insecticides declined in cotton they remain important management options in the Australian grains industry which relies upon broad-spectrum insecticides in part because of the lack of selective options registered in summer and winter pulse crops at that time of this study and also because broad-spectrum insecticides were a cost-effective measure for control of the susceptible species *H. punctigera* (Murray et al. 2013, Brier et al. 2008).The release of high yielding, disease resistant chickpea varieties and expanding export markets for this and other pulses led to increased production in Australia

Figure 1.8.2 Regional frequencies of synthetic pyrethroid resistance in *H. armigera* from 2011-12 to 2014-15: Fenvalerate (A) Bifenthrin (B). Numerical values indicate numbers of insects tested within each season.



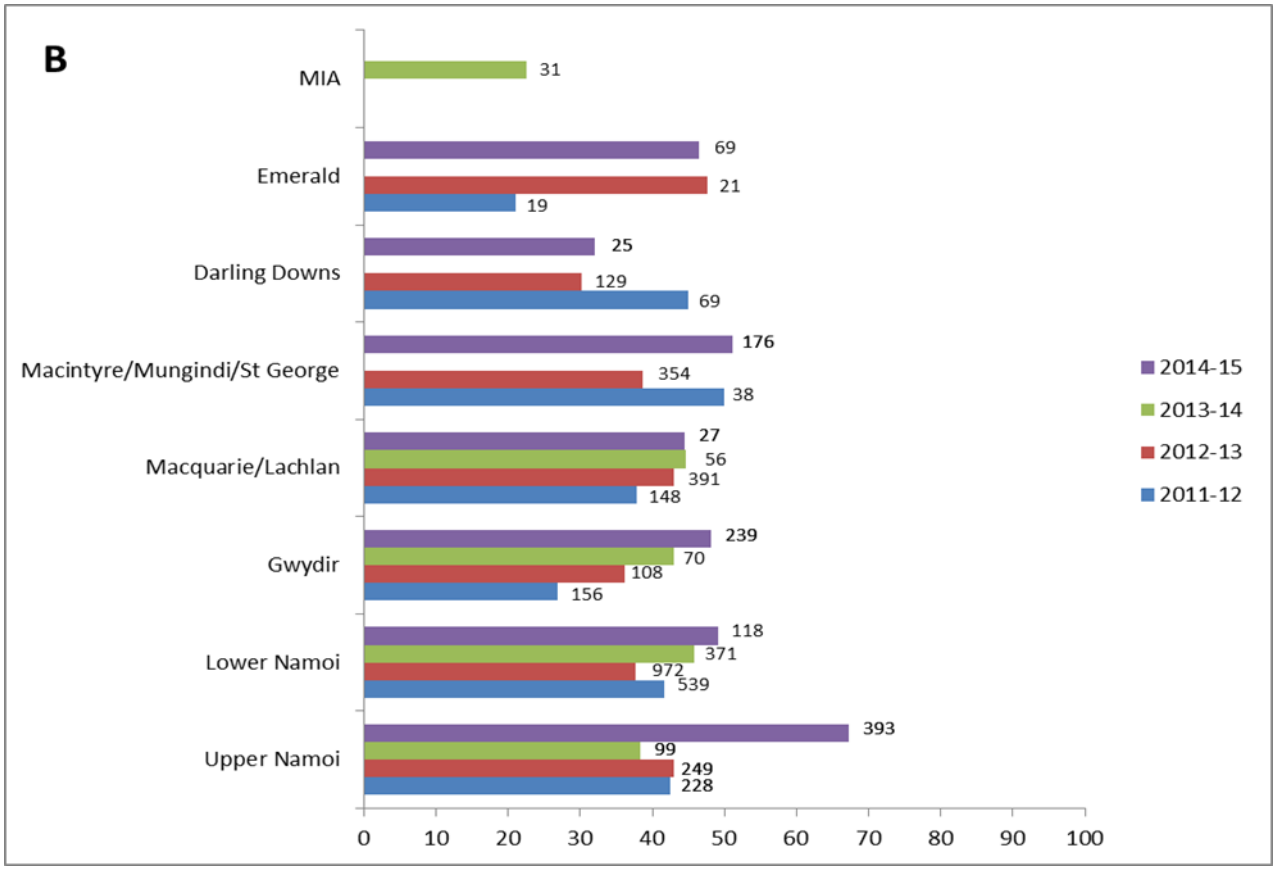
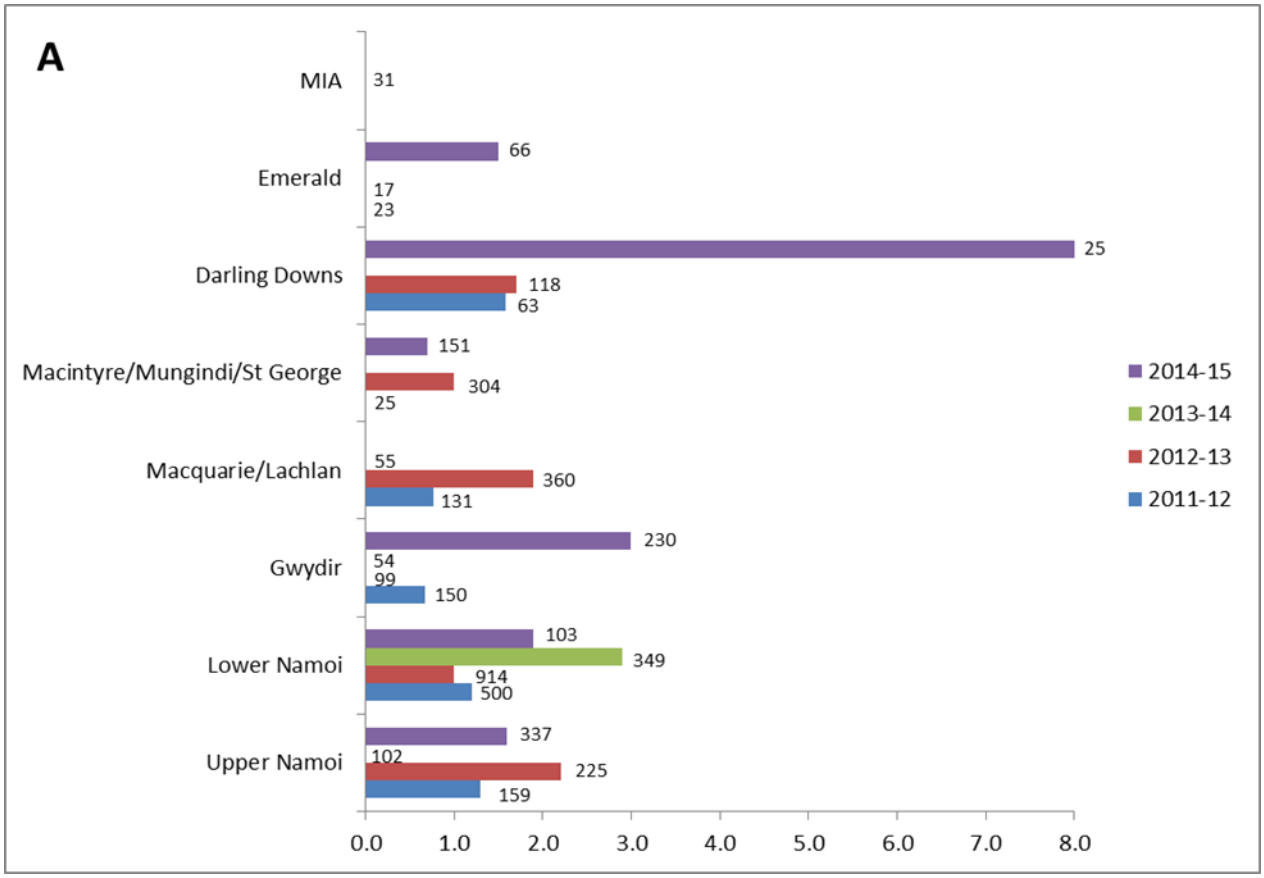
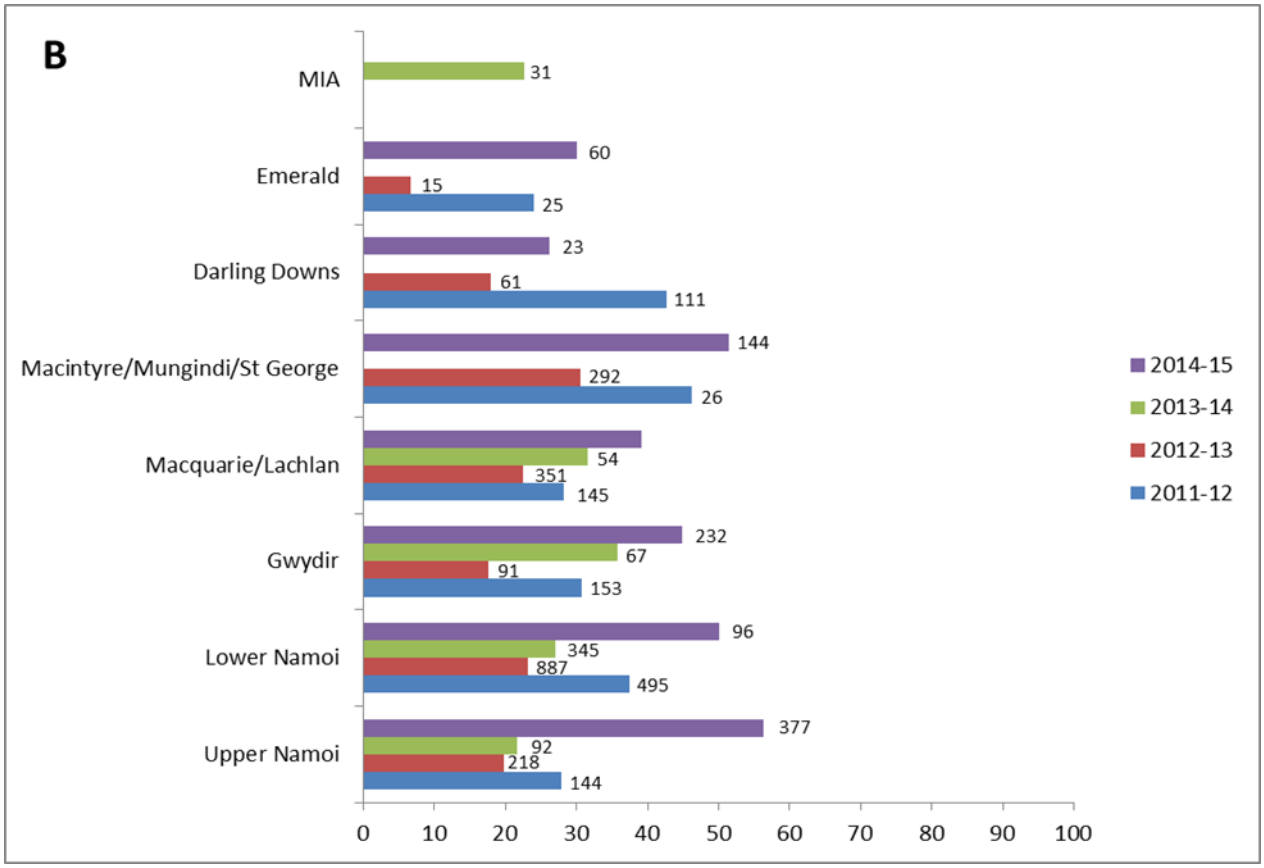


Figure 1.8.3 Regional frequencies of organophosphate and carbamate resistance in *H. armigera* from 2011-12 to 2014-15: Chlorpyrifos (A) Methomyl (B). Numerical values indicate numbers of insects tested within each season.





during the mid-2000's, with pulse hectares peaking in 2010-11 (ABARES 2018). However, pulse crops are hosts for both *H. armigera* and *H. punctigera,* with the crop most at risk from both species during the reproductive phase of plant development (Brier et al. 2008).

A mixed *Helicoverpa* spp. composition in flowering and podding winter pulses is a common occurrence (L.J.B. unpublished data) and often requires insecticidal intervention. The use of pyrethroids and methomyl for *Helicoverpa* spp. control (based either on economic thresholds or used as a prophylactic application) may have provided a scenario for resistance selection in *H. armigera*. Moreover, applications for midge control in sorghum may have also inadvertently selected for resistance in below-threshold populations of *H. armigera* and contributed to the observed increases in resistance frequency to pyrethroids and carbamates.

Whereas resistance management has been supported by the Australian cotton industry over the last 30 years by the development and implementation of a voluntary IRMS, the Australian grains industry has not had access to a formal strategy for mitigating resistance in *H. armigera*. Unregulated insecticide use during the rapid expansion of the pulse industry as a possible source of selection for resistance highlights the urgent need for a strategic approach to insecticide management for preserving the efficacy of other insecticides which are at similar at risk of resistance from over-reliance in grains production systems.

Results from this study also provide evidence that the cotton IRMS alone is not sufficient to delay resistance development in insecticides that are utilized to target *H. armigera* in other commodities. Hence, there is an imperative for the Australian grains industry to support the development and implementation of management strategies to delay the development of resistance to key selective *Helicoverpa* spp. insecticides that are currently at high risk from over-reliance in grains production systems (see Bird 2018, *Appendix 6*).

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Supplementary Milestone 1.9 Comparison of *Helicoverpa* spp. sampling methods: pheromone traps versus egg/larval collections

Introduction and aims

The Crop Consultants Australia (CCA) linked Helicoverpa egg collection project which had previously provided samples to Helicoverpa resistance monitoring projects was discontinued in 2015-16. This presented an opportunity to explore alternative methodologies for sourcing samples for resistance testing.

The aim of this study was to firstly investigate pheromone trapping as an alternative method of field sampling by evaluating the capacity of F_0 moths to generate isofemale lines compared with moths reared from F_0 eggs and larvae. The second aim was to assess whether sampling procedure influences the limits of detection of resistance genes.

Materials and methods

Sampling methods. Sampling methods were compared within seasons where collections were sourced by either visual searches of eggs and larvae, and by the use of pheromone traps (Scentry Biologicals Inc., Billings, MT) baited with *H. armigera* female pheromone lures (InSense PL Cobram, VIC). This occurred in the first two field seasons of the project during 2014-15 and 2015-16. Field-caught male moths were used in favour of field-caught female moths because multiple mating occurs in this species (Coombs et al. 1993) which could lead to misinterpretation of resistance data.

Establishment of F_0, F_1 and F_2 strains. Methods are described in *Milestone 1.4*.

Data analysis. For comparisons of mating success of moths sourced from pheromone traps and moths sourced from the field as eggs or larvae, and comparisons of proportion of positive tests from moths sourced from pheromone traps and moths sourced from the field as eggs or larvae, a logistic regression was used (generalised linear model with logistic transformation) with the two level factor (pheromone male, non-pheromone male) as the explanatory term (R Development Core Team 2012). The change in deviance by adding the explanatory term was compared to a χ^2_1 distribution to determine if proportions were significantly different.

Results

Comparison of methods for producing viable isofemale lines – pooled data

Progression to the F_2 generation of F_0 lines established from eggs and larvae compared with moths is shown in Table 1.9.1. F_2 recovery as a percentage of the number of F_0 isofemale families initiated from families derived from eggs and larvae ranged between 11.0% and 32.5% over the three years during which traditional sampling was conducted (2014-15 to 2016-2017). The average recovery from the pooled sample over the three-year period was 22.7%. On average 33.8% of the F_0 lines established from eggs and larvae produced F_1 progeny. Of these an average of 67.0% went on to produce viable F_2 progeny for testing.

On the other hand, when samples were sourced as moths during the five seasons from 2014-15 and 2018-19 F_2 recovery from F_0 lines ranged between 32.2% (2015-16) and 63.3% (2019-19). This resulted in a 2.2-fold increase in numbers of F_2 isolines from moths (average of pooled sample) compared F_2 isolines from eggs/larvae (average of pooled sample) The increase in F_2 recovery from pheromone trapped moths is attributed to a 26% higher mating efficiency in F_0 moths and a 16% increase in production of viable F_2 progeny from lines that progressed to the F_1 stage.

Although increased F_2 productivity can be attributed to a higher mating efficiency in isofemale families that incorporate field derived moths compared with families that utilize only laboratory reared moths, lower mating efficiency in laboratory reared moths is a comparatively minor factor. Of greater significance is the broader issue of inefficiencies associated with the use of field collected eggs and larvae for generating F_2 lines in determining resistance frequency specifically in *H. armigera* to selective insecticidal classes.

The primary reasons for poor recovery of F_0 material from collections of immature life stages include losses due to egg and larval parasitism, disease, spray drift and infertility of field samples of eggs. A mixed species composition can also result in significant reductions in the size of the *H. armigera* test cohort. However, this is largely overcome by selective sampling in summer cereal crops, particularly sorghum and maize. Temperature-induced stress associated with transportation was also a factor on multiple occasions due to poor transport links from remote locations. Some or all of these factors strongly influenced the size of annual testing cohorts obtained from egg/larval sampling and hence compromised the ability to properly analyse the data and draw meaningful conclusions.

Table 1.9.1 Industry-wide summary of F_2 recovery from egg/larval samples compared with pheromone trap sampling as a proportion of the number of F_0 *H. armigera* isofemale families initiated.

Source and Year	F_0 lines set up	Fertile F_1 pairs	F_2 tests	% recovery from *H. armigera* set up as F_0 lines
H. armigera sourced as eggs/larvae				
2014-15	1362	620	442	32.5
2015-16	1011	268	140	13.8
2016-17	371	42	41	11.0
TOTAL	2744	930	623	22.7
H. armigera sourced as moths				

2014-15	371	254	232	62.6
2015-16	1600	735	515	32.2
2016-17	2223	1245	985	44.3
2017-18	1745	1143	990	56.7
2018-19	1659	1162	1070	64.5
TOTAL	7598	4559	3792	49.9

Comparison of methods for producing viable isofemale lines –annual regional data

2014-15 results. The capacity for generating successful isofemale families from insects collected from the field as eggs and larvae in 2014-15 is shown in Table 1.9.2 (sex ratio of immature life stages = 1). When averaged across all regions, only 2.1% of the total samples collected as eggs and larvae produced viable material for F_2 testing. In contrast, 62.5% of F_0 lines established from pheromone trap-caught male moths and crossed with laboratory susceptible females went on to produce viable F_2 isofemale lines tested for resistance to one or more insecticides (Table 1.9.4).

A mixed species composition was also a major factor that accounted for loss of available insects for resistance testing. However, it is noteworthy that even when collections were strongly dominated by *H. armigera*, as was the case in the upper Namoi/Gwydir, Macintyre and Emerald samples from 2014-15 it was only possible to recover between 3.7% and 9.5% of individuals for F_1 establishment from these samples (Table 1.9.2).

A summary of fertility data from pheromone and non-pheromone trap catches in 2014-15 is shown in Table 1.9.5. The difference between pheromone trap caught males and moths from other sources is highly significant in all cases ($P < 0.0001$) indicating that moths from pheromone traps have a greater degree of mating efficiency, particularly in the F_0 generation, than their male and female counterparts sourced from the field as eggs and larvae.

2015-16 results. The analysis was repeated in 2015-16 and the results presented in Table 1.9.3 which demonstrates the trend is similar to the previous year with a highly significant difference between mating efficiency of pheromone caught moths compared with laboratory reared moths collected from the field as eggs or larvae (sex ratio of immature life stages = 1.1).

A notable difference in the results for 2015-16, only 0.7% of total samples collected as eggs and larvae produced viable offspring for testing and, as a proportion of *H. armigera* cohort collected, only 2.3% was recovered for testing. Notwithstanding the poor F_2 recovery from immature life stages, notable exceptions were obtained in samples from the Tamworth Agricultural Institute (TAI) (25%) and a one-off sample of larvae from chickpeas (technically within the 2016-17 season) (34.7%). In contrast samples, originating from pheromone traps produced 32% of viable F_2 isofemale lines (Table 1.9.4).

Although the number of tests conducted (as a percentage of F_0 lines established) was lower than in the previous year, the results demonstrated a similar increase in capacity for pheromone trap male moths to produce viable F_2 isofemale lines. Significantly more pheromone trap collected moths progressed to the testing stage (32.2%), compared with male moths and female moths that had been collected as immature life stages and reared to adults in the laboratory (15.3 and 12.3%, respectively) ($P < 0.001$) (Table 1.9.6).

2016-17 to 2018-19 results. A summary of results from pheromone trapping during the following three years of the project (2016-2019) are shown in Table 1.9.4. The recovery of F_2 isofemale lines was 44.3%, 56.7% and 64.5% in 2016-17, 2017-18 and 2018-19, respectively.

Comparison of trapping methods for detecting resistance genes

Another key question relates to whether resistance frequency is influenced by sampling procedure. Table 1.9.7 summarises the recovery of resistant individuals from the two sampling techniques in 2014-15. The number of positive tests from pheromone trap caught males (10/232, 4.3%) was the

same as that from the non-pheromone trap caught males (13/305, 4.3%). There was also no significant difference (P = 0.555) between the number of positive tests from the pheromone trap males and all non-pheromone trap moths (male and female moths from egg and larval samples) (15/436, 3.4%). Although the frequency of resistance in female moths was lower (1.5%) than in pheromone or non-pheromone caught males, the difference was not significant (P = 0.255).

Table 1.9.8 summarises the recovery of resistant individuals from different moth sources collected in 2015-16. As in the previous year the number of positive indoxacarb tests from pheromone trap males (2.2%) was not significantly different from the male cohort sourced from eggs and larvae (2.5%) (P = 1.0). There was also no significant difference between the number of positive indoxacarb tests from pheromone trap males and female moths derived from field collected eggs and larvae (3.6%) (P = 0.561). These comparisons suggest the source of moths does not influence presence of resistance alleles and combining resistance monitoring data from F_2 screens where insects have been sourced from moth and egg/larval collections is justified.

Discussion and conclusions

The findings from this study show that utilizing pheromone caught moths greatly enhances mating capacity in the F_1 generation and consequently results in a significantly greater proportion of samples progressing to the critical F_2 stage for inclusion in the testing cohort compared with moths reared from field collected eggs and larvae. In addition, females derived from immature life stages produced significantly fewer F_2 lines than males derived from immature life stages, suggesting progeny of field derived females had lower viability than laboratory adapted females. Adaptation to laboratory environments has also been attributed to improved performance of F_1 progeny of *Ostinia nubilalis* (Stodola et al. 2006).

Inefficiencies associated with traditional visual sampling of eggs and larvae for establishing isofemale families in F_2 screening are clearly supported by the data presented above and highlighted by the fact that an extremely low proportion of the sample collected in 2014-15 (2.1%) and 2015-16 (0.7%) progressed to the testing stage. Considering the human resources and operating costs associated with collecting and processing this volume of samples to achieve a disproportionately small testing cohort suggests that traditional sampling for surveillance of *H. armigera* resistance does not provide a good return on industry investment.

Table 1.9.2 Regional summary of F_2 recovery from egg/larvae collecting 2014-15.

Location	Eggs + Larvae	Total Hatched	Total *H. armigera*	Total *H. punctigera*	% *H. armigera*	Male	Female	F_0 lines set up	Fertile F_1 pairs	F_2 tests	% recovery from total egg/larvae collected	% recovery from *H. armigera* collected
Lower Namoi	2164	1189	838	351	70.5	113	102	215	99	77	3.6	9.2
Upper Namoi	3494	2159	2159	9	100.0	124	123	247	121	79	2.3	3.7
Gwydir	2649	1770	1770	1	100.0	120	120	240	114	83	3.1	4.7
Macquarie	1450	720	296	424	41.1	24	23	47	19	16	1.1	5.4
Mungindi	2806	1398	766	632	54.8	57	59	116	57	38	1.4	5.0
Darling Downs	1577	390	243	147	62.3	46	56	102	32	14	0.9	5.8
St George	2530	1553	223	1330	14.4	66	67	133	59	38	1.5	17.0
Emerald	2090	602	526	76	87.4	57	68	125	60	50	2.4	9.5
Macintyre	1456	705	573	132	81.3	27	35	62	32	24	1.6	4.2
MIA	293	140	11	129	7.9	8	4	12	5	5	1.7	45.5
Kununurra	274	152	82	70	53.9	32	31	63	22	18	6.6	22.0
TOTAL	20783	10892	7497	3395	68.8	674	688	1362	620	442	2.1	5.9
Sex ratio						49.5	50.5					

able 1.9.3 Regional summary of F_2 recovery from egg/larvae collecting 2015-16.

Location	Eggs + Larvae	Total Hatched	Total *H. armigera*	Total *H. punctigera*	% *H. armigera*	Male	Female	F_0 lines set up	Fertile F_1 pairs	F_2 tests	% recovery from total egg/larvae collected	% recovery from *H. armigera* collected
Lower Namoi	5652	2592	1416	1176	54.6	98	65	163	21	8	0.1	0.6
Upper Namoi	8905	4834	4003	863	82.8	162	187	349	115	59	0.7	1.5
TAI	87	24	24	0	100.0	11	13	24	8	6	6.9	25.0
Gwydir	1329	689	621	68	90.1	19	21	40	17	9	0.7	1.4
Macquarie	1645	982	373	609	38.0	49	41	90	22	1	0.1	0.3
Mungindi	2105	1308	211	1097	16.1	20	19	39	5	3	0.1	1.4
Darling Downs	1753	856	481	375	56.2	58	48	106	38	32	1.8	6.7
St George	1787	1079	138	941	12.8	46	35	81	21	16	0.9	11.6
Emerald	1801	900	226	674	25.1	37	12	49	7	4	0.2	1.8
Macintyre	1205	731	258	473	35.3	27	32	59	12	2	0.2	0.8
MIA	615	232	10	222	4.3	4	7	11	2	0	0.0	0.0
TOTAL	**26884**	**14227**	**7761**	**6498**	54.6	**575**	**527**	**1102**	**310**	**181**	**0.7**	**2.3**
Sex ratio						**52.2**	**47.8**					
[†] Emerald	118	118	118	0	100	44	47	91	42	41	34.7	**34.7**

[†] One-off collection of larvae from chickpea 17/07/2016 provided by CSIRO.

Table 1.9.4 Regional summary of F_2 recovery from pheromone trapping.

Year and Location	F_0 lines set up	Fertile F_1 pairs	F_2 tests	% recovery from *H. armigera* moths collected
2014-15				
Upper Namoi	371	254	232	**62.5**
TOTAL	371	254	232	**62.5**
2015-16				
Lower Namoi	213	114	64	30.0
Upper Namoi	1115	489	340	30.5
Tamworth Ag. Institute	272	132	111	40.8
TOTAL	**1600**	**735**	**515**	**32.2**
2016-17				
Lower Namoi	277	175	143	51.6
Upper Namoi	538	273	220	40.9
Gwydir	253	136	70	27.7
Macquarie	219	140	112	51.1
Darling Downs	179	94	55	30.7
Emerald	757	427	385	50.9
TOTAL	**2223**	**1245**	**985**	**44.3**
2017-18				
Lower Namoi	242	176	157	64.9
Upper Namoi	398	263	214	53.8
Gwydir	176	120	109	61.9
Macquarie	223	150	116	52.0
Darling Downs	155	108	97	62.6
Emerald	304	173	156	51.3
Macintyre	225	145	135	60.0
Burdekin	22	8	6	27.3
TOTAL	**1745**	**1143**	**990**	**56.7**
2018-19				
Lower Namoi	225	150	140	62.2
Upper Namoi	109	66	60	55.0
Gwydir	302	238	209	69.2
Darling Downs	265	193	174	65.7
Emerald	175	128	120	68.6
Macintyre	100	80	69	69.0
Burdekin	123	72	66	53.7
Clermont	150	97	94	62.7
Dawson/Callide	210	158	138	65.7
TOTAL	**7598**	**4539**	**3772**	**64.5**

Table 1.9.5 Comparison of fertility in single-pairs established using pheromone trapped male moths and moths sourced from egg and larval samples in 2014-15.

Moth source	Total emerged	Total tested	% Fertile	*P*
Pheromone trap males upper Namoi	371	232	61.7 [a]	
Non-pheromone trap males	674	305	45.3 [b]	<0.0001
Non-pheromone trap females	688	131	19.0 [c]	<0.0001
All non-pheromone trap moths	1362	436	32.0 [bc]	<0.0001
Non-pheromone trap males upper Namoi	124	50	40.3 [b]	<0.0001

Superscript letters after % fertility indicate significant differences ($P < 0.05$).

Table 1.9.6 Comparison of fertility in single-pairs established using pheromone trapped male moths and moths sourced from egg and larval samples in 2015-16.

Moth source	Total emerged	Total tested	% Fertile	*P*
Pheromone trap males	1600	512	32.0 [a]	
Non-pheromone trap males	531	81	15.3 [b]	<0.0001
Non-pheromone trap females	480	59	12.3 [b]	<0.0001
All non-pheromone trap moths	1011	140	13.8 [b]	<0.0001

Superscript letters after % fertility indicate significant differences ($P < 0.05$).

Table 1.9.7 Comparison of positive tests for indoxacarb resistance from pheromone trapped male moths and moths sourced from egg and larval samples in 2014-15.

Moth Source	Total tests	Positive tests	% R	*P*
Pheromone trap male	232	10	4.3	-
Non-pheromone trap male	305	13	4.3	1.0
Non-pheromone trap females	131	2	1.5	0.255
All non-pheromone trap moths	436	15	3.4	0.555

Table 1.9.8 Comparison of positive tests for indoxacarb resistance from pheromone trapped male moths and moths sourced from egg and larval samples in 2015-16.

Moth Source	Total tests	Positive tests	% R	*P*
Pheromone trap male	510	11	2.2	-
Non-pheromone trap male	81	2	2.5	1.0
Non-pheromone trap females	59	3	5.1	0.352
All non-pheromone trap moths	140	5	3.6	0.561

The implementation of a pheromone trapping method to source wild-caught male moths has resulted in vast improvements in efficiency for producing F_2 lines. Increased F_2 production is due to the elimination of losses from egg and larval parasitism, pathogenic infection, infertility, mixed species composition, spray drift, and temperature-induced stress associated with transport of egg and larval samples from remote locations. An additional advantage of a method that uses field-caught moths instead of immature life stages is that it circumvents the need for extended laboratory rearing of insects and hence further enhances the economic efficiency with which samples can be processed (Bolin et al. 1998).

Levels of detection for indoxacarb resistance were not significantly different between pheromone and non-pheromone trapped moths suggesting that the source of moths does not influence the limits of detection for resistance. The increased efficiency of a pheromone-based sampling procedure combined with increased sensitivity of the F_2 screening technique should enhance our ability to identify resistance risk to key selective insecticides for control of *H. armigera*.

In summary, pheromone trapping is an innovative approach in insecticide resistance surveillance which is significantly less labour intensive and provides a more economical methodology for achieving project outcomes compared with traditional egg and larval sampling. Importantly, this method increases the volume of samples from geographically discrete populations and provides greater statistical power for interpretation of regional differences in resistance frequency.

References

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Stodola TJ, Andow DA, Hyden AR, Hinton LJ, Roark JJ, Buschman LL, Porter P and Cronholm GB (2006) Frequency of resistance to *Bacillus thuringiensis* toxin Cry1Ab in southern United States corn belt population of European corn borer (Lepidoptera: Crambidae). *Journal of Economic Entomology* 99:502-507

Supplementary Milestone 1.10 Investigations of alternative trapping systems in Helicoverpa resistance surveillance

Introduction and aims

While the use of Heliothis net traps in resistance surveillance has proved highly successful they are relatively expensive can be difficult to transport to locations remote from the central testing laboratory. Canister (Universal) traps are considerably cheaper (~$15 each) compared with net traps (~$180 each), less cumbersome to operate and more efficient for catching male *H. armigera*. However, canister traps have a solid plastic base which may compromise the quality of moth samples due to heat stress and mechanical damage, thus reducing fitness compared with those caught in net traps which have a more ventilated mesh trapping receptacle. The aim of this study was to investigate a system of trapping that could be used in central and northern Qld that would be more cost-effective than Heliothis net traps while not risking sample quality.

Materials and methods.

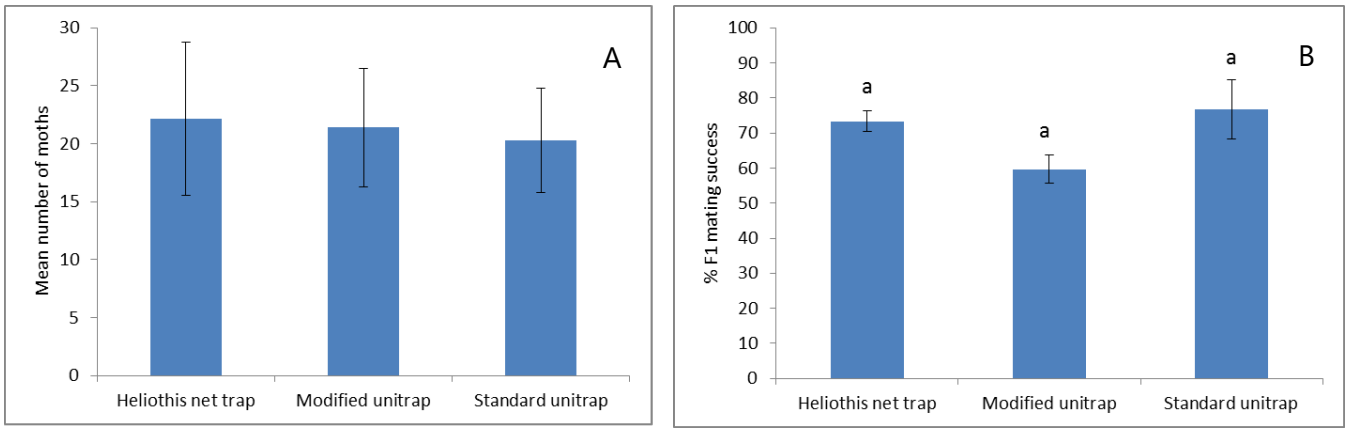
A hybrid trap design was developed comprising the top of a canister trap with a mesh trapping receptacle for greater ventilation (hereafter called modified unitraps). The performance of traditional canister traps (hereafter called standard unitraps) and net traps was evaluated in side-by-side comparisons with modified unitraps conducted on the Liverpool Plains and in the Macquarie Valley during the 2017-2018 season.

Figure 1.10.1 Pheromone trap designs: A Heliothis net trap; B standard unitrap; C modified unitrap with mesh trapping receptacle.

Results

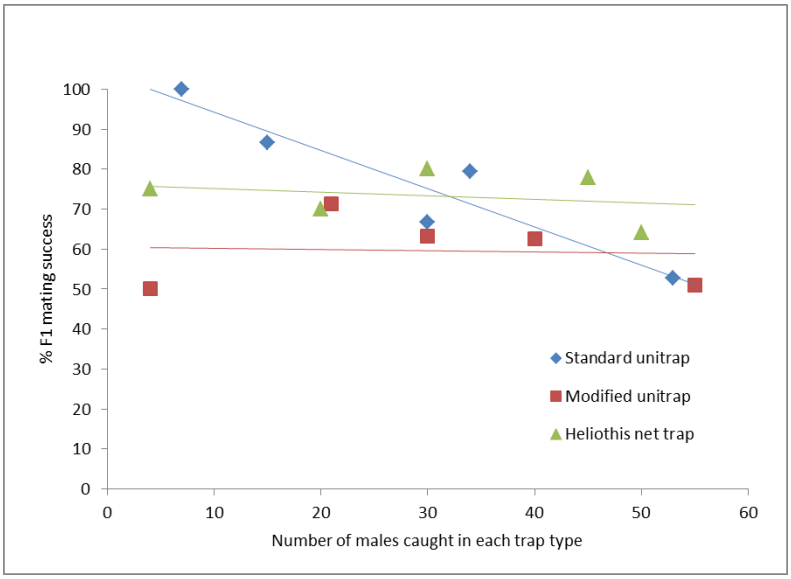
The total number of moths caught in the upper Namoi and Macquarie for evaluating relative effectiveness of the three different trap types during 2017-18 was 1529. The numbers of moths caught in each trap type were similar (n = 510, 527 and 492 in Heliothis net traps, modified unitraps and standard unitraps, respectively) and the average number of moths caught in each trap type was also similar (Figure 1.10.2A). The mating success was compared between males caught in each trap design (Liverpool Plains only) when mated with New GR females in the laboratory. While the mating success of males caught in modified unitraps was slightly lower than in Heliothis net traps and standard unitraps, the differences were not significant (Figure 1.10.2B).

Figure 1.10.2 Mean number of *H. armigera* males caught in three pheromone trap designs: Heliothis net traps, modified unitraps and standard unitraps (A) and mating success of male *H. armigera* caught in three types of pheromone traps in the upper Namoi Valley in 2017-18 (B).



There was a negative relationship between the number of males caught in standard unitraps and mating performance, but not in Heliothis net traps or modified unitraps (Figure 1.10.3). This suggests that as pheromone trap catch increases in standard unitraps, male mating performance decreases.

Figure 1.10.3 Mating success of male *H. armigera* plotted against the total number of moths caught in each trap design for each trapping occasion.



Discussion and conclusions

Evidence from these experiments suggests performance of modified unitraps is comparable with that of the other two trapping systems. Importantly, mating capacity is not reduced in modified unitraps as the number of moth in traps increase. This is in contrast to reduced mating success in standard unitraps when moth numbers increased and is likely due to improved aeration of the holding reciprocal in the field if temperatures increase significantly before traps are collected, during transit of moths from the field to the laboratory and during storage before laboratory processing occurs.

The finding that Texas or modified Unitraps resulted in improved quality of moth samples irrespective of number of moths caught in traps, led to i) continued use of Heliothis net traps in by local collectors and, ii) deployment of the smaller, cost-effective and compact modified unitraps to source and transport samples from grains production areas of central and northern Qld to the testing laboratory at Narrabri.

Objective 2. Isolation and characterisation of mechanisms that confer resistance to selective chemistries in *H. armigera* if detected from the field

Milestone 2.1 Using survivors from F_2 screens of *H. armigera*, establish strains that confer resistance to selective chemistries by multiple backcrossing

Introduction and aims

In 2013, F_2 screening by diet incorporation was introduced for monitoring resistance to selective insecticides in Australian populations of *H. armigera*. This method resulted in increased capacity for detection of low frequency resistance and has led to the first case of genetic indoxacarb resistance isolated from field populations of *H. armigera* in Australia.

A significant advantage of selecting resistant strains recently derived from the field is they are less likely to be confounded by additive effects. To reduce the potentially misleading effects of hybrid vigour the first indoxacarb resistant strain isolated (GY7-39) was serially backcrossed five times to a susceptible strain to produce near-isogenic resistant and susceptible strains with >98% genetic similarity.

Materials and methods

Insect strains. The laboratory strain New GR was established from a cohort of a general laboratory strain GR sourced during the mid-1980s from a series of collections from cotton fields in the Namoi Valley. The New GR strain was susceptible to indoxacarb and monitored regularly by bioassay to evaluate the response to a diagnostic dose of indoxacarb (12µg indoxacarb/ml in a diet incorporation bioassay performed on late 2nd or early 3rd instar larvae) that kills 99.9% of susceptible larvae. The New GR strain was used as the susceptible control in all bioassays and in crosses to the resistant strain. Bioassays were performed on a minimum of four non-synchronous cohorts. As there were no significant differences between cohorts for any of the insecticides tested the results were pooled in the final analysis.

The resistant strain was established from a single *H. armigera* moth collected as an egg from maize near Moree, in November 2013. Progeny from the mating of this moth with a moth from the susceptible New GR strain were subjected to an F_2 screen. The resistant GY7-39 strain was created from F_2 offspring that survived the diagnostic concentration of indoxacarb.

The GY7-39 strain initially had a restricted gene pool because it originated from an isofemale line. To reduce founder effects and maintain indoxacarb resistant and susceptible strains in a common genetic background, the GY7-39 strain was introgressed into the susceptible strain (New GR) by repeated backcrossing (at generations 5, 7 and 12) and reselection to produce near-isogenic resistant and susceptible strains of *H. armigera*. After the first cross, the strain was maintained without selection for one generation and reselected in the subsequent generation with the discriminating dose of indoxacarb. As there was significant survival of F_1 progeny at this concentration the F_1 progeny from the second and third backcrosses were directly subjected to selection.

Insect rearing. Rearing methods used were the procedures previously described. Rearing trays (Tacca Plastics, Sydney, Australia) were covered and heat-sealed with perforated lids (Oliver Products, Grand Rapids, MI). Moths were provided with a 4% honey/sugar solution fed through a cotton wick and housed in containers open at the top and covered with cloth liners secured around the lip of the containers. Eggs were harvested by washing cloth liners in 1% bleach and collecting them on a Whatman No. 54 filter paper by vacuum filtration. Filter papers air dried and placed in sealed plastic bags until neonates hatched. In the larval stage, insect strains were maintained in a laboratory environment of 25 ± 2°C with 14:10 (L: D) h photoperiod and 45-55% RH. Adults were maintained separately under the same conditions of light and temperature and ambient RH.

Resistance screening, bioassays and selection. Resistance was tested by using an F_2 screening procedure which generated isofemale lines from field derived male moths, as described in *Milestone 1.4*. The responses of susceptible and selected strains were measured by performing bioassays on artificial diet into which formulated indoxacarb insecticide (Steward® [15% active ingredient]), DuPont Australia Ltd., Macquarie Park) was incorporated. As above, stock suspension of insecticide was diluted with distilled water to produce six or seven two-fold dilutions; the ratio of diet to insecticide determined the concentration calculated as micrograms of insecticide per millilitre of diet. Serial dilutions were added to 200ml of diet and incorporated by vigorous shaking by hand for 30 seconds to produce a homogenous mixture which was then dispensed into 45-well bioassay trays (Tacca Plastics, Sydney). Late second or early third instars were introduced to trays (one larva per well); untreated diet was used as the control.

Bioassays were performed on three cohorts of insects with individual treatments (insecticide concentrations) of each cohort consisting of a minimum of 20 individuals. Bioassays were maintained under the conditions for larval rearing, described above. The range of concentrations used in each bioassay was expected to induce 1 to 99% mortality. Bioassays were assessed at seven days using standard mortality criteria, described above.

Data analysis. Where necessary, bioassay data were corrected for control mortality (Abbott 1925). Estimates of LC_{50} values and their 95% fiducial limits (FL) were obtained by probit analysis by using POLO-PC software (LeOra Software, Berkeley, CA). Resistance ratios (RR) were calculated by dividing the LC_{50} value of the resistant strain by the LC_{50} value (average of four non-synchronous cohorts) of the laboratory strain (0.147µg/ml) (Bird 2015). Significant differences ($P = 0.05$) between LC_{50} values were determined by the lethal concentration ratio test according to Wheeler et al. (2006) where, if the 95% confidence interval (CI) includes one then the LC_{50}s are not significantly different.

Results

After three generations of selection the field-derived GY7-39 strain (F_5) had 173-fold resistance to indoxacarb compared with the laboratory strain New GR (Table 2.1.1). The GY7-39 strain was then crossed with New GR in the following generation (F4) to create the GY7-39BC_1 strain (Table 2.1.1). Following two generations of resection the strain was crossed on a second occasion when the resistance ratio was 85-fold (Table 2.1.1) and again on a third occasion after five generations of selection when the resistance ratio had reached 141-fold (Table 2.1.2). Reselection in the third backcross resulted in a resistance ratio of >200-fold after four generations (Table 2.1.3). It was estimated (by the principle of Mendelian segregation) that the third backcross had produced a strain of GY7-39 that shared > 93% of its genome with that of the parental strain New GR.

After eight generations of selecting the GY7-39BC_3 strain a fourth outcross was performed with the resistance ratio of this, the GY7-39BC_4 strain, exceeding 300-fold on multiple occasions (Table 2.1.4). After 12 generations of selection the GY7-39BC_4 strain was outcrossed to create the GY7-39OC_5 strain with an estimated genetic similarity to the parental New GR strain of 98.4% (Table 2.1.5).

In 2017-18 a second indoxacarb resistant genotype was isolated by F_2 screening from a field population in the Liverpool Plains region of NSW. The strain was designated UN1U3-10. Serial backcrossing of this strain has commenced and the results shown in Table 2.1.6.

Discussion and conclusions

Quantitative genetic characterisation of resistant strains should be conducted with reference to a near-isogenic laboratory susceptible strain. In other words, the strains of interest should be genetically identical except for the small region of the genome that includes the locus of interest (ffrench-Constant and Bass 2017) to ensure a high probability that observed differences between strains are associated with the resistance allele(s) and not due to differences in hybrid vigour (Hartl and Clark 1997). Moreover, the use of a backcrossing procedure also reduces potentially

confounding effects of inbreeding depression. As the GY7-39 strain originated from a single isofemale family, repeated rounds of

Table 2.1.1 First serial backcross of indoxacarb resistant strain GY7-39 and New GR.

Collection information						
Date: Code: Host		**06.11.13: GY7: maize**				
Generation in lab		**F2**	**F3**	**F4**	**F5**	**F6**
Survival @ 12µg/ml (Total tested)		**10.0 (90)**	**71.6 (545)**	**79.8 (1394)**	**60.9 (294)**	**95.5 (513)**
Probit Analysis Results		LC_{50}	95% FL	Slope	RR	
GY7-39 (F_5)†		25.371	21.333, 30.056	3.0	173	
Generation outcrossed to New GR		F4 (June 2014)				
Generation in lab		F1	F2	F3	F4	F5
Survival @ 12µg/ml (Total tested)		NR	67.2 (702)	85.6 (1323)	88.2 (1258)	87.6 (516)
Probit Analysis Results		LC_{50}	95% FL	Slope	RR	
New GR (Bird 2015)		0.147	0.137, 0.158	3.4	0	
GY7-39 (F5)		25.371	21.333, 30.056	3.0	173	
$BC1_1$ (GY7-39 ♀ x New GR ♂)		6.998	4.674, 11.166	1.6	47	
$BC1_1$ (GY7-39 ♂ x New GR ♀)		9.345	6.314, 14.431	2.1	63	
$BC1_1$ hets combined	F5	7.970	5.889, 11.097	1.8	54	
$BC1_2$‡	F6	12.486	9.633, 15.967	1.8	85	
$BC1_3$		20.799	16.250, 27.302	1.6	141	
$BC1_4$		19.845	15.173, 26.671	2.2	135	
$BC1_5$		29.682	25.856, 34.026	4.3	202	
$BC1_6$		19.900	14.800, 27.334	2.4	135	
$BC1_7$		18.733	13.074, 27.950	2.2	127	
$BC1_8$		Not bioassayed				
$BC1_9$		Colony discarded with significant inbreeding impacting on fitness				

† GY7-39 strain was isolated by F_2 screening and the survivors selected for three generations.
‡ In all tables indicates generation when subsequent backcross occurred.

Table 2.1.2 Second serial backcross of indoxacarb resistant strain GY7-39 and New GR.

Generation backcrossed to New GR: $BC1_2$ (Sept. 2014)					
Generation in lab		**F1**	**F2**	**F3**	**F4**
Survival @ 12µg/ml (Total tested)		**43.7 (1579)**	**NR**	**79.5 (391)**	**NR**
Probit Analysis Results		**LC_{50}**	**95% FL**	**Slope**	**RR**
$BC2_1$ (GY7-39 ♀ x New GR ♂)		5.632	3.207, 9.118	1.4	38
$BC2_1$ (GY7-39 ♂ x New GR ♀)		6.604	4.965, 8.843	1.6	45
$BC2_1$ hets combined	F7	6.574	4.801, 9.032	1.6	45
$BC2_2$	F8	9.799	6.934, 13.756	1.5	67
$BC2_3$	F9	Not bioassayed			
$BC2_4$	F10	16.0	11.398, 23.550	1.9	109
$BC2_5$‡	F11	20.791	16.959, 25.382	2.5	141
$BC2_6$		29.177	24.695, 34.792	2.6	198
$BC2_7$		Not bioassayed			
$BC2_8$		Not bioassayed			
$BC2_9$		23.173	19.226, 27.974	2.7	158
$BC2_{10}$		36.573	23.768, 64.846	1.8	249

Table 2.1.3 Third serial backcross of indoxacarb resistant strain GY7-39 and New GR.

Generation backcrossed to New GR: $BC2_5$ (June 2015)					
Probit Analysis Results		**LC_{50}**	**95% FL**	**Slope**	**RR**
$BC3_1$ (GY7-39 ♀ x New GR ♂)		10.691	8.372, 13.977	2.2	73
$BC3_1$ (GY7-39 ♂ x New GR ♀)		8.768	7.590, 10.128	2.8	60
$BC3_1$ hets combined	F12	9.589	8.355, 11.038	2.4	65
$BC3_2$	F13	5.464	3.602, 8.477	1.0	37
$BC3_3$	F14	19.743	14.371, 28.377	2.2	134
$BC3_4$	F15	31.335	26.305, 37.490	3.5	213
$BC3_5$	F16	32.535	28.027, 37.664	3.8	221
$BC3_6$	F17	16.273	14.378, 18.414	3.3	111
$BC3_7$	F18	33.423	27.631, 40.981	2.9	227
$BC3_8$ ‡	F19	19.458	15.173, 25.493	1.8	132
$BC3_9$		11.094	8.739, 13.809	1.8	75
$BC3_{10}$		16.484	14.474, 18.791	3.5	112
$BC3_{11}$		16.727	13.394, 20.885	1.9	114

Table 2.1.4 Fourth serial backcross of indoxacarb resistant strain GY7-39 and New GR.

Generation backcrossed to New GR: $BC3_8$ (June 2016)					
Probit Analysis Results		**LC_{50}**	**95% FL**	**Slope**	**RR**
$BC4_1$ (GY7-39 ♀ x New GR ♂)		6.743	4.626, 10.112	1.9	46
$BC4_1$ (GY7-39 ♂ x New GR ♀)		8.977	7.203, 11.239	2.1	61
$BC4_1$ hets combined	F20	7.785	6.214, 9.826	1.9	53
$BC4_2$	F21	31.236	21.977, 48.050	2.0	212
$BC4_3$	F22	23.810	20.046, 28.255	2.3	162
$BC4_4$	F23	15.515	13.473, 17.880	2.3	105
$BC4_5$	F24	28.863	23.786, 35.587	2.8	196
$BC4_6$	F25	46.828	26.771, 131.991	2.0	319
$BC4_7$	F26	56.722	49.148, 66.880	3.2	386
$BC4_8$	F27	31.872	27.285, 37.682	2.6	217
$BC4_9$	F28	Not bioassayed			
$BC4_{10}$	F29	34.498	30.944, 46.745	1.9	235
$BC4_{11}$	F30	20.763	17.919, 24.125	2.7	142
$BC4_{12}$ ‡	F31	47.863	40.573, 56.537	2.5	326
$BC4_{13}$		23.189	19.637, 27.723	3.9	158
$BC4_{14}$		33.811	28.086, 40.458	3.8	230
$BC4_{15}$		27.730	22.864, 33.627	3.2	189
$BC4_{16}$		23.365	15.756, 34.257	2.3	166
$BC4_{17}$		19.766	16.060, 24.299	3.1	135
$BC4_{18}$		21.376	18.839, 24.287	3.6	145

Table 2.1.5 Fifth serial backcross of indoxacarb resistant strain GY7-39 and New GR.

Generation backcrossed to New GR: $BC4_{12}$ (July 2018)					
Generation in lab		**F1**	**F2**	**F3**	**F4**
Survival @ 12µg/ml (Total tested)		**50.1 (737)**	**66.0 (2340)**	**NR**	**88.7 (495)**
Probit Analysis Results		**LC_{50}**	**95% FL**	**Slope**	**RR**
$BC5_1$ (GY7-39 ♀ x New GR ♂)		10.430	9.308, 11.682	4.7	71
$BC5_1$ (GY7-39 ♂ x New GR ♀)		12.245	10.075, 14.787	4.1	83
$BC5_1$ hets combined	F32	11.328	10.168, 12.602	4.3	77
$BC5_2$	F33	6.498	5.557, 7.469	2.0	44
$BC5_3$	F34	19.549	11.229, 37.322	2.8	133
$BC5_4$	F34	19.089	16.986, 21.460	4.4	130
$BC5_5$	F35	20.138	16.756, 24.242	3.5	134
$BC5_6$	Not bioassayed				

Table 2.1.6 First serial backcross of indoxacarb resistant strain UN1U3-10 and New GR.

Collection information					
Date: Code: Host		**18.10.17: UN1U3: chickpeas**			
Generation in lab		**F2**	**F3**	**F4**	**F5**
Survival @ 12µg/ml (Total tested)		**28.9 (180)**	**52.6 (352)**	**93.6 (419)**	**95.6 (295)**
Probit Analysis Results		**LC_{50}**	**95% FL**	**Slope**	**RR**
GY7-39 (F4) †		82.219	67.261, 100.980	2.7	560
Generation outcrossed to New GR (date)		F4 (Aug. 2018)			
Generation in lab		F1	F2	F3	F4
Survival @ 12µg/ml (Total tested)		96.6 (899)	NR	NR	NR
Probit Analysis Results		LC_{50}	95% FL	Slope	RR
New GR (Bird 2015)		0.147	0.137, 0.158	3.4	0
UN1U3-10 (F4)		82.219	67.261, 100.980	2.7	560
UN1U3-10 (F5)		110.635	94.821,130.229	2.7	753
BC_1 (UN1U310 ♀ x New GR ♂)		53.265	47.491, 59.840	4.4	362
BC_1 (UN1U310 ♂ x New GR ♀)		57.481	48.826, 67.996	3.9	391
$BC1_1$ hets combined	F5	55.339	50.215, 61.068	4.1	376
$BC1_2$	Not bioassayed				

recombination by backcrossing and reselection reduced the risk of genetic bottlenecks in the GY7-39 strain that may have occurred through loss of genetic variance (Hartl and Clark 1997). As a result of this program of serial backcrossing, the fifth serial backcross of the GY7-39 strain (GY7-39BC5) had >98% isogenicity with the susceptible parental strain New GR.

An important part of building a risk assessment profile of indoxacarb resistance is to undertake studies to investigate the degree of dominance of resistance genes and whether fitness costs are associated with resistance. While genetic dominance is a key factor for influencing the rate at which resistance will develop in a population, fitness costs are also considered to be important because

they can reduce the coefficient of selection, favouring susceptible individuals relative to resistant individuals in the absence of selection which helps to decrease the spread and frequency of resistance alleles in field populations (Carrière and Tabashnik 2001).

Many studies have identified an association between fitness costs and resistance. However, only a small number attribute deleterious pleiotropic effects to resistance (e.g. reduced survival, increased development time and decreased reproductive performance) in near-isogenic strains. Studies that identify fitness costs using comparisons of potentially genetically divergent strains are not conclusive because they can be strongly influenced by factors not related to resistance. For example, fitness costs were found to be associated with indoxacarb resistance in a Chinese strain of *H. armigera* (Cui et al. 2018). This laboratory selected strain had lower growth rate, lower fecundity and reduced relative fitness (0.67) compared with an unselected strain. However, use of non-isogenic strains was a significant limitation in the Chinese study and may have resulted in a misinterpretation of the extent of a fitness differential observed between the two strains. On the other hand, high level of isogenicity achieved between resistant and susceptible strains in the present study significantly reduces the risk of false or inconclusive results.

The recent isolation of a strain (UN1U3-10) with a different mechanism of indoxacarb resistance suggests a diverse molecular basis of indoxacarb resistance in *H. armigera*. Characterisation of this strain is in the preliminary stages and is described in Milestone 2.2.

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Milestone 2.2 Characterise the genetic basis of resistance for novel mechanisms isolated from survivors of F_2 screens using specific crosses and bioassays

Introduction and aims

Indoxacarb is an important option for selective control of *H. armigera* in a range of crops that play host to this species. The first isofemale family with resistance to indoxacarb was collected from a maize field in the Gwydir valley in November 2013 (DAN1204 project). This strain was subsequently selected at the discriminating concentration of indoxacarb and serially outcrossed to the parental laboratory strain to create near isogenic lines for quantitative genetic analysis, as described above. This strain, designated GY7-39, was then characterized by comparison with a near-isogenic indoxacarb-susceptible laboratory strain to determine inheritance and synergism of indoxacarb resistance. This section of the report summarises findings from the analysis.

The aim of the present study was to characterise indoxacarb resistance in the GY3-39 strain of *H. armigera* isolated by F_2 screening. This involved: 1) investigating the inheritance of indoxacarb resistance by quantitative genetic analysis, where specific crosses and bioassays were used to determine sex-linkage, dominance, and the number of loci involved in resistance and 2) investigating the synergistic effects of PBO on indoxacarb resistance in *H. armigera*. Results provide an evidence-based assessment of the resistance risk posed by the presence of indoxacarb resistance alleles in the *H. armigera* population which can be utilized to improve the effectiveness of resistance management strategies.

A second aim was to initiate characterisation of the newly isolated form of indoxacarb resistance in a strain collected from chickpeas on the Liverpool Plains of NSW in 2017. This strain was designated as the UN1U3-10 strain. Preliminary results of characterisation by quantitative genetic analysis are presented below, with further work required to determine the causal mechanism of resistance.

Materials and methods

Insect strains. The strains used were the near-isogenic strains described in *Milestone 2.1* and maintained in the laboratory according rearing methods described in *Milestone 1.4*.

Bioassays. The responses of susceptible and resistant strains to indoxacarb as performed by diet incorporation bioassay using formulated indoxacarb insecticide developed by Bird (2015) and described in *Milestone 2.1.*

Synergism bioassays were based on the methods of Forrester et al. (1993) and performed by firstly exposing larvae to a range of concentrations of indoxacarb using the diet incorporation bioassay method, described above. After five days of exposure, larvae within a weight range of 30-40mg were selected for testing. Piperonyl butoxide (PBO) was dissolved in analytical grade acetone at a concentration known to cause no mortality in *H. armigera* (50µg/µl) (Forrester et al. 1993). Larvae were then treated with 1µl of the PBO/acetone solution by topical application to the dorsal thorax using a 50 µl micro-syringe in a repeating dispenser (Hamilton Company, Reno, NV); PBO (50 µg/µl) alone was used as the control.

In all cases, bioassays were performed on three cohorts of insects with individual treatments (insecticide concentrations) of each cohort consisting of a minimum of 20 individuals. Bioassays were maintained under the same conditions described for larval rearing. Diet incorporation bioassays were assessed at seven days and topical bioassays were assessed at three days using the mortality criteria, described above.

Genetics of resistance. Each of the various crosses established to determine the genetic basis of resistance comprised a minimum of 30 female and 30 male moths in each of three cohorts. The response of F_1 progeny from reciprocal mass crosses between the susceptible laboratory strain New GR, and the indoxacarb resistant strain GY7-39 were used to examine degree of dominance of

resistance (*see Text Box 2.1.2*). Data from these tests of dominance were also used to examine whether resistance was autosomal or sex-linked and controlled by a single or multiple factors (*see Text Box 2.1.2*).

Text Box 2.1.1 Method for determining degree of dominance.

Degree of dominance for the 50% lethal concentration (LC_{50}) (D_{LC}) was calculated as follows:

$$D_{LC} = (\log LC_{RS} - \log LC_{SS}) / (\log LC_{RR} - \log LC_{SS})$$

where LC_{RR}, LC_{RS}, and LC_{SS} are lethal concentrations for the resistant, F_1 hybrid and susceptible strains, respectively. The resulting parameters range from 0 (completely recessive resistance) to 1 (completely dominant resistance) (Bourguet et al. 2000).

Effective dominance (D_{ML}) was calculated from mortality values at a range of single concentrations as follows:

$$D_{ML} = (ML_{RS} - ML_{SS}) / (ML_{RR} - ML_{SS})$$

where ML_{RR}, ML_{RS}, and ML_{SS} are the mortality levels (ML) at a particular concentration for the resistant, F_1 hybrid, and susceptible strains, respectively. The D_{ML} values range from 0 (completely recessive) to 1 (completely dominant) (Bourguet et al. 2000).

Text Box 2.1.2 Method for determining number of genes involved in resistance.

The number of loci influencing resistance was estimated by a direct test for a monogenic inheritance based on the observed and expected mortalities from a reciprocal backcross to each single dose (Tabashnik 1991). This test was based on the goodness-of-fit χ^2 between the F_1 backcross and the New GR susceptible strain, with expected values calculated as described by Sokal and Rohlf (1981) as follows

$$:\chi^2 = (F_1 - pn)^2 / pqn$$

where F_1 is the observed number of dead larvae in the reciprocal backcross at dose x, p is the expected proportion of larvae dead, n is the number of backcross progeny exposed to dose x, and $q = 1 - p$. The null hypothesis was rejected if the test resulted in a significant deviation ($P < 0.05$), comparable with a χ^2 distribution with one degree of freedom.

If resistance is monogenic and strains are homozygous, then New GR are SS, GY7-39 are RR, F_1 progeny are RS, the backcross of F_1 × New GR produces progeny that are 50% SS and 50% RS, and the backcross of F_1 × GY7-39 produces progeny that are 50% RR and 50% RS.

The monogenic model was tested by the method of Tabashnik (1991) which involved calculating the average of mortality probabilities for RS and SS individuals (Y_x) to each single dose as follows:

$$Y_x = 0.5 \times (W_{F1} + W_{SS})$$

where W_{F1} is the observed mortality for F_1 pooled progeny of reciprocal mass crosses of the resistant GY7-39 strain and the susceptible New GR strain, and W_{SS} is the mortality of the susceptible strain New GR, at each concentration.

Data analysis. Where necessary, bioassay data were corrected for control mortality (Abbott 1925). Estimates of LC_{50} values and 95% fiducial limits (FL) were obtained by probit analysis by using POLO-PC software (LeOra Software, Berkeley, CA). Resistance ratios (RR) were calculated by dividing the LC_{50} value of the resistant strain by the LC_{50} value (average of four non-synchronous cohorts) of the laboratory strain. Significant differences ($P = 0.05$) between LC_{50} values were determined by the lethal dose ratio test according to Wheeler et al. (2006) where, if the 95% confidence interval (CI) includes 1 then the LC_{50}s are not significantly different. Synergistic ratio (SR) was calculated by dividing the LC_{50} of the strain tested with indoxacarb alone by LC_{50} of the strain tested with indoxacarb + PBO.

Results

Genetics of resistance to indoxacarb in the GY7-39 strain

Sex linkage and dominance. Bioassays of indoxacarb against the F_1 progeny from reciprocal mass crosses between GY7-39 and New GR were conducted on the third backcrossing event during the creation of near isogenic strains. At the time of the crossing experiment the resistance ratio for the GY7-39 strain was 198-fold (Table 2.2.1). The LC_{50} values of the F_1 progeny of the reciprocal crosses between the resistant and susceptible strains were similar based on a ratio test ($P = 0.05$) suggesting inheritance of resistance in the GY7-39 strain was autosomal, with no evidence of sex linkage or

maternal effects. The resistance ratio decreased to 65-fold (pooled from the reciprocal crosses) in the F_1 progeny from the third backcross event (Table 2.2.1). The degree of dominance (D_{LC}) for the pooled reciprocal crosses was 0.80 indicating indoxacarb resistance in the GY7-39 strain was inherited as an incomplete dominant trait.

Table 2.2.1 Responses to indoxacarb of the resistant strain (GY7-39), laboratory strain (New GR), and progeny of crosses to indoxacarb following the third backcross.

Strain/cross	LC_{50} [µgAI/ml diet] (95% FL)	Slope ± SE	RR	Lethal dose ratio (95% CI)	D_{LC}
New GR (susceptible)	0.148 (0.137, 0.158)	3.4 ± 0.2			
GY7-39 (resistant)	29.18 (24.695, 34.792)	2.6 ± 0.2	198		
F_1: GY7-39♀ x New GR♂	10.69 (8.372, 13.977)	2.1 ± 0.2	72		0.81
F_1: GY7-39♂ x New GR♀	8.77 (7.590, 10.128)	2.8 ± 0.2	59	1.219 (0.972, 1.530)	0.77
F_1: Pooled	9.55 (8.198, 11.174)	2.4 ± 0.1	65		0.80
Backcross F_1 x New GR	2.20 (1.855, 2.612)	1.2 ± 0.1			

Estimation of effective dominance (D_{ML}) for six concentrations tested showed that dominance was dose dependent and dominance increased with concentration (Table 2.2.2). Resistance was functionally recessive at the highest concentration of 48µg/ml (D_{ML} = 0.07), partially dominant at intermediate concentrations, and functionally dominant at the lowest concentration of 0.75µg/ml (D_{ML} = 1).

Table 2.2.2 Dominance of indoxacarb resistance in *H. armigera* as a function of indoxacarb concentration.

Concentration [µgAI/ml diet]	Survival (%)			Effective Dominance (D_{ML})
	New GR	F_1	GY7-39	
0.75	2	94	100	1.00
1.5	0	87	100	0.96
3	0	74	100	0.87
6	0	52	95	0.78
12	0	12	78	0.67
24	0	2	73	0.16
48	0	1	25	0.07

Number of genes involved. Results from a direct test of monogenic inheritance suggest that one (or a few closely linked) loci conferred resistance to indoxacarb in the GY7-39 strain. The backcross of F_1 × New GR was preferred to the backcross of F_1 × GY7-39 because the F_1 differed more from the susceptible strain than from the resistant strain, thus increasing the power of the backcross for distinguishing among modes of inheritance. The test showed no significant deviation between observed and expected mortality at six of the nine concentrations tested (Table 2.2.3). A significant deviation may be either an artefact of the bioassay or because the concentrations were too low to discriminate between the susceptible strain and the F_1 backcross progeny or may indicate the presence of low-level polygenic resistance conferred by generalist mechanisms.

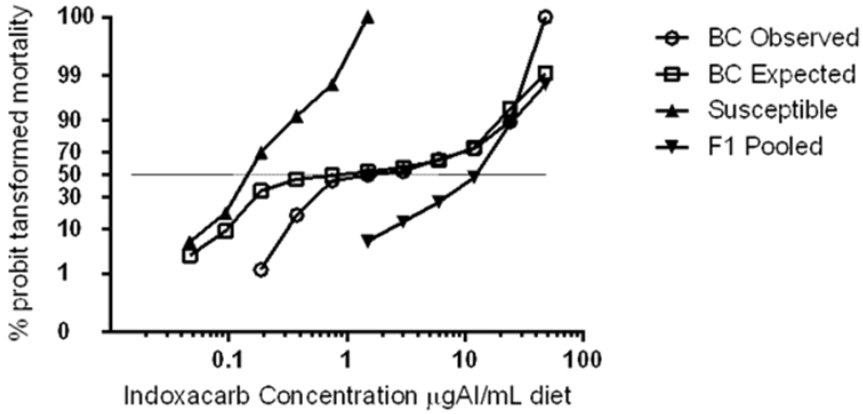
Table 2.2.3 A direct test of monogenic inheritance for resistance to indoxacarb by comparing expected and observed mortality of the backcross (F_1 × New GR) of *H. armigera.*

Concentration [µgAI/ml diet]	No. of larvae tested	Observed mortality (proportion)	Expected mortality (Y_x)	χ^2 (df = 1)	*P*
0.1875	240	3 (0.01)	0.35	120.68	< 0.0001
0.375	240	41 (0.17)	0.46	79.67	< 0.0001
0.75	240	106 (0.44)	0.49	2.39	0.12
1.5	238	118 (0.50)	0.53	1.06	0.30

3	237	126 (0.53)	0.57	1.18	0.28
6	240	154 (0.64)	0.63	0.16	0.69
12	240	175 (0.73)	0.74	0.09	0.77
24	238	213 (0.89)	0.94	8.56	0.03
48	240	240 (1.00)	0.99	2.02	0.16

The slope of the concentration-mortality response for backcross progeny (F_1 backcross × New GR) was 1.2 ± 0.1, which was 3-fold lower than the slope of the susceptible strain (3.4 ± 0.2), and 2-fold lower than the slope for the resistant strain and F_1 (2.6 ± 0.2 and 2.4 ± 0.1, respectively) (Table 2.2.1). This pattern of slopes combined with a strong inflection point at the median lethal concentration (Figure 2.1.1) suggests increased genetic variation in the backcross compared with the parental and F_1 strains and provides additional evidence in support of a single gene model of indoxacarb resistance in *H. armigera*.

Figure 2.1.1. Response of indoxacarb of *H. armigera* from susceptible and resistant strains, F_1 progeny (pooled data from reciprocal crosses) and backcross progeny (F_1 × susceptible). The backcross (BC) observed curve was plotted using the observed mortality at each concentration. The BC expected curve was plotted with expected mortality calculated from a single locus model (see Text Box 2.1.1, above).



Synergism of indoxacarb resistance in the GY7-39 strain. At the time of testing for synergism the resistance ratio for indoxacarb was 140-fold in the GY7-39 strain compared with the New GR strain (Table 2.2.4). Resistance was reduced from 20.8-fold to 0.92-fold by the addition of PBO to actively feeding *H. armigera* larvae resulting in a synergistic ratio of 23-fold. A small but significant synergistic effect was observed in the New GR strain (Table 2.2.4). Results suggest PBO-suppressible metabolic processes are important mechanisms conferring resistance to indoxacarb in *H. armigera*.

Table 2.2.4 Toxicity of indoxacarb with and without PBO (50µg/µl) on laboratory (New GR) and resistant (GY7-39) strains of *H. armigera.*

Insecticide	Strain	LC_{50} [µg/ml diet] (95% FL)	Slope ± SE	RR	SR (95% CI)
Indoxacarb	New GR	0.148 (0.137, 0.158)	3.4 ± 0.2		
Indoxacarb + PBO	New GR	0.112 (0.086, 0.154)	8.3 ± 1.0		1.3 (1.201, 1.455)
Indoxacarb	GY7-39	20.791 (16.959, 25.382)	2.5 ± 0.2	140	
Indoxacarb + PBO	GY7-39	0.918 (0.748, 1.132)	1.8 ± 0.1	6	22.6 (18.020, 28.466)

Genetics of resistance to indoxacarb in the UN1U3-10 strain: preliminary results

Sex linkage and dominance. Bioassays of indoxacarb against the F_1 progeny from reciprocal mass crosses between UN1U3-10 and New GR were conducted on the first backcrossing event during the creation of near isogenic strains. At the time of the crossing experiment the resistance ratio for the UN1U3-10 strain was 110.6-fold (Table 2.2.5). The LC_{50} values of the F_1 progeny of the reciprocal crosses between the resistant and susceptible strains were similar based on a lethal dose ratio test (P = 0.05) suggesting inheritance of resistance in the UN1U3-10 strain was autosomal, with no evidence of sex linkage or maternal effects. These results support observations of survival in the initial F_2

screen where the proportion of larvae that survived the diagnostic concentration of indoxacarb was statistically greater than survival expected if resistance is recessive (i.e. 6.25%) (proportion observed = 27.8, $\chi^2 = 79.1$, $P < 0.0001$).

The resistance ratio decreased to 55.3-fold (pooled from the reciprocal crosses) in the F_1 progeny from the first backcross event (Table 2.2.5). The degree of dominance (D_{LC}) for the pooled reciprocal crosses was 0.92 indicating indoxacarb resistance in the UN1U3-10 strain was inherited as an incomplete dominant trait and has higher genetic dominance than the GY7-39 strain (Table 2.2.1). There was also significantly higher survival and faster larval development in the UN1U3-10 strain at the discriminating concentration of indoxacarb compared with the previously characterised indoxacarb resistant strain, GY7-39. This combined with high genetic dominance suggests high heritability is associated with the UN1U3-10 form of indoxacarb resistance.

Table 2.2.5 Responses to indoxacarb of the resistant strain (GY7-39), laboratory strain (New GR), and progeny of crosses to indoxacarb following the third backcross.

Strain/cross	LC_{50} [µgAI/ml diet] (95% FL)	Slope ± SE	RR	Lethal dose ratio (95% CI)	D_{LC}
New GR (susceptible)	0.148 (0.137, 0.158)	3.4 ± 0.2			
UN1U3-10 (resistant)	110.63 (94.821, 130.229)	2.7 ± 0.3	753		
F_1: UN1U3-10 ♀ x New GR♂	53.26 (47.491, 59.840)	4.4 ± 0.4	362		0.92
F_1: UN1U3-10 ♂ x New GR♀	57.48 (48.826, 67.996)	3.9 ± 0.4	391	0.927 (0.782, 1.098)	0.93
F_1: Pooled	55.34 (50.215, 61.068)	4.1 ± 0.3	376		0.92

The analysis also estimated effective dominance (D_{ML}) from a bioassay of ten indoxacarb concentrations. The results demonstrate that dominance is dose dependent and increased as concentration decreased. Resistance was functionally recessive at the two highest concentrations of 192 and 384 µg/ml ($D_{ML} = 0$), partially dominant at intermediate concentrations, and functionally dominant at discriminating concentration of 12µg/ml ($D_{ML} = 1$) (Table 2.2.6), indicating a high degree of heritability is associated with the UN1U3-10 form of resistance.

Table 2.2.6 Dominance of indoxacarb resistance in *H. armigera* as a function of indoxacarb concentration.

Concentration	Survival (%)			Effective Dominance
[µgAI/ml diet]	New GR	F_1	UN1U3-10	(D_{ML})
0.75	2	100	100	1.00
1.5	0	100	100	1.00
3	0	99	100	1.00
6	0	98	100	1.00
12	0	99	98	1.00
24	0	91	97	0.94
48	0	67	83	0.80
96	0	16	53	0.30
192	0	0	28	0.00
384	0	0	8	0.00

Molecular analysis confirmed that the GY7-39 and UN1UN3-10 strains are not allelic and resistance in each strain is likely conferred by multiple distinct metabolic detoxification mechanisms. This is also consistent with a preliminary finding that indoxacarb resistance in UN1U3-10 is partially suppressible by the metabolic inhibitor piperonyl butoxide (data not shown). Further work is needed to confirm the magnitude of metabolic inhibition in this strain.

Discussion and conclusions

Inheritance of resistance is a critical factor for determining rate of resistance evolution (Bourguet 2000). Reciprocal crosses between the GY7-39 strain and the New GR strain indicated resistance to

indoxacarb in GY7-39 was inherited autosomally and as an incompletely dominant trait. Similarly, deltamethrin resistance in *H. armigera* was also incompletely dominant (Daly and Fisk 1992) and resulted in rapid development of pyrethroid resistance in Australia (Forrester et al. 1993).

In contrast to pyrethroids, indoxacarb resistance is relatively low in the Australian *H. armigera* population (Bird et al. 2017). Alternatively, the presence of fitness costs associated with resistance could delay the spread of resistance alleles under certain conditions. If resistance alleles are rare, as is the case with indoxacarb resistance in *H. armigera* in Australia, development of resistance depends not only on level of exposure to insecticide, but also on the relative fitness of the heterozygotes compared with the susceptible genotype (Roush and McKenzie 1987). At low frequencies, resistance alleles are present primarily in heterozygotes and dominant fitness costs would favour a delay in resistance development (Carrière and Tabashnik 2001). Investigations to determine if fitness costs are involved in delaying indoxacarb resistance in *H. armigera* are discussed in *Supplementary Milestone 2.5*, below.

It is generally assumed that monogenic resistance is more likely to spread than polygenic resistance (Roush and Hoy 1981). Results from a monogenic test of inheritance suggests that resistance to indoxacarb was controlled by a single major (or a few tightly linked) loci.

High levels of genetic dominance can contribute to the spread of resistance under selection pressure, with the expression of dominance depending upon the dose of insecticide applied (Bourguet 2000). Dominant resistance is considered to favour an increase in resistance heritability because, if the dose applied in the field is not high enough to kill heterozygous individuals, incompletely dominant resistance becomes a functionally dominant trait. On the other hand when resistance is dominant, theory predicts that susceptible genes can persist for much longer in the population as heterozygotes and therefore slow the time to fixation (Georghiou and Taylor 1992). The degree of dominance of indoxacarb resistance was concentration-dependent (resistance was recessive at the highest concentration and dominant at the lowest concentration tested) suggesting the risk for indoxacarb resistance is high due to enhanced survival of heterozygotes compared with susceptible individuals.

The most common mechanisms associated with resistance to insecticides in *H. armigera* are mediated by oxidation attributed to cytochrome P450 monooxygenases, and hydrolysis and/or sequestration by carboxylesterases (McCaffrey 1998). The involvement of metabolic mechanisms in indoxacarb resistance has been reported in several insect species based on synergistic effects of metabolic inhibitors on indoxacarb toxicity (Sayyed and Wright 2006, Sayyed et al. 2008, Nehare et al. 2010, Gao et al. 2014). Observed suppression of resistance in the GY7-39 strain by the metabolic inhibitor PBO indicates the likely involvement of metabolic detoxification enzymes in indoxacarb resistance in *H. armigera*.

The isolation of a second unique indoxacarb resistant strain suggests indoxacarb resistance is genetically diverse in the Australian *H. armigera* population. This is not surprising given the strong track record of *H. armigera* to develop metabolic pathways to detoxify a broad range of insecticidal chemistries. Results from recent molecular analysis of the UN1U3-10 strain confirmed that resistance is not associated with the *kdr* gene, a common mutation that confers target site resistance to insecticides in *H. armigera*. The *kdr* gene was also not present in the GY7-39 strain. This supports findings from previous inhibition studies and molecular characterisation suggesting the putative mechanism of indoxacarb resistance in Australian populations of *H. armigera* is mediated by multiple metabolic detoxification systems. Further genetic and molecular characterisation of this new form of indoxacarb resistance is planned.

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Milestone 2.3 Investigate patterns of cross-resistance in strains with novel resistance to selective chemistries

Introduction and aims

An important component of assessing resistance risk associated with commercial use of insecticides is the evaluation of cross-resistance profiles of resistant strains. The aim of this study was to determine the efficacy of insecticides with registration on *H. armigera* against the indoxacarb resistant strain GY7-39.

Materials and methods

Insect strains. The strains used was the near-isogenic third backcross of GY7-39 strain described in *Milestone 2.1* and the parental susceptible strain New GR. Strains were maintained in the laboratory according general rearing methods described in *Milestone 1.4*.

Insecticides. Commercial insecticide formulations were used in diet incorporation bioassays: chlorantraniliprole (Altacor® [35% active ingredient]), DuPont Australia Ltd., Macquarie Park, emamectin benzoate (Affirm® [1.9% active ingredient]), Syngenta Crop Protection, Macquarie Park. Fenvalerate (95.3%) provided by Sumitomo Chemical, Sydney. The Cry1Ac toxin was produced from the HD73 strain by GeneSearch (Arundel, Qld). A recombinant clone of the *cry2Ab* gene in *B. thuringiensis* was used for the production of Cry2Ab toxin. The Cry2Ab *B. thuringiensis* strain was grown in nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, UK), with 10µg/ml tetracycline, for 3–4 days in a 28 °C orbital shaker. When sporulation and crystal formation was completed, the suspension was centrifuged and the pellet washed three times. The pellet was resuspended in distilled water and sonicated. A sample of the final suspension was transferred into SDS sample buffer and electrophoresed on a 10% polyacrylamide gel with four known concentrations of bovine serum albumin (BSA). The concentration of Cry2Ab was estimated by scanning the gel and analysing the density of the Cry2Ab band relative to the BSA standard using Scion Image 1.62 software (Scion Corporation, Frederick, MD).

Bioassays. The responses of indoxacarb susceptible and resistant strains to chlorantraniliprole and emamectin benzoate were measured by bioassay on artificial diet into which formulated insecticide was incorporated according to the method described in *Milestone 2.1*. Late second or early third instars were introduced to trays (one larva per well); untreated diet was used as the control.

Cross-resistance to Cry1Ac and Cry2Ab was evaluated using a surface treatment bioassay. Artificial diet was dispensed into 24-well plates (Falcon, Cowley, UK). A stock suspension of the Cry toxins was diluted with distilled water to produce eight two-fold dilutions; concentrations were calculated as µg of toxin per cm^2 of diet surface. A pipette was used to transfer 50µl aliquots of serially diluted Cry toxin to the surface of the diet. The toxin solution was distributed over the diet surface and the residual surface liquid was allowed to evaporate in a cool air flow. One unfed neonate larva was introduced to each well; distilled water was used as the control.

Cross-resistance to fenvalerate was tested by topical bioassay. Insecticide solutions were prepared from technical material dissolved in analytical grade acetone to produce six two-fold serial dilutions. Larvae between 30-40 mg were treated with 1µl of acetone/insecticide solution by topical application to the dorsal thorax using a 50 µl micro-syringe in a repeating dispenser (Hamilton Company, Reno, NV); acetone alone was used as the control. In all cases, bioassays were performed on three cohorts of insects with individual treatments (insecticide concentrations) of each cohort consisting of a minimum of 20 individuals. Bioassays were maintained under the same conditions described for larval rearing. Diet incorporation bioassays were assessed at seven days and topical bioassays were assessed at three days using the mortality criteria, described above.

Data analysis. Where necessary, bioassay data were corrected for control mortality (Abbott 1925). Estimates of LC_{50} values and their 95% fiducial limits (FL) were obtained by probit analysis by using POLO-PC software (LeOra Software, Berkeley, CA). Resistance ratios (RR) were calculated by dividing

the LC_{50} value of the resistant strain by the LC_{50} value (average of four non-synchronous cohorts) of the laboratory strain.

Results

The response of the GY7-39 strain to chlorantraniliprole was similar to that of the New GR strain based on the ratio test ($P = 0.05$) (Table 2.3.1), suggesting a lack of cross resistance. Emamectin benzoate, fenvalerate, Cry1Ac and Cry2Ab had greater toxicity to the GY7-39 strain than to the New GR strain based on the ratio test ($P = 0.05$) (Table 2.3.1) suggesting indoxacarb resistant *H. armigera* may be more sensitive to these insecticides.

Table 2.3.1 Resistance levels to five insecticides in the resistant (GY7-39) and laboratory strain (New GR).

Insecticide	Population	LC_{50} [µg/ml diet] (95% FL)	Slope ± SE	RR (95% CI)
Indoxacarb	New GR	0.147 (0.137, 0.158)	3.4 ± 0.19	
	GY7-39($BC3_3$) †	24.734 (20.443, 30.316)	2.4 ± 0.21	168
Chlorantraniliprole	New GR	0.020 (0.0173, 0.0234)	4.4 ± 0.48	
	GY7-39	0.024 (0.0200, 0.0277)	2.6 ± 0.26	1.2 (0.958, 1.428)
Emamectin benzoate	New GR	0.009 (0.0777, 0.1061)	5.2 ± 0.56	
	GY7-39	0.006 (0.0472, 0.0646)	4.8 ± 0.54	0.6 (0.518, 0.706)
Fenvalerate	New GR	55.828 (40.006, 79.495)	3.3 ± 0.32	
	GY7-39	44.587 (31.110, 63.774)	3.1 ± 0.29	0.8 (0.656, 0.972)
Cry1Ac	New GR	45.624 (36.715, 56.729)	1.8 ± 0.13	
	GY7-39	17.636 (15.804, 19.675)	2.8 ± 0.17	0.4 (0.318, 0.470)
Cry2Ab	New GR	52.312 (44.442, 61.418)	2.0 ± 0.15	
	GY7-39	41.751 (33.322, 51.621)	1.6 ± 0.09	0.8 (0.643, 0.991)

† GY7-39($BC3_3$) strain was backcrossed to New GR three times and then selected for three generations.

Discussion and conclusions

Knowledge of insecticide cross-resistance is important for developing strategies to delay the evolution of field resistance. In the present study indoxacarb resistance in the GY7-39 strain did not extend cross-resistance to other insecticides specific for *H. armigera*. There was enhanced sensitivity of the GY7-39 strain to emamectin benzoate and the two Cry toxins from *Bacillus thuringiensis* currently deployed in commercial transgenic cotton varieties. Each of these insecticides has a unique mode of action and distinct from that of indoxacarb (Kass et al. 1980, Cordova et al. 2006, Caccia 2010).

The GY7-39 strain was also more sensitive to fenvalerate. The target site for both indoxacarb and pyrethroid is voltage-dependent sodium channels. Although increased target site insensitivity (knockdown resistance or *kdr*) has contributed to pyrethroid resistance at various times and geographical locations in Australia (Daly 1993, Gunning 1996) pyrazolines block sodium channels in a manner different from pyrethroids (Tsurubuchi 2001) and would therefore not be expected to compromise resistance risk for indoxacarb. Notwithstanding the rapid selection of indoxacarb resistance in the GY7-39 strain (see *Milestone 2.2*) a lack of cross-resistance to other Helicoverpa insecticides including Bt toxins (Bird and Downes 2014, *Milestone 3.1*), indicates that resistance could be managed effectively by the use of rotational strategies which incorporate transgenic technologies.

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Milestone 2.4 Investigate molecular basis of resistance

Introduction and aims

Understanding the molecular basis of resistance is essential for DNA-based monitoring of resistance in field populations. Recent availability of the *H. armigera* genome enables application of high throughput genetic mapping approaches for identifying resistance mechanisms in this species. The aim of this work was to utilize such mapping techniques to identify the region of the *H. armigera* genome containing the major locus conferring indoxacarb resistance in the GY7-39 strain by advanced crossing (conducted at ACRI) and genotype-by-sequencing (GBS) analysis (conducted at EMAI).

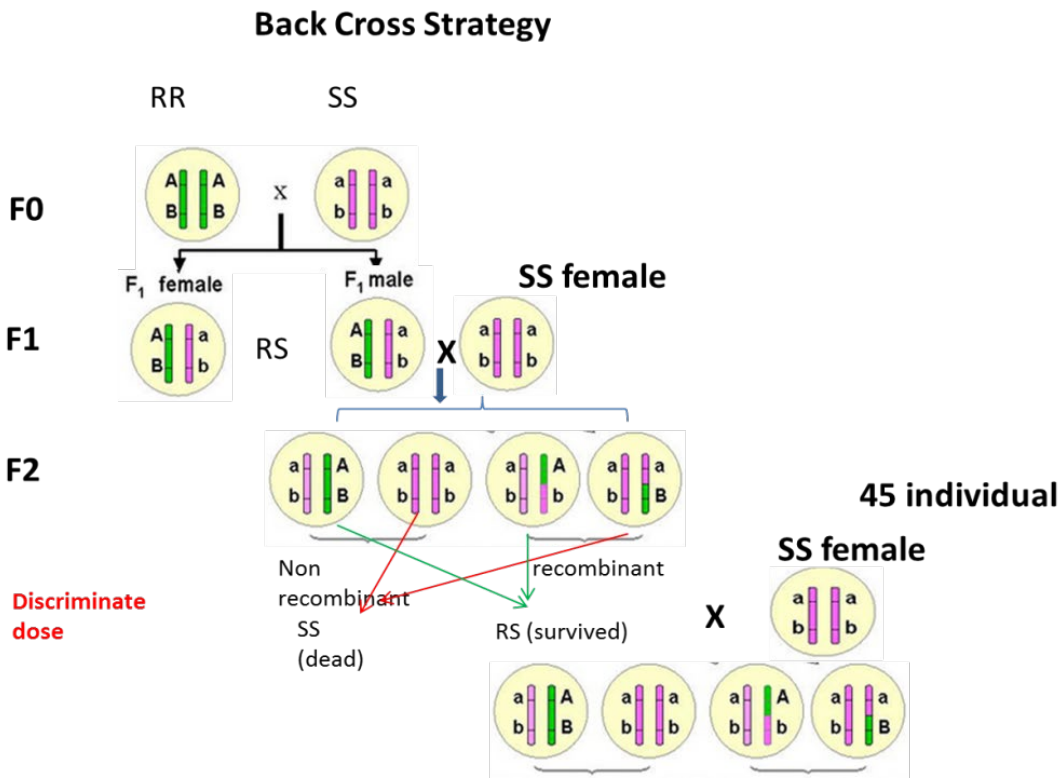
Materials and methods

Insect strains and specific crosses. *S*trains used in the backcrossing program were the near-isogenic third backcross of GY7-39 described in *Milestone 2.1* and the parental susceptible strain New GR. Strains were maintained in the laboratory according general rearing methods described in *Milestone 1.4*.

The process for identifying the locus associated with indoxacarb resistance involved two sequential backcrossing processes. Firstly, linkage mapping was utilised to locate the resistance gene(s) to a particular linkage group and, secondly a sequencing approach was used to very precisely map the resistance gene in the *H. armigera* genome.

Linkage mapping. The initial F_0 cross comprised a resistant *AA* male and a susceptible *aa* female (Figure 2.4.1). The resistant allele *A* is close to its marker *B* and the susceptible allele *a* is close to its marker *b*. Therefore, the resistant strain is *AABB* and the susceptible is *aabb*. The F_1 hybrid progeny from this initial cross were heterozygous carriers of a single resistance allele (Aa).

Figure 2.4.1 Specific crosses of indoxacarb susceptible and resistant moths to produce recombinants for linkage mapping.



The next step was to backcross an F_1 male (*Aa*) to a female moth from the susceptible strain (*aa*) to produce *Aa* and *aa* genotypes which were differentiated by discriminating dose bioassay (Bird 2016). As each allele had a linked marker (*B*), if there was no recombination the heterozygote will stay *AaBb*. However, if recombination occurred the resulting genotype would be *Aabb*.

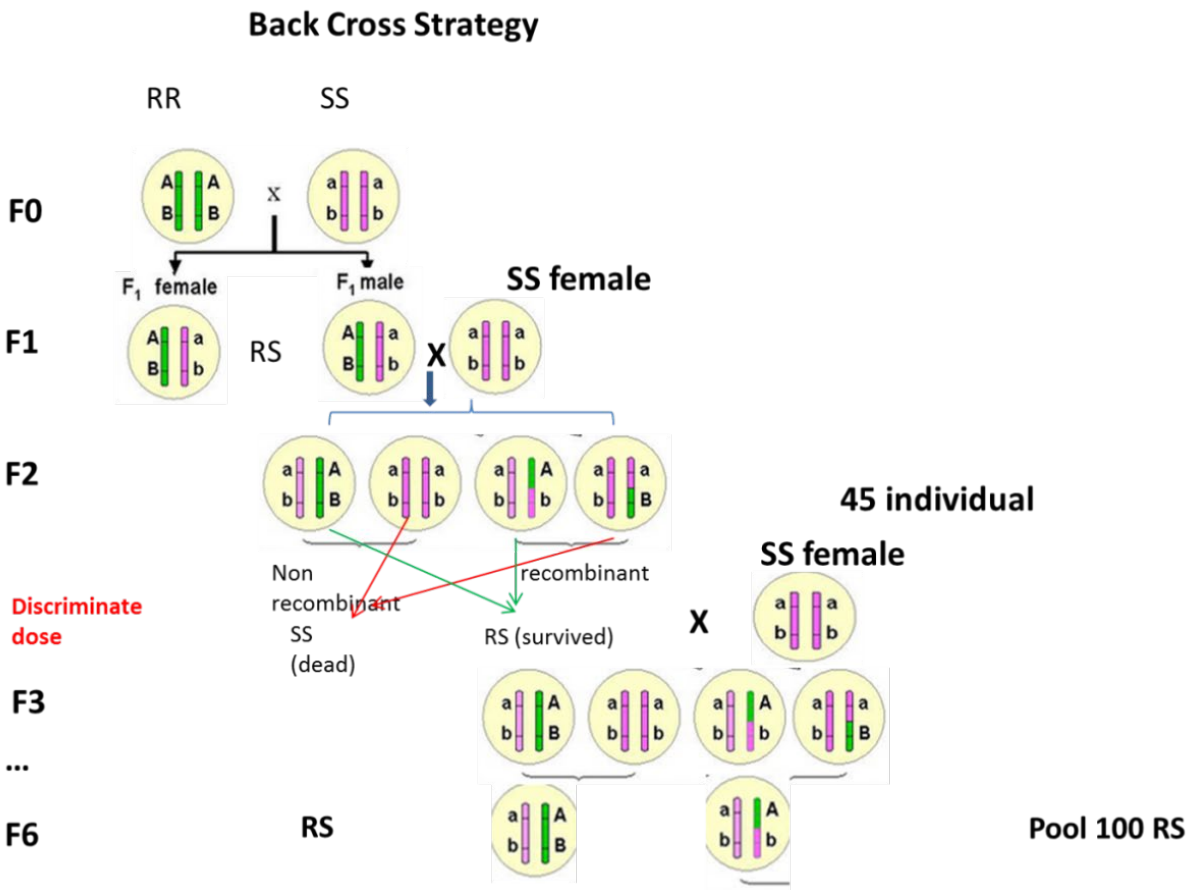
Likewise, no recombination would produce *aabb* with recombination producing *aaBb*. Theoretically, if marker *B* is linked to the resistance gene *A* the ratio of recombinants divided by total progeny will be <0.5. If the marker is not linked to the resistance gene, the ratio will be exactly 0.5. To extrapolate this theory, if we have many markers we can find groups where markers co-segregate with the resistance gene to form a linkage group. Markers on the same chromosome will form a linked group and their order on the chromosome can be estimated based on the rate of recombination between their markers.

Although this approach was not expected to pinpoint the resistance gene(s) it was expected to provide groups of markers linked to the resistance gene which could be mapped to the *H. armigera* genome sequence to potentially to locate the resistance gene(s).

Sequencing. The aim of the sequencing approach was to pinpoint the location of the resistance gene by precisely mapping it in the *H. armigera* genome. To achieve this, the program of serial backcrossing continued for a further three generations and involved crossing resistant *Aa* male progeny (selected at a discriminating dose of indoxacarb) with susceptible *aa* females from the parent culture (Figure 2.4.2). Three independent lineages were established with each producing more than 100 F_6 resistant progeny. The F_6 resistant larvae were collected and stored in RNAlater® at -20 °C prior to being pooled for DNA extraction and sequencing.

DNA extraction and GBS. DNA was extracted with a DNeasy® Blood & Tissue kit (Qiagen) from adult or larval head tissue (Woolley et al. 2017). A total of 95 DNA samples plus one negative (no DNA) were placed in a 96-well plate. Samples included eight individuals from the susceptible New GR strain

Figure 2.4.2 Serial backcross of indoxacarb susceptible and resistant moths to produce F_6 recombinants for sequencing.



(including two susceptible F_0 females), 14 resistant GY7-39 insects (including two resistant F_0 males), and 73 resistant F_6 insects from two families; one family with 35 and another family with 38 F_6. All DNA samples were quantified by spectrophotometry and further analysed by gel electrophoresis.

GBS library construction and sequencing was carried out at the Cornell genomic diversity facility (http://www.biotech.cornell.edu). The Cornell output produced millions of short sequence reads called tags that were approx. 100bp long and mapped to regions within the *H. armigera* genome. Insects with identical sequences at a given position had the same unique tag sequence. For that reason the analysis focused on the sequence tags which were found in resistant *H. armigera* only.

Processing sequence reads and tag mapping. Unique sequence tags were aligned to the *H. armigera* genome which incorporated a linkage mapped chromosome length genome provided pre-publication (T. Walsh personal communication). Individual tag read counts were obtained and the filtered tags were aligned to a *H. armigera* reference genome. A subsequent genome-wide association (GWA) analysis between the tag for indoxacarb resistance in both resistant and susceptible individuals was performed.

Fine mapping of indoxacarb resistance. Sequence tags were grouped into haplotypes based on the tag mapping position. Here we assumed the haplotype linked to indoxacarb resistance should be present only in resistant individuals and absent from susceptible individuals. Additionally, the haplotype linked to indoxacarb resistance should be present in most of the F_6 resistant individuals. For each haplotype, two resistant and two susceptible individuals were sequenced to confirm the haplotype sequence using a total of 11 primers flanking the regions of high association to indoxacarb resistance.

Genotype testing. A TaqMan SNP (single nucleotide polymorphism) assay was designed based on the aligned sequences for susceptible and resistant strains at position HaChr16:6101072. All primers and probes were synthesized by Biosearch Technologies Inc. (Novato USA).

Validation of haplotypes by Sanger sequencing. For each resistance associated haplotype, tag sequences were imported into SEQUENCHER 5.4.6 (Gene Codes, Ann Arbor, MI, USA) and aligned

with the *H. armigera* reference genome sequence. Primer pairs were designed to amplify the identified region.

Results

Indoxacarb resistance phenotype segregation in backcrosses. At the discriminating concentration of indoxacarb approximately two-thirds of F_1 progeny survived a discriminating dose (68% to 79.9%). For F_2 progeny, the survival rate declined to approx. 45% (40.5% to 47.2%) and by generation F_6, progeny survival continued to be stable at approx. 43% (Table 2.4.1). This ratio of resistance was consistent with our previous finding that the indoxacarb resistance allele in GY7-39 is partially dominant.

Sequencing and identification of informative tags. A total of 258,100,460 reads from 95 DNA samples were obtained. These mapped tags were distributed across 31 chromosomes on the *H. armigera* genome with 8,697 unique locations.

GWA analysis of indoxacarb resistance. There were 131 tags identified as having a significant association with indoxacarb resistance with 53 tags having high association ($P = 3.28 \times 10^{-11}$). These tags were present in all 14 resistant individuals and absent from the eight susceptible individuals. Forty-two out of 53 tags were located in a 3.6 MB region on chromosome 16 (Figure 2.4.1).

Fine mapping. Sixteen haplotypes were found on chromosome, 16 spanning the 3.6 MB region (5922895 - 9528852). All of these haplotypes were represented in the 14 resistant GY7-39 individuals but not in New GR individuals. In Family 1, the entire 3.6MB region was co-segregated with the resistant genotype in the F_6 progeny. Thirty-two out of 35 F_6 resistant progeny carried these haplotypes. In the region HaChr16:6294314-8558542, six haplotypes were co-segregated 100% with the resistance phenotype. However, in Family 2 only one haplotype (H3 HaChr16:6101017) was co-segregated with resistant phenotype and all 38 F_6 resistant progeny carried this haplotype.

Table 2.4.1 Number of larvae surviving a discriminating concentration of indoxacarb (12µg/ml indoxacarb in a diet-incorporation bioassay) at backcross F_1, F_2 and F_6.

Family	No. tested	No. dead	% survival
F_1 (♂ GY7-39 x ♀ New GR)			
1	657	177	72.1
2	658	206	67.3
3	199	46	76.9
F_2 (♂ F_1 x ♀ New GR)			
1	2208	1032	46.7
2	2523	1022	40.5
3	1146	541	47.2
F_6 (♂ F_5 x ♀ New GR)			
1	2032	882	43.4
2	2878	1224	42.5
3	2155	951	44.1

Figure 2.4.3 Results of Genotype-By-Sequencing (GBS) to map the indoxacarb resistance gene by GWA analysis to a narrow the region of the *H. armigera* genome (in HaChr116:5922895-8549350) containing six candidate genes (including the *CYP6AE* gene cluster which encodes for metabolic detoxification enzymes).

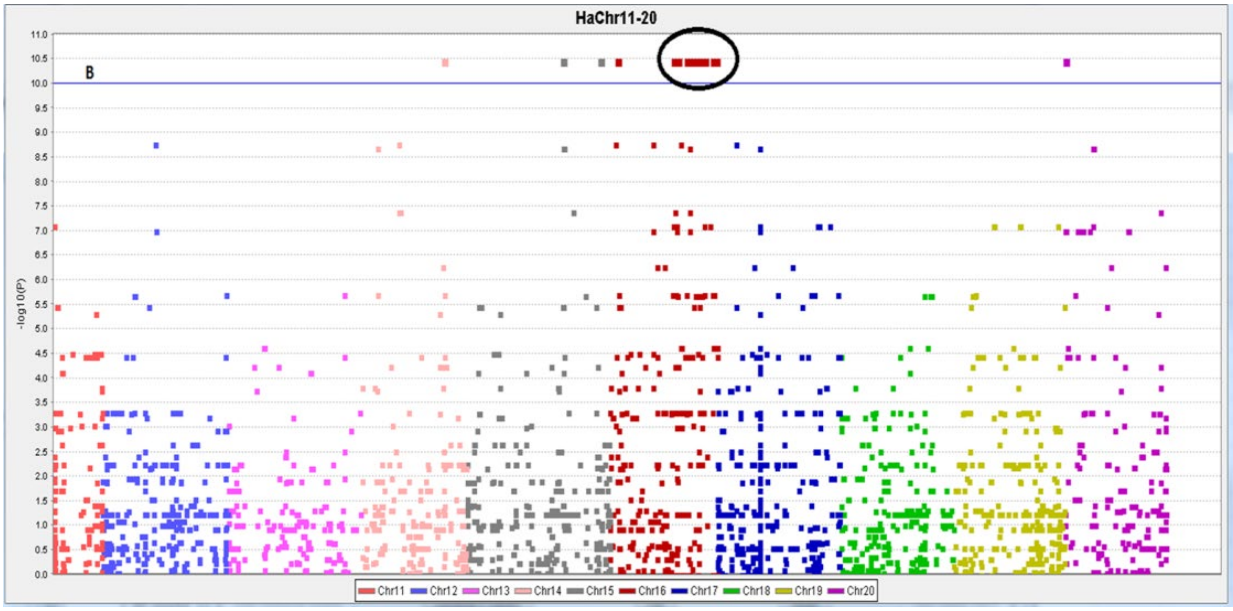


Table 2.4.2 lists the genotypes of SNP at HaChr16:6101072 from a TaqMan assay for the F_0 GY7-39 resistant strain comprising the founder F_0 resistant males from the two crosses, F_0 New GR susceptible strain and F_6 resistant progeny including the third family which was not used for the GBS analysis. All F_0 GY7-39 individuals were homozygous for the allele ATTT and the susceptibles from the New GR strain were homozygous for the allele ATTTT. The F_1 resistant male progeny in all three families were heterozygotes. F_6 resistance progeny were 50% heterozygotes in Family 1, 90% heterozygotes in Family 2 and 100% heterozygotes in Family 3.

Table 2.4.2 Genotype HaChr16 :6101072 in *Helicoverpa armigera* resistant and susceptible individuals.

Strain	Homozygote (ATTT)*	Heterozygote (ATTT/ATTTT)*	Homozygote (ATTT/ATTTT)*
GY7-39 indoxacarb resistant	6	0	0
New GR indoxacarb susceptible	0	0	12
F_1 resistant male	0	5	0
F_2 resistant progeny	0	5	1
F_6 Family 1 resistant progeny	0	5	5
F_6 Family 2 resistant progeny	0	9	1
F_6 Family 3 resistant progeny	0	10	0

*ATTT allele from GY7-39 and ATTTT allele from New GR.

Discussion and conclusions

This study provides a practical example of GBS for mapping an insecticide resistance gene(s) where little linkage information was available. To identify the causal gene for indoxacarb resistance in GY7-39, a program of multi-generation backcrossing was performed where males that survived a discriminating dose of indoxacarb were used in crosses to a susceptible strain, New GR.

GBS was performed on 95 *H. armigera* samples (73 F_6 indoxacarb resistant, eight susceptible and 14 resistant GY7-39) generating 13203 tags with 8697 unique locations on the *H. armigera* genome. By a process of GWA analysis and fine mapping with segregation analysis using only haplotypes unique to the resistant GY7-39 strain, the indoxacarb resistance gene were mapped to a narrow 2.5MB region located on chromosome 16 (HaChr16:6083884- 6223558). There are two indoxacarb resistance genes in this region; *IndoR1* on HaChr16:6083884-6023558 and *IndoR2* on HaChr16:6294314-8558542. The analysis suggests that GY7-39 F_0 individuals carry both *IndoR1* and *IndoR2* in approx. a 2.5 Mbp segment that likely originated from a single F_0 female moth. In subsequent crosses, some F_6 resistant progeny lost the *IndoR1* gene (e.g. Family 1) but all the F_6 resistant progeny in Family 1 had *IndoR2*. In Family 2, all the F_6 resistant progeny had *IndoR1*, but lost *IndoR2*. This confirms initial genetic analysis of indoxacarb resistance which suggest that one (or a few closely linked) loci conferred indoxacarb resistance in GY7-39 (Bird 2016).

The mechanism of GY7-39 indoxacarb resistance studied here is consistent with cytochrome P450 monooxygenases determined from inhibition studies using piperonyl butoxide (Bird 2016). Mutations at the voltage-gated sodium channel (VGSC) were involved in indoxacarb and metaflumizone resistance in *Plutella xylostella* (Wang et al. 2016) and in *Tuta absoluta* (Roditakis et al. 2017). Our results suggest that the VGSC could be excluded as the cause of indoxacarb resistance in GY7-39. The VGSC locus is located on HaChr15 in *H. armigera* genome and there is no tag near the VGSC locus associated with indoxacarb resistance phenotype found in this study.

A cluster of nine P450 genes located on Hachr16, namely CYP6AE14, CYP6AE20, CYP6AE19, CYP6AE18, CYP6AE17, CYP6AE16, CYP6AE11, CYP6AE15 and CYP6AE12, span approx. 85kb (HaChr16: 8451146-8531656). Wang (2018) used CRISPR gene editing to knockout the CYP6AE gene cluster in *H. armigera* causing reduced insect survival when exposed to phytochemicals and insecticides so confirming cluster involvement in xenobiotic detoxification. In particular, CYP6AE17 and CYP6AE18 are able to metabolise indoxacarb by recombinant P450s and we consider both CYP6AE17 and CYP6AE18 to be the primary candidate detoxifiers for I*ndoR2*. However, a number of gaps in the current *Helicoverpa* spp. genome assembly still remain, particularly on HaChr16, and a possible re-arrangement in gene order cannot be discounted. Nevertheless, these results provide background data which will be useful for future genetic investigations including fine mapping of indoxacarb resistance genes and the eventual development an effective DNA based diagnostic to support resistance management.

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Supplementary Milestone 2.5 Investigate fitness costs and stability of resistance

Introduction and aims

An important part of building a risk assessment profile of indoxacarb resistance is to determine whether fitness costs are associated with resistance. Fitness costs are considered important for influencing the development of resistance and determining the magnitude and degree of dominance of such costs could be valuable for improving resistance management strategies (Carrière and Tabashnik 2001).

Ideally, fitness comparisons should be made between near-isogenic strains which are genetically identical except for the locus of interest. This ensures a high probability that observed differences between strains are associated with the resistance allele(s) and are not the result of genetic variance (see *Milestone 2.1*).

Relative fitness is measured by two methods, either directly comparing fitness components (e.g. survival, development rate or weight) between insecticide resistant and susceptible strains in the absence of insecticide, or by evaluating stability of resistance to insecticides in heterogeneous laboratory strains by taking into account the cumulative effect of all fitness components over many generations. Fitness costs are detected by the first method when one or more fitness components are significantly lower in the resistant strain than in the susceptible strain and by the second method when the frequency or level of resistance declines significantly over time in the absence of selection (Gassmann et al. 2009).

The aim of this study was to use both methods to determine the presence of differential fitness between near-isogenic indoxacarb resistant and susceptible strains. Firstly, comparisons of fitness components in life-history trait analyses were conducted on glasshouse-grown cotton and artificial diet in the laboratory. Secondly, population cage experiments were conducted in the laboratory in which strains with known resistance frequencies were maintained with and without selection for six generations and screened each generation to test for stability of resistance.

Materials and methods

Insect strains, bioassays and selection procedures. Bioassays were performed on four non-synchronous cohorts of New GR, and results were pooled in the final analysis because there were no significant differences between cohorts tested. The New GR strain was used as the susceptible control in all bioassays and in crosses to the resistant strain. The resistant strain GY7-39 was established from a single *H. armigera* moth collected as an egg from a maize field near Moree in 2013 (see *Milestone 2.2*). The strain was selected from F_2 offspring that survived the diagnostic dose by diet incorporation of indoxacarb (12 µg per ml in a diet incorporation bioassay performed on late second or early third instar larvae) (described in *Milestone 2.1*) that kills 99.9% of susceptible larvae (Bird 2015). Bioassays were performed on all iterations of the GY7-39 strain every generation.

Creation of near-isogenic strains by backcrossing. Because it had originated from an isofemale line the GY7-39 strain initially had a restricted gene pool. To reduce risk of genetic divergence as a result of founder effect and/or genetic drift, and to maintain the indoxacarb resistant and susceptible strains in a common genetic background, the GY7-39 strain was introgressed into the New GR strain by repeated backcrossing (at generations 5, 7, 12 and 20) and reselected to produce a near-isogenic resistant strain of *H. armigera* (described in *Milestone 2.1* and summarised in Table 2.5.1). After the first cross (BC1) the F_1 strain was maintained without selection for one generation and reselected in the subsequent generation with the diagnostic dose of indoxacarb (Table 2.1.1). As there was significant survival of F_1 progeny at this concentration, the F_1 progeny from the second (BC2) (Table 2.1.2) and third (BC3) (Table 2.1.3) backcrosses were directly subjected to selection. The fitness comparisons reported here were performed on the near-isogenic fourth backcross of GY7-39BC4 (Table 2.1.4). A summary of the relevant backcross events is shown in Table 2.5.1.

Life table construction. The relative performance of strains reared to pupation on whole cotton plants and artificial diet was tested by comparing the near-isogenic fourth serial backcross of the resistant strain GY7-39BC4, the susceptible strain New GR, and F_1 progeny from the reciprocal crosses of the two strains.

Table 2.5.1 Response of the resistant strain (GY7-39) during selection of near-isogenic lines compared with the susceptible New GR strain.

Genotype	*G* †	LC_{50} [µg/ml diet] (95% FL) ‡	Fit of probit line			RR
			Slope ± SE	X^2 (df)	*P*	
New GR		0.15 (0.144, 0.165)	3.1 ± 0.1	6.66 (4)	0.155	-
GY7-39 (F_5) §	3	25.02 (21.418, 29.251)	2.6 ± 0.2	6.47 (3)	0.091	167
GY7-39 (BC1)	2	15.21 (7.593, 25.631)	2.6 ± 0.5	14.02 (3)	0.003	101
GY7-39 (BC2)	5	22.86 (19.547, 26.698)	2.6 ± 0.2	2.96 (3)	0.398	152
GY7-39 (BC3)	8	22.68 (19.426, 26.447)	2.6 ± 0.2	2.68 (3)	0.444	151

GY7-39 (BC4)	13	22.31 (5.555, 67.154)	2.6 ± 0.8	12.59 (3)	0.006	149

†Number of generations selected between each backcross.
‡ Response to indoxacarb at the time of each backcross.
§ GY7-39 strain was isolated by F_2 screening and the survivors selected for three generations.

Adults were reared, as described above, and progeny allocated to either a plant diet in the glasshouse, or an artificial diet in the laboratory. The laboratory cohorts were apportioned to either standard rearing conditions or simulated overwintering conditions (see below).

Cotton seed used in glasshouse experiments was the conventional variety Sicot 71RRF. Seeds were planted into 20 cm pots containing a soil mix consisting of 60% garden soil and 40% perlite with approx. 20g per pot, of Osmocote slow-release fertilizer. Plants were grown in a clear-roofed glasshouse. Maximum and minimum temperatures were recorded daily and ranged between 23 and 31°C. Larvae were introduced onto the terminals of plants 10 weeks after germination when plants were at the 10-12 leaf stage of development with fruiting structures ranging from squares to flowers.

Insect survival and development. Neonate larvae were fed on artificial diet for 24 hours before being introduced to cotton plants. Fifty larvae from each genotype were transferred onto the terminal leaves of 10-week old cotton plants (one larva per plant). Larvae were contained on plants using fabric mesh cages and plants arranged in a randomized design in the glasshouse. In diet experiments 180 neonates from the New GR, GY7-39BC4 and GY7-39 female x New GR male strains and 125 neonates from the GY7-39 male x New GR female strain were fed on artificial diet and maintained under the rearing conditions. Larval growth was measured using a development index where a numerical value was assigned to each larval stage (Bird and Akhurst 2004). Growth and mortality was recorded every two or three days until pupation. Pupation was calculated as the number of larvae that failed to reach the pupal stage divided by the total number of larvae tested in each strain.

Pupal weights and adult emergence. Fifth instar larvae were removed from the glasshouse after they had ceased feeding and had taken on a characteristic pre-pupal appearance. Pre-pupae were placed on artificial diet and observed daily until pupation. After 72 hours pupae from cotton and diet experiments were sorted by gender and weighed. Adult emergence was recorded daily and the percentage of emergence was calculated in each strain as the number of moths that had successfully emerged as a proportion of the number of pupae. Survival from neonate to reproductive adult was calculated as the number of fertile adults as a proportion of the total number of larvae within each strain.

Reproductive potential. Upon emergence, each adult was paired with a single moth of opposite gender from the diet-reared New GR colony. The paired moths were provided with a 4% honey/sucrose solution and each pair maintained in a 500 mL plastic container lined with paper towels. A layer of fine cloth was placed between the container and the lid covering the aeration hole. Both the cloth and the paper served as oviposition substrates and were replaced every two days. Cage liners were incubated at 25°C overnight. Eggs that did not reach the characteristic 'brown ring' stage within 24 hours were considered infertile. Liners containing at least some eggs that showed signs of development were placed at 4°C to prevent hatching. The total numbers of fertile and infertile eggs laid by each were counted. Realized fecundity was calculated for each strain as the mean number of fertile eggs laid per female. Egg viability was calculated as the number of fertile eggs/total number of eggs laid.

Text Box 2.5.1 Calculation of intrinsic rate of natural increase.

Net replacement rate (R_o), representing the average number of female offspring produced by each female during its lifetime was calculated for each strain as:

$$R_o = (n \times l_e \times l_a) \times p$$

where n is the mean number of eggs per female, l_e is the proportion of fertile eggs, l_a is the proportion of reproductive adults and p is the proportion of fertile females in each strain (Birch 1948). The net replacement rate was used to calculate intrinsic rate of population increase as:

$$r_m = (\ln R_o)/T$$

where T is the development time from egg to adult eclosion (Birch 1948). The relative fitness of the resistant and F_1 strains was calculated as the ratio of R_o of the resistant and F_1 strains to the R_o of the susceptible strain.

Survival of post-diapaused adults. Resistant, susceptible and F_1 strains were reared from neonate to the early fifth instar on artificial diet. The number of larvae used to initiate diapause were 1170, 2020, 1980 and 855 in the New GR, F_1 (GY7-39 male x New GR female), F_1 (GY7-39 female x New GR male) and GY7-39BC4 strains, respectively. Diapause was induced in each strain by incubating early fifth instar larvae in a diapause chamber at 18°C with the photoperiod of 11:13 (L:D) (Murray and Wilson 1990).

Pupae were kept under simulated overwintering conditions for another three to four weeks before being removed from the incubator and examined for eyespot migration. Linear retention of eyespots across the postgenal eye of pupae was used as confirmation of diapause (Cullen and Browning 1978).

Diapaused pupae were sorted by gender and weighed. Pupal mortality and the emergence of non-diapaused adults were recorded. Surviving pupae were incubated at 4°C for 14 d to break diapause. Pupae were returned to normal adult rearing conditions and adult emergence was recorded. Survival of larvae to adult and pupae to adult was adjusted to exclude the small number of moths that emerged under diapausing conditions.

Stability of resistance. After 12 generations of selection of the fourth serial backcross, resistance had stabilised at approx. 150-fold compared with the New GR strain. At this point selection was discontinued in a sub-population of the GY7-39BC4 strain, designated the GY7-39BC4 Unsel strain, and was reared for a further six generations on untreated diet with no further exposure to insecticide.

The selected GY7-39BC4 strain was exposed to a diagnostic dose in bioassays using diet-incorporated indoxacarb in each subsequent generation. After seven days the larvae of the GY7-39BC4 strain were transferred onto untreated diet to complete development.

The selected and unselected strains were reared under general laboratory conditions described in *Milestone 2.1*. Approximately 380 pupae of uniform size and age were used to initiate each generation and the response of selected and unselected strains was measured and compared in each generation by diet incorporation bioassay, as described in *Milestone 2.1*.

Data analysis. Where necessary, bioassay data were corrected for control mortality (Abbott 1925). Probit regressions to estimate LC_{50}, ET_{50} (median effective time to reach 50% pupation and emergence), associated slope values and 95% fiducial limits were calculated using stand-alone software (Barchia 2001).

Resistance ratios (RR) were calculated by dividing the LC_{50} value of the resistant strain by the LC_{50} value of the laboratory strain. Significant differences ($P = 0.05$) between LC_{50} values were determined by the lethal concentration ratio test (Wheeler et. al 2006) where if the 95% confidence interval (CI) includes 1 then the LC_{50}s are not significantly different.

Mean pupal weights, realized fecundity and moth longevity were compared by one-way analysis of variance (ANOVA) and Tukey-Kramer honestly significant difference multiple comparison tests. Proportional data of survival, fertility and sex ratio were analysed using χ^2 distribution. All analyses were performed using SPSS statistical software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp., 2013).

Results

Larval survival and development. Survival and development parameters on cotton are shown in Table 2.5.2 and on diet on Table 2.5.3. There were no significant differences in survival from first instar larvae to pupation between susceptible, resistant or F_1 strains on cotton ($\chi^2 = 1.572$; df = 3; $P = 0.666$) or diet ($\chi^2 = 4.935$; df = 3; $P = 0.177$). There were no significant differences in survival to

emergence on cotton (χ^2 = 0.745; df = 3; P = 0.863) or diet (χ^2 = 0.147; df = 3; P = 0.986) or in the number of reproductive adults produced from each strain on cotton (χ^2 = 1.061; df = 3; P = 0.786) or diet (χ^2 = 4.601; df = 3; P = 0.203). Larvae developed to pupae 2-3 days faster on diet compared with cotton. Similar rates of larval development in susceptible, resistant and the F_1 strains occurred on cotton and diet (Figure 2.5.1). There were small (<1 day) but significant delays in pupation rate in the resistant strain compared with the susceptible strain on cotton and a similar delay in larval development between the two F_1 strains.

Pupal weight and moth emergence. These developmental parameters on cotton are shown in Table 2.5.2 and on diet on Table 2.5.3. Pupal weight on cotton was similar in males between strains (F = 2.66, df = 3, 7; P = 0.055) and significantly different in females between strains (F = 3.57; df = 3, 80; P = 0.018) due to significantly smaller (P < 0.05) pupae in the GY7-39BC4 compared with the F_1 (GY7-39 male x New GR female) strain. On diet pupal weights were significantly different between strains for males (F = 11.44; df = 3, 301; P < 0.01) and females (F = 5.57; df = 3, 270; P = 0.001). This was because both male and female pupae of the New GR strain were significantly larger than pupae from all other strains on diet (P < 0.05).
Higher pupal mortality in the GY7-39BC4 strain eliminated individuals with slower larval development and resulted in a significantly shorter mean generation time compared with the New GR strain on cotton (Table 2.5.2). On diet, pupation rate was similar between strains (pupation not recorded in F_1 (GY7-39 male x New GR female)). Generation time was also similar between GY7-39BC4, New GR and the F_1 (GY7-39 female x New GR male) strain, and significantly faster in the F_1 (GY7-39 male x New GR female) strain (Table 2.5.3).

Reproductive potential. Life history traits relating to reproductive capability on cotton are shown in Table 2.5.2 and on diet in Table 2.5.3. The proportion of females present in each strain was similar on cotton (P = 0.415) and diet (P = 0.626). There was no significant between-strain differences in female fertility on cotton (P = 0.345) or diet (P = 0.322). Egg viability was also similar between strains on cotton (P = 0.933) and diet (P = 0.661). Although realised fecundity was lower in females from the F_1 (GY7-39 male x New GR female) strain reared on cotton the difference was not significant (F = 2.21; df = 3, 58; P = 0.096). This contributed to a 28% reduction in net replacement rate (R_o) and a reduction in intrinsic rate of natural increase in this strain (r_m = 0.134) compared with New GR (r_m = 0.146). Reproductive fitness was highest in the F_1 reciprocal cross (r_m = 0.169) and GY7-39BC4 (r_m = 0.167). There was low magnitude differential fitness between strains demonstrated by a comparison of r_m of the susceptible strain where GY7-9BC4 and reciprocal crosses (GY7-39BC4 male x New GR, GY7-39BC4 female x New GR) were 1.1, 0.9 and 1.1, respectively, indicating no major disadvantage on cotton in strains with genes for indoxacarb resistance.

Significant differences in fecundity on diet (F = 4.04; df = 3, 101; P = 0.009) was the result of reduced egg laying capacity in the GY7-39BC4 strain and led to a reduction in R_o (Table 2.5.3). However, r_m was only slightly reduced compared with the susceptible strain due to similar rates of development on diet. A comparison of r_m of the susceptible strain showed relative fitness of GY7-9BC4 and reciprocal crosses (GY7-39BC4 male x New GR, GY7-39BC4 female x New GR) were 0.9, 1.1 and 1.0 on diet, respectively.

Male fertility was significantly lower (P < 0.05) in the GY7-39BC4 and F_1 (GY7-39 male x New GR female) strains compared with the New GR and F_1 (GY7-39 female x New GR male) strains on cotton (Table 2.5.2) and diet (Table 2.5.3).

There was no significant difference in longevity of female moths when reared on cotton (F = 1.89; df = 3, 75; P = 0.138). On diet female longevity was significantly different (F = 3.68; df = 3, 121; P = 0.014) because the F_1 (GY7-39 male x New GR female) longevity was reduced. However, longevity in the GY7-39BC4 and New GR strains was similar (Table 2.5.3). In contrast, the longevity of male moths was significantly different on cotton (F = 4.57; df = 3, 72; P = 0.006) and diet (F = 27.4; df = 3, 152; P < 0.001). Male longevity in the GY7-39BC4 strain was significantly (P < 0.05) reduced by 2.8 days on cotton (Table 2.5.2) and on diet by 5.4 days (Table 2.5.3), compared with the New GR strain.

Table 2.5.2 Mean fitness parameters for New GR, GY7-39BC4 and F_1 progeny from a reciprocal backcross of the two strains on 10 week-old glasshouse-grown cotton

Life History Trait	New GR	F_1 strains		GY7-39
		GY7-39 male x New GR female	GY7-39 female x New GR male	
% Survival				
Pupation	97.8[a]	84.2[a]	100[a]	95.6[a]
Adult emergence	93.3[a]	100[a]	95.7[a]	88.4[a]
Reproductive adult	91.3[a]	84.2[a]	95.7[a]	84.4[a]
Development time (days)				
ET_{50} pupation (FL)	18.6 (18.38, 18.91) [bc]	19.2 (18.50, 19.84) [ab]	18.3 (17.86, 18.64) [c]	19.5 (18.99, 20.03) [a]
ET_{50} emergence (FL)	36.7 (35.99, 37.31) [a]	37.0 (36.09, 39.04) [a]	34.4 (33.92, 34.97) [b]	34.3 (33.53, 35.09) [b]
Mean pupal weight (mg)				
Female (*n*)	319.1 ± 8.3[ab] (20)	299.7 ± 12.0[ab] (18)	324.9 ± 6.7[a] (25)	292.5 ± 8.0[b] (22)
Male (*n*)	330.1 ± 5.8[a] (25)	314.7 ± 8.2[a] (16)	301.0 ± 9.1[a] (19)	307.2 ± 10.3[a] (17)
Reproductive potential				
% female fertility	73.3[a]	66.7[a]	88.0[a]	81.0[a]
% male fertility	80.0[a]	50.0[b]	84.2[a]	56.3[b]
% egg viability	86.8[a]	86.7[a]	94.0[a]	91.5[a]
Realized fecundity	939 ± 154[a]	602 ± 81[a]	979 ± 108[a]	953 ± 84[a]
Proportion of females	0.43[a]	0.56[a]	0.53[a]	0.56[a]
Net replacement rate (R_o)	243	165	332	312
Intrinsic rate of population increase (r_m)	0.146	0.134	0.169	0.167
Relative fitness [††]	1.0	0.9	1.1	1.1
Adult longevity (days)				
Female	14.4 ± 0.6[a]	14.9 ± 1.0[a]	16.1 ± 0.8[a]	13.7 ± 0.8[a]
Male	13.6 ± 0.6[a]	14.5 ± 1.1[a]	13.9 ± 0.5[a]	10.8 ± 0.7[b]

Superscript letters after means within life history traits indicate significant differences ($P < 0.05$) between the means.
[†]Relative fitness = R_o (Gy7-39 or F_1 progeny) / R_o (New GR).

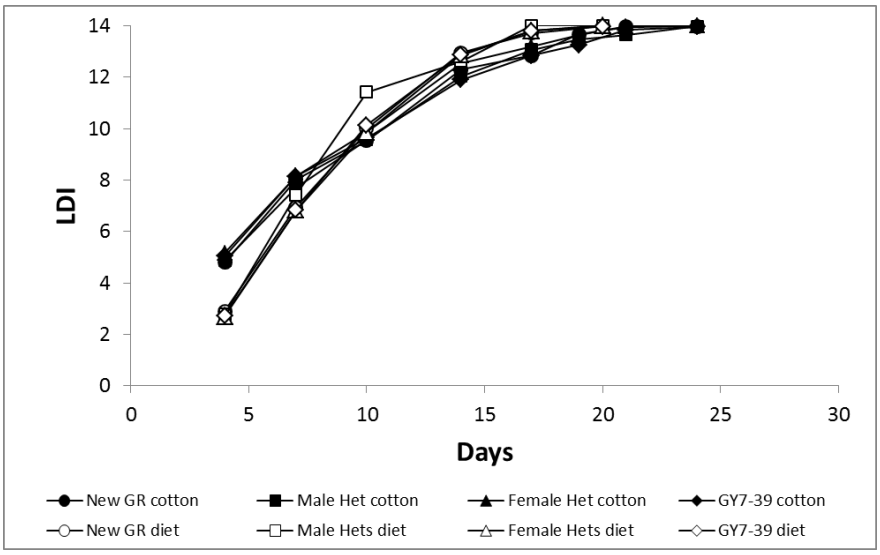
Table 2.5.3 Mean fitness parameters for New GR, GY7-39BC4 and F_1 progeny from a reciprocal backcross of the two strains on artificial diet

Life History Trait	New GR	F_1 strains		GY7-39
		GY7-39 male x New GR female	GY7-39 female x New GR male	
% Survival				
Pupation	96.6[a]	73.6[a]	97.7[a]	80.4[a]
Adult emergence	100[a]	97.8[a]	95.4[a]	100[a]
Reproductive adult	96.6[a]	71.9[a]	93.2[a]	80.4[a]
Development time (days)				
ET_{50} pupation (FL)	16.3 (16.08, 16.52) [a]	NR	16.1 (15.59,16.64) [a]	16.2 (16.12, 16.35) [a]
ET_{50} emergence (FL)	32.1 (31.85, 32.29) [a]	30.4 (28.64, 31.98) [b]	31.7 (31.04, 32.30) [ab]	32.2 (31.91, 32.44) [a]
Mean pupal weight (mg)				
Female (*n*)	417.1 ± 3.4[a] (72)	398.3 ± 5.8[b] (46)	397.9 ± 4.0[b] (81)	397.2 ± 4.0[b] (75)
Male (*n*)	416.3 ± 2.9[a] (101)	388.2 ± 5.7[b] (43)	392.5 ± 3.5[b] (92)	399.7 ± 4.1[b] (69)
Reproductive potential				
% female fertility	75.0[a]	63.3[a]	78.9[a]	85.7[a]
% male fertility	91.7[a]	60.0[b]	97.6[a]	60.5[b]
% egg viability	84.5[a]	85.9[a]	89.5[a]	74.0[a]
Realized fecundity	1009 ± 100[ab]	1367 ± 151[a]	1087 ± 91[ab]	810 ± 96[b]
Proportion of females	0.42[a]	0.52[a]	0.47[a]	0.52[a]
Net replacement rate (R_o)	189	298	221	155
Intrinsic rate of population increase (r_m)	0.158	0.181	0.165	0.152
Relative fitness [†]	1.0	1.1	1.0	0.9
Adult longevity (days)				
Female	17.7 ± 0.7[ab]	15.0 ± 1.0[a]	17.4 ± 0.6[ab]	18.4 ± 0.7[b]
Male	16.2 ± 0.4[a]	16.0 ± 0.5[a]	18.2 ± 1.2[a]	10.8 ± 0.5[b]

Superscript letters after means within life history traits indicate significant differences ($P < 0.05$) between the means.

[†]Relative fitness = R_o (Gy7-39 or F_1 progeny) / R_o (New GR)

Figure 2.5.1 Larval development of indoxacarb resistant, susceptible and F_1 strains determined on 10 week-old whole cotton plants grown in a glasshouse and in the laboratory on artificial diet. New GR susceptible (●), GY7-39BC4 resistant (■), F_1 (GY7-39 male x New GR female) (▲) and F_1 (GY7-39 female x New GR male) (♦) on cotton (open symbols), and artificial diet (corresponding closed symbols).



Survival of adults following an artificially induced diapause. Fitness components of resistant, susceptible and hybrid F_1 strains measured throughout an artificially induced diapause are shown in Table 2.5.4. Survival of larvae to pupation when reared in overwintering conditions was lowest in the GY7-39BC4 strain followed by the New GR strain and the hybrid F_1 strains but these differences were not significant ($\chi^2 = 1.312$; df = 3; $P = 0.726$) (Table 2.5.4).

Survival of larvae to adult was significantly different between strains ($\chi^2 = 21.242$; df = 3; $P = 0.0001$). The highest proportion of larvae that entered diapause and then survived to adult was in the F_1 (GY7-39 male x New GR female) strain at 63.9% (Table 4). Although this was higher than survival in the New GR strain, the difference was not significant ($\chi^2 = 3.472$; df = 1; $P = 0.062$). Larval survival to adult in the resistant strain (22.9%) was significantly lower than in the F_1 (GY7-39 male x New GR female) strain ($\chi^2 = 19.366$; df = 1; $P < 0.001$) and the New GR strain ($\chi^2 = 6.922$; df = 1; $P = 0.008$), with larval survival significantly different between the F_1 strains ($\chi^2 = 7.666$; df = 1; $P = 0.006$) (Table 4). The proportion of adults that emerged from diapaused pupae was similar between strains ($\chi^2 = 3.727$; df = 3; $P = 0.292$) (Table 2.5.4).

Differences between pupal weight of strains in diapause were highly significant for males ($F = 74.15$; df = 3, 2419; $P < 0.001$) and females ($F = 62.80$; df = 3, 2107; $P < 0.001$). Male pupae of the F_1 (GY7-39 male x New GR female) strain were significantly larger ($P < 0.01$) than male pupae of New GR strain. Similarly, female pupae of the F_1 (GY7-39 male x New GR female) strain were significantly larger ($P < 0.01$) than female pupae of New GR strain. Pupae of the F_1 (GY7-39 female x New GR male) and GY7-39BC4 strains were similar. Male pupae from these two strains were significantly smaller compared with the reciprocal F_1 and New GR strain. However, the difference was only significant between the two F_1 strains ($P < 0.01$) (Table 2.5.4).

The proportion of females present after diapause in each strain was similar ($\chi^2 = 0.406$; df = 3; $P = 0.940$) (Table 2.5.4). Longevity of moths that survived diapause was significantly different between strains in both males ($F = 16.86$; df = 3,611; $P < 0.001$) and females ($F = 12.60$; df = 3,543; $P < 0.001$) with male and female moths of the F_1 (GY7-39 female x New GR male) strain living significantly ($P < 0.05$) longer than all other strains.

The pattern of mortality through diapause and the significant reduction in weight of post-diapaused pupae in the resistant strain and male pupae of an F_1 hybrid strain suggests that there may be a

partial non-recessive survival cost associated with indoxacarb resistance in overwintering in *H. armigera*.

Stability of resistance. The dose response to indoxacarb of selected and unselected strains are shown table 2.5.5. In the first generation following relaxation of selection there was a significant difference between the LC_{50} of the selected and unselected strain (CI 0.549, 0.839). Nevertheless, the resistance ratio of the unselected strain remained at 147-fold. Over the following six generations without exposure to indoxacarb resistance in the GY7-39BC4 Unsel strain was stable with no further significant decline in LC_{50} based on comparison with the selected strain of GY7-39BC4.

Discussion and conclusions

Evaluation of fitness costs and stability is important for determining resistance risk associated with the use of insecticides and can be useful for formulating insecticide resistance management strategies. Here, relative fitness of near-isogenic indoxacarb resistant, susceptible and F_1 hybrid strains of *H. armigera* previously isolated from survivors from an F_2 screen were compared in life history trait analyses and selection experiments to determine stability of resistance.

There was no evidence of a major cost associated with survival, development time or female reproductive potential in indoxacarb resistant *H. armigera*. Smaller female pupae in the resistant strain did not result in reduced fecundity or female longevity on cotton compared with the susceptible or hybrid F_1 strains. On diet lower pupal weight was correlated with lower fecundity in the resistant strain. However, pupal weight was not correlated with fecundity in the hybrid F_1 strains. Male fertility was lower in the resistant strain compared with the susceptible strain with male fertility also significantly reduced in one of the F_1 hybrid strains reared on cotton and diet suggesting a non-recessive fitness cost may be a force for delaying resistance through reduced reproductive capacity in males with alleles for indoxacarb resistance. However, the finding that net reproductive rate is not adversely affected by the presence of genes for indoxacarb resistance lends further support to a lack of a strong fitness cost which would not favour an overall decline in resistance frequency (Carrière and Tabashnik 2001). In contrast, strains of *Heliothis virescens* and *H. armigera* selected for indoxacarb resistance in the laboratory had lower growth rate, lower fecundity and reduced relative fitness (0.06 and 0.67, respectively) than an unselected strain (Sayyed et al. 2008, Cui et al. 2018). However, unlike the present study, these comparisons were not made between near-isogenic strains.

The finding that individual fitness components in indoxacarb resistant and heterozygous strains of *H. armigera* was not reduced to any large extent on cotton further supports a lack of fitness costs of indoxacarb resistance because the magnitude of costs are generally exacerbated by a plant diet compared with artificial diet (Gassmann et al. 2009). Moreover, the relative overall fitness of the resistant strain, which takes into account the cumulative effect of individual fitness parameters, was slightly higher in the GY7-39BC4 strain and one of the reciprocal crosses compared with the New GR strain, which also demonstrated that strains with resistance alleles were not at a significant disadvantage on cotton. However, it is possible that fitness in resistant individuals may be impacted to a greater degree under more challenging environmental conditions (e.g. Raymond et al. 2005).

The gene(s) for indoxacarb resistance in the GY7-39BC4 strain is inherited as an incompletely dominant trait (*Milestone 2.2*, Bird 2016). Because the level of resistance in the GY7-39BC4 strain was stable when the strain was maintained under selection we have assumed that, by the principle of Hardy-Weinberg equilibrium, the gene for indoxacarb resistance is fixed in the GY7-39BC4 strain and the frequency of *rr*, *rs* and *ss* was constant. Our finding that there was no significant decline in indoxacarb resistance in the absence of insecticide indicates a high level of stability and is consistent with a lack of fitness cost demonstrated in the life-history trait analysis.

Winter diapause is an adaptive strategy which allows survival of *H. armigera* populations in temperate regions of eastern Australia when host plants are scarce and temperatures are generally too low to allow successful development. A 52% reduction in survival of larvae to post-diapaused adults in the

GY7-39BC4 strain compared with the New GR strain suggests a reduced physiological capability of indoxacarb resistant *H. armigera* to survive in overwintering conditions.

Table 2.5.4 Effect of artificially induced diapause on survival of New GR, GY7-39BC4 and F_1 progeny from a reciprocal cross of New GR and GY7-39BC4 reared on artificial diet.

Life History Trait	New GR	F_1 strains		GY7-39
		GY7-39 male x New GR female	GY7-39 female x New GR male	
% pupae that entered diapause	97.2[a]	99.7[a]	95.8[a]	99.1[a]
Survival				
% survival of larvae to pupae	83.4[a]	80.8[a]	79.7[a]	70.0[a]
% survival of larvae to adult[†]	44.5[ab]	63.9[a]	36.2[bc]	22.9[c]
% emergence from diapaused pupae	83.7[a]	92.5[a]	70.0[a]	74.6[a]
Mean pupal weight (mg)				
Female (*n*)	378.9 ± 2.6[b] (414)	420.5 ± 3.4[a] (347)	369.5 ± 2.5[bc] (702)	367.4 ± 2.6[c] (648)
Male (*n*)	388.4 ± 2.3[b] (517)	430.1 ± 3.3[a] (342)	379.1 ± 2.0[c] (828)	376.2 ± 2.3[c] (736)
Proportion of females	0.45[a]	0.50[a]	0.46[a]	0.47[a]
Adult longevity (days)				
Female	17.1 ± 0.4[bc]	16.0 ± 0.6[c]	19.7 ± 0.4[a]	18.4 ± 0.4[ab]
Male	15.6 ± 0.3[c]	17.9 ± 0.5[ab]	19.0 ± 0.3[a]	17.3 ± 0.4[b]

Superscript letters after means within life history traits indicate significant differences ($P < 0.05$) between the means.
[†] Survival excludes the number of moths that emerged under overwintering conditions.

Table 2.5.5 Response of the GY-39BC4 strain to indoxacarb in the absence of insecticide selection

Generation of GY7-39BC4	LC_{50} [µg/ml diet] (95% FL)	Fit of probit line			Lethal dose ratio (95% CI)	RR
		Slope ± SE	X^2 (df)	*P*		
F14 Sel	32.56 (27.859, 37.948)	2.8 ± 0.3	5.68 (2)	0.058		217
F14 Unsel	22.08 (19.111, 25.561)	2.8 ± 0.2	2.35 (4)	0.572	0.678 (0.549, 0.839)	147
F15 Sel	27.73 (11.044, 53.634)	3.2 ± 0.6	6.33 (2)	0.042		185
F15 Unsel	31.40 (27.761, 35.467)	3.2 ± 0.3	2.39 (2)	0.303	1.133 (0.854, 1.501)	209
F16 Sel	23.86 (13.175, 42.658)	3.6 ± 0.7	14.34 (3)	0.003		159
F16 Unsel	24.17 (21.169, 27.526)	3.6 ± 0.3	6.47 (3)	0.091	1.013 (0.739, 1.388)	163
F17 Sel	19.89 (17.208, 23.022)	2.8 ± 0.2	3.43 (4)	0.489		133
F17 Unsel	23.42 (20.256, 27.138)	2.8 ± 0.2	9.16 (4)	0.057	1.177 (0.956, 1.449)	156
F18 Sel	21.54 (18.649, 24.929)	2.8 ± 0.2	7.11 (4)	0.130		144
F18 Unsel	16.33 (11.002, 24.515)	2.8 ± 0.4	12.56 (4)	0.014	0.758 (0.563, 1.021)	109
F19 Sel	19.32 (16.672, 22.401)	2.7 ± 0.2	2.29 (4)	0.683		130
F19 Unsel	19.55 (16.876, 22.676)	2.7 ± 0.2	9.02 (4)	0.061	1.012 (0.820, 1.249)	130
F20 Sel	20.29 (17.880, 23.018)	3.7 ± 0.4	5.49 (3)	0.139		135
F20 Unsel	23.06 (20.327, 26.159)	3.7 ± 0.4	5.39 (3)	0.145	1.136 (0.950, 1.359)	154

† Survival at the diagnostic dose (DD) of indoxacarb, 12µg / ml of artificial diet.

However, the cost appears to be largely recessive and would provide limited benefits from a resistance management perspective. This is because recessive costs are a relatively weak force for driving a decrease in resistance allele frequency in early-stage of resistance when heterozygotes are the main carriers of resistance alleles (Carrière and Tabashnik 2001).

Comparisons can be made with pyrethroid resistance in the 1990's where reduced fitness as a result of larval exposure to overwintering conditions were thought to be associated with a decline in the frequency of pyrethroid resistance in *H. armigera* observed following an artificially induced diapause in the laboratory (Daly and Fisk 1995) and in overwintering field populations (Daly and Fitt 1990). Survival costs associated with overwintering also selected against dieldrin and diazinon resistance in *Lucilia cuprina* (McKenzie 1996), and resistance to Bt toxins in *Pectinophera gossypiella* (Carrière et al. 2001) and *H. armigera* (Bird and Akhurst 2004).

Notwithstanding the finding from the present study that reproductive capacity in male moths with resistance alleles was reduced compared with the susceptible strain, overall fitness was not reduced when the resistant strain was reared for six generations in the absence of insecticide. However, costs may be more deleterious under field conditions when combined with other pleiotropic effects. For example, male reproductive costs may be more effective for delaying resistance in regions of Australia where diapause occurs and where overwintering survival in *H. armigera* is already reduced. In this study we did not investigate possible cumulative effects of survival costs in diapause and reduced male reproductive capacity in the indoxacarb resistant strain. Further studies are warranted to investigate possible impacts from multiple fitness costs on stability of indoxacarb resistance in regions where diapause occurs.

Reduced fitness in resistant strains is usually associated with energetically costly processes associated with gene amplification or up-regulation of metabolic enzymes (Devonshire and Field 1991) or with target site resistance mechanisms involving point mutations which result in functional constraints at the modified target site (ffrench-Constant and Bass 2016). The putative mechanism of indoxacarb resistance in a precursor strain of GY7-39BC4 was associated with the CYP6AE gene cluster (Chen et al. 2017, 2019) which is consistent with findings from inhibition experiments (Bird 2016) and from CYP6AE gene cluster knockout experiments which restored susceptibility to indoxacarb in *H. armigera* (Wang et al. 2018). The lack of a fitness cost in GY7-39BC4 suggests that either detoxification of indoxacarb is not energetically costly, or that there could be involvement of fitness modifiers that ameliorate the cost of resistance (e.g. McKenzie 1996).

Indoxacarb resistance is at relatively low levels in Australian populations of *H. armigera* (see *Milestone 1.4*). However, use of selective products such as indoxacarb in grains production systems

for control of *H. armigera* has increased and the cotton IRMS is not sufficiently effective for managing resistance in other commodities (Bird 2018). The fitness cost of indoxacarb resistance is, at best, small and transient. Moreover, dominant inheritance of a metabolic mechanism (Bird 2016, Chen et al. 2017, 2019) exacerbates resistance risk because as dominance of resistance increases the effect of the dominance of the fitness cost diminishes (Tabashnik et al. 2004) and management tactics for delaying resistance under these circumstances are more difficult to implement (Roush 1989, ffrench-Constant and Bass 2016). Therefore, it is imperative that effective resistance management strategies that rely on chemical rotations are adopted to prolong efficacy of indoxacarb in farming systems that play host to *H. armigera*. The finding that fitness cost may be associated with survival through a winter diapause suggests there is a greater imperative for adoption of management strategies in northern regions of Australia where diapause does not occur.

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Objective 3. Conduct studies to determine patterns of cross-resistance between Bt-resistant Helicoverpa strains and conventional chemistries

Milestone 3.1 Collaboration with CSIRO, to test the dose response of VIP3A resistant strains of *H. armigera* and *H. punctigera* against conventional chemistries registered for use against *Helicoverpa* spp.

Introduction and aims

Widespread uptake of *Bt* technology in Australia imposes continuous selection pressure on target pests. In other parts of the world at least three lepidopteran species subjected to prolonged exposure in granary, field, or greenhouse environments have developed conspicuous resistance to *Bt* toxins (Tabashnik et al. 2013). High exposure to Bt toxins expressed in transgenic cotton combined with higher than expected resistance frequencies to Cry toxins *Helicoverpa* spp. (Mahon et al. 2007, Downes et al. 2009) may increase resistance risk for Bt and for other insecticides.

The aim of this study was to test the response of isogenic Vip3A resistant and susceptible strains of *H. armigera* (SP477) and *H. punctigera* (Hp8-48) to conventional insecticides registered for control of *Helicoverpa* spp. to determine existence of cross-resistance.

Materials and methods

Insect strains. The susceptible *H. armigera* laboratory strain (New GR) (see *Milestone 2.1*). The susceptible *H. punctigera* laboratory strain (LHP) was established from a population from uncultivated hosts in south-west Queensland in the spring of 1999. Susceptibility to Vip3A was monitored regularly by evaluating the response to a concentration of Vip3A toxin that kills > 95% of susceptible neonate larvae; 10µg/cm^2 and 2µg/cm^2 as diet surface contamination, for *H. armigera* and *H. punctigera*, respectively (Mahon et al. 2012).

The Vip3A resistant *H. armigera* strain SP477 was isolated during the summer of 2009-10 from individuals collected as eggs on non-Bt cotton. The Vip3A resistant *H. punctigera* strain Hp4-48 was isolated during the summer of 2008-09 from individuals collected as eggs. Both strains originated from populations from the Gwydir region (Mahon et al. 2012).

Both the *H. armigera* and *H. punctigera* resistant strains were isolated using a previously described F_2 screen where a Vip3A clone in *E. coli* was the source of toxin (Sena et al. 2009). Cells of *E. coli* were grown at 37°C in a shaking incubator overnight in Luria-Bertani medium. Expression of Vip3A protein was induced by the addition of isopropyl-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM at OD_{600}. Cells were further incubated at 28°C overnight, harvested by centrifugation for 15 minutes at 5000 g and then resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO4, 1.5 mM KH_2PO_4, pH 7.4) and sonicated. Purified Vip3A was prepared from a portion of the sonicated cell lysate using a HIS SELECT 1 ml cartridge (Sigma) and was examined for purity and stability by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein concentration of the purified sample was quantified by using the Bradford method with BSA as a standard (Mahon et al. 2012).

Bioassays. Broad-spectrum contact insecticides (pyrethroids, chlorpyrifos and methomyl) were tested by topical application of insecticide using the methods of Forrester et al. (1993) described in *Milestone 1.8*. Selective insecticide (indoxacarb, chlorantraniliprole and emamectin benzoate) were tested by diet incorporation bioassay using the methods of Bird (2015) bioassay in *Milestone 2.1*.

Insecticides. Insecticide solutions used in topical bioassays were prepared from technical material dissolved in analytical grade acetone to produce two-fold serial dilutions corresponding to six or seven insecticide concentrations which were expected to induce 1 to 99% mortality. Fenvalerate

(95.3%) (Sumitomo Chemical, Sydney); bifenthrin (93.3%) (FMC, Brisbane); chlorpyrifos (99.8%) (Dow AgroScience, Sydney), methomyl (98%) (Bayer CropScience, Melbourne).

Commercial insecticide formulations used in diet incorporation bioassays were: indoxacarb (Steward® [15% active ingredient]), chlorantraniliprole (Altacor® [35% active ingredient]), DuPont Australia Ltd., Macquarie Park, emamectin benzoate (Affirm® [1.9% active ingredient]), Syngenta Crop Protection, Macquarie Park.

Data analysis. For each species cross-resistance was examined by comparing the dose-responses of Vip3A-resistance strains with those of susceptible strains. Slopes, LD_{50}/LC_{50} and associated 95% fiducial limits were calculated by probit analysis using the POLO-PC software (LeOra Software, Berkeley, CA). Dose responses were corrected for control mortality (Abbott 1925). For each species/insecticide combination, a resistance ratio was calculated by dividing the LD_{50} value of the resistant strains by the LD_{50} of the susceptible laboratory strains. Differences in dose-responses were considered to be significant if the 95% fiducial limits of median lethal concentrations did not overlap.

Results

The dose-response of the Vip3A resistant and susceptible strains of *H. armigera* were established for four classes of broad-spectrum insecticides (Table 3.1.1) and three classes of selective insecticides (Table 3.1.2). There was no significant cross-resistance to synthetic pyrethroids and minimal cross-resistance to chlorpyrifos (RR = 2.2-fold). Negative-cross resistance was observed between SP477 and methomyl (RR = 0.5-fold).

The dose-response of the Vip3A resistant and susceptible strains of *H. punctigera* were established for four classes of broad-spectrum insecticides (Table 3.1.3) and three classes of selective insecticides (Table 3.1.4). Results indicate that the 8-48Hp strain was significantly more susceptible to fenvalerate, and methomyl. However, the magnitude of the increase in sensitivity is small (1.6 and 2-fold for fenvalerate and methomyl, respectively). There was no cross-resistance to bifenthrin, chlorpyrifos or indoxacarb with very low magnitude cross-resistance (< 2-fold) to chlorantraniliprole and emamectin benzoate.

Table 3.1.1 Resistance levels to four broad-spectrum insecticides in the Vip3A resistant strain of *H. armigera* (SP477) and laboratory strain (New GR).

Insecticide	Strain	LD_{50} [µg ai/larva]	95% fiducial limits Lower	95% fiducial limits Upper	Slope ± SE	RR†
Fenvalerate	New GR	0.054	0.0430	0.068	2.6 ± 0.16	
	SP477	0.051	0.0400	0.065	2.7 ± 0.27	0.9
Bifenthrin	New GR	0.017	0.0140	0.020	3.1 ± 0.19	
	SP477	0.029	0.0190	0.044	2.8 ± 0.31	1.7
Chlorpyrifos	New GR	3.179	2.673	3.772	2.4 ± 0.15	
	SP477	7.113	5.523	9.533	1.9 ± 0.21	2.2*
Methomyl	New GR	0.968	0.806	1.198	1.8 ± 0.13	
	SP477	0.491	0.343	0.673	2.3 ± 0.21	0.5 *

† RR = resistance ratio.
* Indicates significant difference between strains.

Table 3.1.2 Resistance levels to three selective insecticides in the Vip3A resistant strain on *H. armigera* (SP477) and laboratory strain (New GR).

Insecticide	Strain	LC_{50} [µl/ml diet]	95% fiducial limits Lower	95% fiducial limits Upper	Slope ± SE	RR†
Indoxacarb	New GR	0.1472	0.1366	0.1585	3.4 ± 0.19	
	SP477	0.1577	0.1127	0.2144	3.0 ± 0.27	1.1
Chlorantraniliprole	New GR	0.0143	0.0129	0.0159	3.5 ± 0.30	
	SP477	0.0175	0.0142	0.0216	5.1 ± 0.56	1.2
Emamectin benzoate	New GR	0.0074	0.0060	0.0090	5.1 ± 1.00	
	SP477	0.0091	0.0079	0.0105	4.3 ± 0.45	1.2

† RR = resistance ratio.

Table 3.1.3 Resistance levels to four broad-spectrum insecticides in the Vip3A resistant strain of *H. punctigera* (Hp8-48) and laboratory strain (LHP).

Insecticide	Strain	LD_{50} [µg ai/larva]	95% fiducial limits Lower	Upper	Slope ± SE	RR†
Fenvalerate	LHP	0.0095	0.0080	0.0112	3.9 ± 0.4	
	Hp8-48	0.0059	0.0500	0.0068	6.1 ± 0.8	0.6*
Bifenthrin	LHP	0.0069	0.0049	0.0092	3.1 ± 0.3	
	Hp8-48	0.0071	0.0058	0.0088	3.4 ± 0.3	1.0
Chlorpyrifos	LHP	0.371	2.673	0.529	4.8 ± 0.6	
	Hp8-48	0.272	5.523	0.327	4.8 ± 0.6	0.7
Methomyl	LHP	0.721	0.806	1.058	1.6 ± 0.2	
	Hp8-48	0.357	0.343	0.454	1.7 ± 0.2	0.5 *

† RR = resistance ratio.
* Indicates significant difference between strains.

Table 3.1.4 Resistance levels to four broad-spectrum insecticides in the Vip3A resistant strain of *H. punctigera* (Hp8-48) and laboratory strain (LHP).

Insecticide	Strain	LC_{50} [µl/ml diet]	95% fiducial limits Lower	Upper	Slope ± SE	RR†
Indoxacarb	LHP	0.1701	0.1065	0.2653	3.3 ± 0.3	
	Hp8-48	0.1253	0.1053	0.1486	4.1 ± 0.4	0.7
Chlorantraniliprole	LHP	0.0041	0.0034	0.0050	4.6 ± 0.5	
	Hp8-48	0.0076	0.0063	0.0091	3.2 ± 0.3	1.9*
Emamectin benzoate	LHP	0.0048	0.0044	0.0052	8.1 ± 1.2	
	Hp8-48	0.0078	0.0068	0.0090	5.9 ± 0.7	1.6*

† RR = resistance ratio.
* Indicates significant difference between strains.

Discussion and conclusions

The use of insecticide sprays for *Helicoverpa* spp. control remains relevant not only for the small proportion of growers that plant non-*Bt* cotton varieties but also for around 15% of the area planted to Bollgard cotton which for short periods can carry susceptible *Helicoverpa* spp. larvae at densities above the recommended threshold level for insecticidal control (Downes and Mahon 2012). Moreover, in many regions cotton is preceded by extensive plantings of winter pulses and coarse grain crops such as sorghum which can require insecticide sprays to control *Helicoverpa* spp. larvae (Murray et al. 2013).

Cross-resistance between insecticide sprays and *Bt* toxins may seem unlikely given their different modes of action and the likelihood that the molecular basis of resistance will also be diverse. The molecular basis of resistance to Cry toxins in *Helicoverpa* spp. is likely to be associated with modifications at the toxin receptor binding site (Akhurst et al. 2003) whereas target site resistance in *H. armigera* to organophosphates and carbamates is mediated by insensitive acetylcholinesterase, and to pyrethroids by modifications to the voltage-gated sodium channel of nerve cells (McCaffery 1998). High level cross-resistance between Cry1Ac and a range of conventional broad-spectrum and selective insecticides has been documented (Alvi et al. 2012). Moreover, it is possible that increased metabolic capacity of detoxification insect enzymes favours development of resistance across a diverse range of insecticide classes including *Bt* toxins (Li et al. 2007).

At this stage the target site for Vip3A proteins is not known, and therefore the mechanism of resistance is not clearly understood (Chakroun et al. 2014). Notwithstanding gaps in current

knowledge regarding the molecular basis of Vip3A resistance in *Helicoverpa* spp., results of this work concluded minimal cross resistance exists between the broad-spectrum insecticides tested and high-level Vip3A resistance conferred in the SP477 strain of *H. armigera* and the Hp8-48 strain of *H. punctigera.*

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Objective 4. Preparing for new product options

Milestone 4.1 Using appropriate bioassay techniques, accumulate baseline data for new Helicoverpa insecticides pending registration

NOTE: *There were no new insecticides pending registration during the life of the project. However, there was no published data available to describe the baseline response of H. punctigera to selective insecticides. Therefore this milestone was addressed by evaluating susceptibility to indoxacarb, chlorantraniliprole and emamectin benzoate in this species.*

Introduction and aims

A prolonged period of drought between 2001 and 2009 reduced the prevalence of *H. punctigera* native hosts in inland Australia (Gregg et al. 2016, Gregg 2017). The subsequent decline in *H. punctigera* populations migrating from the inland to cropping areas (Baker and Tann 2016) may have reduced the capacity for resistance to be diluted which, in turn could be a driver of resistance to insecticides used in the management of *H. punctigera* such as chlorantraniliprole, emamectin benzoate and indoxacarb. These insecticidal classes are important rotational options in *Helicoverpa* spp. control in a range of pulse and oilseed crops because of their selectivity in comparison to broad-spectrum insecticides (Torres and Bueno 2018) and low resistance allele frequency (Bird et al. 2017). Resistance to selective insecticides was initially monitored in *Helicoverpa* spp. by topical application (Rossiter et al. 2008). Although these insecticides have some contact activity in Lepidoptera, ingestion is considered the primary route by which insects accumulate a lethal dose of insecticide (Bird 2016). Previous findings in *H. armigera* were that delivery of these insecticides by contact may not be the optimal method for measuring dose response of insecticides where a lethal dose of insecticide is accumulated more efficiently by ingestion (Bird 2015).

In the light of reported changes in population dynamics of this species, this study readdresses the potential for *H. punctigera* to develop insecticide resistance and assess the suitability of methods for discriminating between resistant and susceptible phenotypes as a pre-emptive step in resistance management in this species. The objectives of this study were to: (1) evaluate insecticide-incorporated diet feeding bioassay method for determining the toxicity of the selective insecticides to *H. punctigera* as an alternative to the traditional topical method of bioassay; (2) accumulate baseline susceptibility data to determine the range of intra-specific tolerance in field populations of *H. punctigera* collected across cropping areas of eastern Australia; (3) use the baseline data generated from these bioassays to establish discriminating concentrations for emamectin benzoate, chlorantraniliprole and indoxacarb and (4) compare susceptibility of *H. punctigera* and *H. armigera* to these insecticides.

Materials and methods

Insect Strains. A laboratory susceptible strain of *H. punctigera*, designated LHP, was used to check for consistency of bioassay results during this study. This strain was originally established from a population collected from uncultivated hosts in southwest Qld in the spring of 1999.

Field populations of *H. punctigera* were sampled from major cropping areas located across NSW and in southern and central Qld. They were collected as eggs and larvae between September 2013 and January 2016 from cotton, pigeon pea, and a range of pulse crops; a minimum of 50 field collected individuals constituted each geographically distinct strain. Each strain of *H. punctigera* was tested within two generations of establishment in the laboratory. Rearing methods for laboratory and field strains were the same as described for *H. armigera* in Bird (2015).

Insect Bioassays. The dose responses of laboratory and field strains were determined from bioassays on artificial diet into which formulated insecticide was incorporated, as described in Bird (2015) and described in *Milestone 1.4*. As in previous bioassays commercial insecticide formulations

were used: indoxacarb (Steward® [15% active ingredient], Du Pont Australia Ltd., Macquarie Park), emamectin benzoate (Affirm® [1.9% active ingredient], Syngenta Crop Protection) and chlorantraniliprole (Altacor® [35% active ingredient], Du Pont Australia Ltd., Macquarie Park).

Each insecticide was tested on non-synchronous cohorts of laboratory LHP strain on two occasions between November 2013 and December 2014. As there were no significant differences between cohorts for any of the insecticides tested the bioassay results were pooled to generate an average LC_{50} for the LHP strain.

All bioassays were maintained under the same conditions used for insect rearing and were assessed for mortality at 7 days. As in a previously published study of *H. armigera* susceptibility to these insecticides (Bird 2015) exposure of larvae to insecticide-incorporated diet for 7days was found to be the optimal time for assessment of mortality because this provided a high level of discrimination between larvae that survived and larvae that were killed at each insecticide concentration, hence ensuring mortality would not be underestimated if insects recovered from initial knockdown effects of the insecticide. The criteria for mortality were the same as for *H. armigera* and included one or more of the following: larvae unable to perform coordinated movement when prodded; paralysis of prolegs; larvae very slow to right themselves (time exceeding 3 seconds).

Data analysis. Dose responses of larvae to insecticides were corrected for control mortality using the formula of Abbott (1925). Probit regressions including slope, LC_{50}, $LC_{99.9}$, LD_{50} and associated 95% fiducial limits were calculated using software developed by Barchia (2001).

Toxicity ratio for field strains was calculated by dividing the LC_{50} of each field population by the LC_{50} of the laboratory strain LHP (pooled result from bioassays of two non-synchronous cohorts). Toxicity ratio of insecticides was compared in laboratory strains of *H. punctigera* and *H. armigera* by dividing the LC_{50} of the laboratory strain (LHP) by the LC_{50} of a laboratory strain of *H. armigera* (LC_{50} determined from four non-synchronous cohorts described in Bird (2015)).

Toxicity ratio of insecticides was compared in field strains of *H. punctigera* and *H. armigera* by dividing the LC_{50} *H. punctigera* field strains (pooled data of 12, 11 and 14 strains tested against chlorantraniliprole, emamectin benzoate and indoxacarb, respectively) by the LC_{50} of *H. armigera* field strains (pooled data of 21, 20 and 22 strains tested against chlorantraniliprole, emamectin benzoate and indoxacarb, respectively) (Bird 2015).

Results

Toxicity of insecticides in laboratory strains. Chlorantraniliprole had the highest toxicity when tested by diet incorporation bioassay (LC_{50} = 4.2 µg/L) (Table 4.1.1). Emamectin benzoate had a similar level of toxicity to the laboratory strain using this method of insecticide delivery (LC_{50} = 4.8 µg/L) (Table 4.1.2), and indoxacarb had the lowest toxicity (LC_{50} = 147.2 µg/L) (Table 4.1.3). There was goodness-of-fit of the response in diet incorporation bioassays to the probit binomial model for emamectin benzoate (P = 0.9976) and indoxacarb (P = 0.0681), while there was a slight deviation from the model for chlorantraniliprole (P = 0.0347).

Toxicity of insecticides in field strains. There was a narrow variation in response to chlorantraniliprole between the 12 field strains of *H. punctigera* tested (1.9-fold) and dose responses were accompanied by high slopes (3.9 ± 0.2 from pooled data of field strains). High susceptibility to chlorantraniliprole was indicated by low toxicity ratios ranging between 0.7 and 1.3. The most tolerant strains included a population collected from pigeon pea at Bellata with an LC_{50} value of 5.6µg/L and a strain from St George which had the highest $LC_{99.9}$ of 31.3µg/L (Table 4.1.1).

The response *H. punctigera* in field populations to emamectin benzoate also showed a narrow range of intra-specific variation between the 11 field populations tested (2.4-fold). Pooled slope values of field strains were 5.0 ± 0.5. Toxicity ratios, ranging between 0.7 and 1.8, indicate high susceptibility of field populations to this insecticide. A population established from chickpea and faba bean fields at Moree had the highest level of tolerance with an LC_{50} value of 8.7µg/L, while a strain originating from cotton fields at Wee Waa had the highest $LC_{99.9}$ value of 26.1µg/L (Table 4.1.2).

Table 4.1.1 Bioassay on 12 field strains and one laboratory susceptible strain of *H. punctigera* tested as late 2nd/early 3rd instars on diet incorporated chlorantraniliprole (350g/kg) and assessed for mortality at 7 days.

Collection data							Fit of probit line			% Mortality (*n*) [c]	
Origin of field strains (G[a])	Host	Collection date	LC_{50} [µg/L diet]	(95% FL)	$LC_{99.9}$ [µg/L diet]	Toxicity ratio [b]	Slope ± SE	X^2 (df)	*P*	15.6 µg/L diet	31.2 µg/L diet
Emerald QLD (F_1)	chickpea	Sept 2016	2.9	(2.56 - 3.52)	13.0	0.7	4.7 ± 0.6	12.87 (9)	0.1686	100 (60)	100 (59)
Breeza NSW (F_1)	canola	Sept 2013	2.9	(2.46 - 3.35)	28.7	0.7	3.1 ± 0.4	17.35 (10)	0.0670	-	-
Goondiwindi QLD (F_2)	pigeon pea	Jan 2016	3.0	(2.67 - 3.42)	15.3	0.7	4.4 ± 0.5	4.37 (9)	0.8854	100 (60)	100 (59)
Kingaroy QLD (F_1)	cotton	Dec 2015	3.2	(2.83 - 3.68)	19.4	0.8	4.0 ± 0.5	6.42 (9)	0.6973	100 (60)	100 (60)
Mungindi NSW (F_1)	cotton	Dec 2015	3.6	(3.16 - 4.21)	24.7	0.9	3.7 ± 0.4	13.76 (12)	0.3163	96.7 (60)	100 (60)
Griffith NSW (F_1)	cotton	Dec 2015	3.8	(3.42 - 4.23)	14.0	0.9	5.5 ± 0.6	5.11 (9)	0.8246	100 (60)	100 (60)
St George QLD (F_1)	cotton	Nov 2015	3.8	(2.82 - 4.91)	31.3	0.9	3.4 ± 0.6	18.10 (8)	0.0205	98.3 (60)	100 (60)
Emerald QLD (F_1)	cotton	Nov 2015	3.9	(3.48 - 4.44)	20.7	0.9	4.3 ± 0.5	8.40 (8)	0.3954	96.7 (60)	100 (60)
Wee Waa NSW (F_2)	cotton	Jan 2014	4.0	(3.53 - 4.44)	19.1	1.0	4.6 ± 0.5	5.15 (10)	0.8809	100 (60)	100 (60)
Goondiwindi QLD (F_1)	cotton	Dec 2015	4.1	(3.34 - 4.95)	20.6	1.0	4.4 ± 0.7	19.69 (10)	0.0323	100 (60)	100 (60)
Trangie NSW (F_2)	cotton	Jan 2016	4.2	(3.66 - 4.79)	32.3	1.0	3.5 ± 0.4	4.03 (11)	0.9690	98.3 (60)	100 (60)
Bellata NSW (F_2)	pigeon pea	Jan 2016	5.6	(5.02 - 6.27)	24.8	1.3	4.8 ± 0.5	6.18 (11)	0.8611	98.3 (60)	100 (60)
Pooled			3.7	(3.46 - 3.92)	22.8		3.9 ± 0.2			98.9 (660)	100 (658)
Laboratory strain LHP			4.2	(3.72 - 4.71)	26.4		3.9 ± 0.4	36.73 (23)	0.0347	100 (120)	100 (120)

[a] Generation tested.
[b] Toxicity ratio = LC_{50} of field population/LC_{50} of LHP strain (average of two non-synchronous LHP cohorts).
[c] Mortality at highest concentration tested.

Table 4.1.2 Bioassay on 11 field strains and one laboratory susceptible strain of *H. punctigera* tested as late 2nd/early 3rd instars on diet incorporated emamectin benzoate (19g/L) and assessed for mortality at 7 days.

Collection data							Fit of probit line			% Mortality (*n*) [c]	
Origin of field strains (G[a])	Host	Collection date	LC_{50} [µg/L diet]	(95% FL)	$LC_{99.9}$ [µg/L diet]	Toxicity ratio [b]	Slope ± SE	X^2 (df)	*P*	11.9 µg/L diet	23.7 µg/L diet
Bellata NSW (F_2)	pigeon pea	Jan 2016	3.6	(3.20 - 3.95)	13.3	0.7	5.1 ± 0.6	12.22 (10)	0.2706	100 (60)	100 (60)
Trangie NSW (F_2)	cotton	Jan 2016	3.7	(3.32 - 4.14)	16.0	0.8	4.9 ± 0.6	17.05 (11)	0.1064	98.3 (60)	100 (60)
Wee Waa NSW (F_1)	chickpea	Oct 2013	5.2	(4.80 - 5.69)	11.9	0.9	8.7 ± 1.4	4.89 (8)	0.7693	100 (60)	100 (60)
Emerald QLD (F_1)	chickpea	Sept 2016	5.5	(5.03 - 6.04)	14.6	1.2	7.3 ± 1.0	2.72 (7)	0.9096	100 (60)	100 (60)
Goondiwindi QLD (F_2)	pigeon pea	Jan 2016	5.6	(5.07 - 6.22)	19.2	1.2	5.8 ± 0.7	13.90 (9)	0.1259	95.0 (60)	100 (60)
St George QLD (F_1)	cotton	Nov 2015	5.7	(5.15 - 6.24)	16.7	1.2	6.6 ± 0.8	8.38 (9)	0.4964	100 (59)	100 (60)
Griffith NSW (F_1)	cotton	Dec 2015	5.8	(5.24 - 6.40)	18.6	1.2	6.1 ± 0.7	5.20 (9)	0.8165	98.3 (60)	100 (60)
Wee Waa NSW (F_2)	cotton	Jan 2014	5.9	(5.31 - 6.65)	26.1	1.2	4.8 ± 0.6	12.04 (8)	0.1494	93.3 (60)	100 (60)
Kingaroy QLD (F_1)	cotton	Dec 2015	6.4	(5.77 - 7.11)	23.3	1.3	5.5 ± 0.6	12.53 (11)	0.3252	96.7 (60)	100 (60)
Breeza NSW (F_1)	canola	Sept 2013	8.5	(7.85 - 9.25)	16.9	1.8	10.4 ± 1.2	5.89 (7)	0.5527	93.3 (60)	100 (60)
Moree NSW (F_1)	chickpea/faba bean	Sept 2013	8.7	(7.99 - 9.41)	16.5	1.8	11.1 ± 1.3	1.65 (7)	0.9767	93.3 (60)	100 (60)
Pooled			5.6	(5.04 - 6.25)	23.0		5.0 ± 0.5			97.1 (659)	100 (660)
Laboratory strain LHP			4.8	(4.47 - 5.06)	12.0		7.7 ± 0.7	6.67 (20)	0.9976	100 (120)	100 (120)

[a] Generation tested.
[b] Toxicity ratio = LC_{50} of field population/LC_{50} of LHP strain (average of two non-synchronous LHP cohorts).
[c] Mortality at highest concentration tested.

Table 4.1.3 Bioassay on 14 field strains and one laboratory susceptible strain of *H. punctigera* tested as late 2nd/early 3rd instars on diet incorporated indoxacarb (150 g/L) and assessed for mortality at 7 days.

Collection data							Fit of probit line			% Mortality (*n*) [c]	
Origin of field strains (G[a])	Host	Collection date	LC_{50} [µg/L diet]	(95% FL)	$LC_{99.9}$ [µg/L diet]	Toxicity ratio [b]	Slope ± SE	X^2 (df)	*P*	1500 µg/L diet	3000 µg/L diet
Bellata NSW (F_2)	pigeon pea	Jan 2016	133.2	(114.6 - 153.9)	1560	0.9	2.9 ± 0.3	12.33 (13)	0.5008	100 (60)	100 (60)
Goondiwindi QLD (F_1)	cotton	Dec 2015	135.4	(109.6 - 167.5)	935	0.9	3.7 ± 0.6	19.70 (10)	0.0322	100 (60)	100 (60)
Emerald QLD (F_1)	cotton	Nov 2015	142.5	(124.0 - 163.2)	1306	1.0	3.2 ± 0.3	11.48 (13)	0.5707	98.3 (60)	100 (60)
Wee Waa NSW (F_2)	cotton	Jan 2014	148.2	(127.6 - 171.5)	1823	1.0	2.8 ± 0.3	23.21 (14)	0.0569	100 (59)	100 (60)
Breeza NSW (F_1)	canola	Sept 2013	148.7	(110.8 - 195.8)	1083	1.0	3.6 ± 0.7	34.83 (11)	0.0003	100 (60)	100 (60)
Kingaroy QLD (F_1)	cotton	Dec 2015	148.8	(127.5 - 172.6)	2032	1.0	2.7 ± 0.2	13.83 (16)	0.6114	100 (60)	100 (60)
St George QLD (F_1)	cotton	Nov 2015	152.2	(134.6 - 172.5)	946	1.0	3.9 ± 0.4	12.48 (11)	0.3287	100 (60)	100 (60)
Goondiwindi QLD (F_2)	pigeon pea	Jan 2016	153.3	(130. 5- 178.9)	2396	1.0	2.6 ± 0.2	8.31 (15)	0.9108	100 (59)	100 (60)
Mungindi NSW (F_1)	cotton	Dec 2015	163.9	(121.4 - 224.1)	1013	1.1	3.9 ± 0.8	48.85 (12)	0.0001	100 (60)	100 (60)
Emerald QLD (F_1)	chickpea	Sept. 2016	193.0	(168. 1- 221.5)	1885	1.3	3.1 ± 0.3	17.75 (15)	0.2760	100 (60)	100 (60)
Trangie NSW (F_2)	cotton	Jan 2016	214.1	(184.2 - 248.8)	3008	1.5	2.7 ± 0.2	16.11 (15)	0.3748	100 (59)	100 (60)
Griffith NSW (F_1)	cotton	Dec 2015	220.8	(189.8 - 256.9)	3229	1.5	2.7 ± 0.2	17.73 (15)	0.2771	98.3 (60)	100 (60)
Moree NSW (F_1)	chickpea/faba bean	Sept 2013	243.9	(212.0 - 280.8)	2585	1.7	3.0 ± 0.3	21.51 (15)	0.1213	96.7 (60)	100 (60)

Wee Waa NSW (F_1)	chickpea	Oct 2013	248.7	(213.3 - 289.6)	3991	1.9	2.6 ± 0.2	18.43 (17)	0.3622	96.7 (60)	100 (60)
Pooled			172.2	(160.6 - 184.6)	2159		2.8 ± 0.1			99.3 (837)	100 (840)
Laboratory strain LHP			147.2	(135.3 - 160.1)	825		4.1 ± 0.3	33.80 (23)	0.0681	99.2 (120)	100 (120)

[a] Generation tested.
[b] Toxicity ratio = LC_{50} of field population/LC_{50} of LHP strain (average of two non-synchronous LHP cohorts).
[c] Mortality at highest concentration tested.

The response of *H. punctigera* to indoxacarb showed a narrow (2-fold) intra-specific variation in the 14 field strains tested with high susceptibility demonstrated by low toxicity ratios ranging between 0.9 and 1.9. The least sensitive strains had $LC_{99.9}$ values > 3000µg/L. In the case of a strain established from chickpea fields near Wee Waa, the $LC_{99.9}$ was 3991µg/L (Table 4.1.3).

General goodness-of-fit to the probit binomial model was indicated by a significant deviation from the model in only two of the 12 field strains tested with chlorantraniliprole (St George F_1 cotton, P = 0.0205; Goondiwindi F_1 cotton P = 0.0323) and the laboratory strain LHP (P = 0.0347) (Table 4.1.1), and three of the 14 field strains tested with indoxacarb (Goondiwindi F_1 cotton P = 0.0322; Breeza F_1 canola P = 0.0003; Mungindi F_1 cotton P < 0.0001) (Table 4.1.3). The dose response of emamectin benzoate demonstrated a goodness-of-fit to the probit model in all 11 strains tested (Table 4.1.2).

Establishment of discriminating doses. To validate the suitability of candidate diagnostic concentrations for use in resistance monitoring, strains were assessed at two doses at the extreme upper end of the dose range which were expected to cause 90 to 100% mortality. For chlorantraniliprole, mortality at the two highest concentrations tested of 15.6 and 31.2µg/L was 98.9% and 100%, respectively (Table 4.1.1). The empirical result from bioassays was consistent with the predicted $LC_{99.9}$ response of the most highly tolerant strains, and only slightly exceeded the highest dose tested in two strains collected; one from cotton at Trangie and the other from cotton at St George (Table 4.1.1). Therefore, the recommended diagnostic concentration for monitoring resistance to chlorantraniliprole in *H. punctigera* is 0.032µg/ml.

The two highest concentrations of emamectin benzoate tested were 11.9 and 23.7µg/L and resulted in 97.1% and 100% mortality, respectively (Table 4.1.2). Predicted $LC_{99.9}$ values exceeded the highest dose tested in two strains originating from chickpea and cotton fields at locations near Wee Waa (Table 4.1.2). Based on both the empirical and theoretical data, the recommended diagnostic concentration for monitoring resistance to emamectin benzoate in *H. punctigera* is 0.026µg/ml.

The highest concentrations of indoxacarb tested were 1500 and 3000µg/L, which produced 99.3% and 100%, respectively. The most tolerant strains exceeded the highest concentration tested in three cases; Trangie cotton, Griffith cotton and Wee Waa chickpeas (Table 4.1.3). Based on the $LC_{99.9}$ estimates of mortality in the most tolerant strains and, considering empirical mortality, the recommended discriminating concentration for indoxacarb in *H. punctigera* is 4µg/ml.

Toxicity of feeding bioassays in H. punctigera compared with H. armigera. Comparisons were made between the response of *H. punctigera* observed in this study and the response of *H. armigera* reported in Bird (2015) and shown in Table 4.1.4. In laboratory susceptible strains, chlorantraniliprole was significantly (P < 0.05) more toxic (toxicity ratio = 0.292; 95% CI 0.251 - 0.340) to *H. punctigera* (LC_{50} 0.0042µg/ml; Table 4.1.1) than *H. armigera* (LC_{50} 0.014µg/ml; Bird 2015). Similarly, a comparison of the dose response of emamectin benzoate shows this insecticide was significantly more toxic (toxicity ratio = 0.644; 95% CI 1.274 - 1.890) to *H. punctigera* (LC_{50} 0.0048µg/ml; Table 4.1.2), than to *H. armigera* (LC_{50} 0.007µg/ml; Bird 2015). The LC_{50} for indoxacarb to *H. punctigera* (LC_{50} 0.1472µg/ml; Table 4.1.3) was not significantly different (toxicity ratio = 1.0; 95% CI 0.893 - 1.112) from *H. armigera* (LC_{50} 0.147µg/ml; Bird 2015).

Field strains of *H. punctigera* were significantly more susceptible than field strains of *H. armigera* to chlorantraniliprole (toxicity ratio = 0.147; 95% CI 0.134 - 0.162), emamectin benzoate (toxicity ratio = 0.549; 95% CI 0.484 - 0.624) and indoxacarb (toxicity ratio = 0.591; 95% CI 0.533 - 0.656).

Response of H. punctigera to insecticides in feeding bioassays compared with topical bioassays. Table 4.1.5 shows *H. punctigera* toxicity data from topical bioassays of the LHP strain of *H. punctigera* obtained from Bird and Downes (2014) and LJB (unpublished). In the laboratory susceptible strain chlorantraniliprole, emamectin benzoate and indoxacarb were more toxic when administered orally in a feeding bioassay compared with contact by topical administration. The LD_{50} of chlorantraniliprole, emamectin benzoate and indoxacarb when administered topically was 0.335, 6.96 and 1.456µg/ml, respectively (Table 4.1.4). Hence, chlorantraniliprole, emamectin benzoate and indoxacarb were 80-,

1450- and 10-fold, respectively, more toxic by ingestion than by contact in the laboratory *H. punctigera* strain.

Table 4.1.4 Toxicity of insecticides on strains of *H. armigera* tested using a diet incorporation method of bioassay [data from Bird (2015): LC_{50} of laboratory susceptible strain determined from four non-synchronous cohorts; LC_{50} of field strains determined from pooled data of 20, 21 and 22 strains tested against emamectin benzoate, chlorantraniliprole and indoxacarb, respectively].

Insecticide	LC_{50} [µg/ml] (95% FL)	Slope ± SE	Toxicity ratio[†]
Laboratory susceptible strain			
Chlorantraniliprole	0.014 (0.013 –0.016)	3.5 ± 0.3	0.292*
Emamectin benzoate	0.007 (0.006 – 0.009)	5.1 ± 1.0	0.644*
Indoxacarb	0.147 (0.137 – 0.158)	3.4 ± 0.2	1.0
Field strains (pooled data)			
Chlorantraniliprole	0.025 (0.023 – 0.027)	2.5 ± 0.1	0.147*
Emamectin benzoate	0.010 (0.009 – 0.011)	3.5 ± 0.2	0.549*
Indoxacarb	0.291 (0.270 – 0.214)	2.3 ± 0.1	0.591*

[†] Toxicity ratio = *H. punctigera* LC_{50} / *H. armigera* LC_{50}.
* Indicates significant difference ($P < 0.05$) between response of *H. armigera* and *H. punctigera.*

Table 4.1.5 Toxicity of insecticides on a laboratory susceptible strain of *H. punctigera* using a topical method of bioassay.

Insecticide	LC_{50} [µg/ml] (95% FL)	$LC_{99.9}$ [µg/ml]	Fit of probit line			Reference
			Slope ± SE	X^2 (df)	*P*	
Chlorantraniliprole	0.335 (0.297-0.378)	1.749	4.3 ± 0.5	14.40 (11)	0.2116	Bird and Downes (2014)
Emamectin benzoate	6.960 (5.945-8.128)	126.816	2.5 ± 0.92	20.77 (18)	0.2912	L.J. Bird unpublished data
Indoxacarb	1.456 (1.231-1.702)	21.603	2.6 ± 0.3	9.45 (12)	0.6641	Bird and Downes (2014)

Discussion and conclusions

The use of a diagnostic concentration of insecticide to discriminate between resistant and susceptible phenotypes is a highly efficient method for monitoring resistance in insect populations (Roush and Miller 1986) and has been used in *Helicoverpa* spp. resistance monitoring in Australia since the mid-1980s. This technique was originally developed for broad-spectrum insecticides whereby toxicity is mediated primarily through contact mode of entry (Forrester et al. 1993). More recently, selective insecticidal classes have been introduced for which intoxication occurs primarily by ingestion and bioassay methods designed for contact insecticides may not be appropriate for measuring the response of insecticides where a lethal dose of insecticide is accumulated more efficiently by ingestion.

This study, along with a similar study of *H. armigera* (Bird 2015), demonstrates selective insecticides are more toxic when administered by ingestion than by contact. Both studies also demonstrated that high slope values and goodness-of-fit to a probit model were associated with the dose response regressions generated from feeding bioassays. Therefore, delivery of insecticide by ingestion using a diet incorporation bioassay technique could increase discrimination between resistant and susceptible genotypes and provide a highly appropriate method for monitoring insecticide resistance in these species than traditional topical bioassays (ffrench-Constant and Roush 1990).

Toxicity of chlorantraniliprole and emamectin benzoate was similar and approx. 30-fold higher than indoxacarb when insecticides were administered orally. However, emamectin benzoate was 21-fold less toxic than chlorantraniliprole and 5-fold less toxic than indoxacarb when insecticides were administered by contact. Emamectin benzoate and analogues of this compound such as abamectin were shown to have relatively weak contact activity compared with stomach toxicity in other noctuid

larvae (Anderson et al. 1986, Venkateswari et al. 2008) possibly due to poor cuticular penetration of these insecticides in some species (Deecher et al. 1990).

There was differential inter-species toxicity in both laboratory and field strains with significantly higher LC_{50} values for chlorantraniliprole and emamectin benzoate in *H. armigera* compared with *H. punctigera*. Similarly, fenvalerate was found to be 2-fold more efficacious on *H. punctigera* than on *H. armigera*, with endosulfan having similar toxicity to both species (Forrester et al. 1993).

There was high susceptibility to chlorantraniliprole, emamectin benzoate and indoxacarb, based on comparisons with the laboratory strain, and a narrow range of intra-specific variation between the dose responses of geographically diverse populations. Low intra-specific variation in baseline sensitivity to emamectin benzoate and indoxacarb was also found amongst populations of *H. armigera* sampled from similar cotton and grains regions of eastern Australia (Bird 2015). However, a narrow range of susceptibility among conspecific populations may not preclude the potential of populations to respond to selection and developing resistance that results in the failure of insecticides to provide field control (Tabashnik 1994). For example, pyrethroid resistance was detected in field populations of *H. punctigera* at low frequencies (<5%) during periods of high usage of these products in consecutive years during the mid to late 1980s, presumably due to intensive selection from insecticide use in the cotton industry (Forrester et al. 1993). In a subsequent study, pyrethroid resistance in *H. punctigera* was detected at elevated levels (17-fold) in a field population established from cotton fields in the Macquarie valley in 1994 (Gunning et al. 1997).

Metabolic studies suggest that *H. punctigera* and *H. armigera* have a similar capacity to develop metabolic detoxification systems (Collins and Hooper 1984, Gunning et al. 1994, 1997) and is further supported by laboratory selection of *H. punctigera* which resulted in a strain with 10.5-fold resistance to fenvalerate and 17.4-fold resistance to deltamethrin (Forrester et al. 1993). Resistance in this strain was partially suppressible by the metabolic inhibitor PBO, suggesting involvement of a metabolic resistance mechanism. Notwithstanding similar metabolic profiles in the two species, the induction of metabolic enzymes in *H. punctigera* may not necessarily be associated with specific functions associated with detoxification (Collins 1985). This is because elevated activity of enzymes commonly associated with resistance (such as esterases, mixed-function oxidases or glutathione transferases) is often used to infer the presence of causal mechanisms. This can be misleading because only specific enzymes within each class are involved in resistance to different insecticides in the same chemical group (Sawicki 1987).

The most common explanation for the non-persistent nature of insecticide resistance in *H. punctigera* is dispersal ecology associated with migratory behaviour (Forrester et al. 1993). Establishment of populations on native hosts in southwestern Qld after summer-autumn rain (Gregg et al. 2016) and is typically followed by an annual influx of moths into cropping landscapes of eastern Australia, usually in early spring (Baker et al. 2011, Baker and Tann 2016). These immigrants are thought to mix with resident *H. punctigera* populations in cropping areas thus diluting any resistance alleles that may have been selected for through exposure to insecticide (Forrester et al. 1993). In addition, *H. punctigera* has a complex diapause (Murray 1992) and cohorts which avoid exposure to insecticides through quiescence could be an important source of dilution for resistance. However, as noted by Gunning and Easton (1994) changes in environment and habitat may influence the population ecology of *H. punctigera,* which could in turn favour resistance development in this species.

Recent studies provide evidence that the annual spring migration of *H. punctigera* from inland regions into cropping areas of eastern Australia was considerably impacted by the millennium drought (2001-2009) which severely affected availability of *H. punctigera* native hosts in inland Australia (Gregg et al. 2016, Gregg 2017). The impact on *H. punctigera* breeding habitat consequently reduced the magnitude of moth migration (Baker and Tann 2016). Since the end of the millennium drought, irregular summer and autumn rainfall has occurred in parts of the inland but the abundance of primary *H. punctigera* hosts has not yet recovered to pre-drought levels (Le Mottee 2015, Gregg 2017). Consequently, the size of the spring *H. punctigera* immigrant population originating from the inland remains low and this could have serious implications for resistance management. This study

provides information that could be used to develop a surveillance program for *H. punctigera* if resistance risk in this species continues to increase.

The determination of discriminating concentrations was based on a theoretical estimate of highest $LC_{99.9}$ values and taking into account the empirical mortality observed at the upper limits of the dose response curve (Robertson et al. 2007). Based on these criteria we propose the use of concentrations of 0.032, 0.026 and 4 µg of insecticide/ml of diet for chlorantraniliprole, emamectin benzoate and indoxacarb, respectively, to discriminate between resistant and susceptible *H. punctigera*.

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Objective 5. Develop collaboration with Bayer for investigating cross-resistance in recently isolated Bt resistant strains of *H. punctigera*

Milestone 5.1 Test the dose response of Bt resistant strains of *H. punctigera* against conventional chemistries registered for use against Helicoverpa

Cross-resistance between insecticides has the potential to compromise efficacy of products. In particular, the presence of generalist mechanisms (e.g. metabolic detoxification) has the potential to reduce the efficacy of pivotal selective products such as indoxacarb and chlorantraniliprole and emamectin benzoate.

Therefore, evaluating patterns of cross-resistance is a key component for determining the risk profile of insect pests and provides important background knowledge for developing resistance management plans which rely on rotational strategies.

In July 2018 the project leader, with support from the R and D Manger (CRDC), approached Bayer Crop Science with a proposal to develop a collaboration aimed at assessing the cross-resistance profile in recently isolated strains of *H. punctigera* with resistance to toxins produced by the bacteria *Bacillus thuringiensis* and which are currently deployed in commercial varieties of Bt transgenic cotton. This work was designed to expand on similar studies previously undertaken in collaboration with CSIRO to investigate efficacy of conventional insecticides against *H. armigera* and *H. punctigera* strains resistant to Cry2Ab (Bird and Downes 2014) and Vip3A (*Milestone 3.1*, above).

A Materials Transfer Agreement (MTA) was drafted by Bayer Crop Science in mid-2018 and provided to NSW DPI Contracts and IP personnel for comment. It was recommended that the MTA should be amended to reflect NSW DPI's obligations under the CRDC agreement (DAN1506 contract), regarding IP licences and publication obligations. Dr Kristen Knight provided details of these recommendations to Bayer's legal representative who was asked to include DPIs draft recommendations in a revised MTA.

Dr Knight was contacted on many occasions for advice on progress of the MTA. However, this advice was not forthcoming in time to complete the work before the end of the DAN1506 project. Dr Knight has indicated that Bayer is willing to progress this collaboration, albeit within a time frame past the end date of the DAN1506 project and subject to the drafting of a new MTA aligned with the a new Research Agreement. NSW DPI now anticipates that this milestone will be completed during the life of a subsequent project.

Objective 6. Undertake project review and gather industry feedback regarding work completed and establish whether there are new industry research priorities

A three-year review of this project was conducted in Brisbane on October 18th 2017. Participants in the review meeting were Susan Mass (R and D Manager, CRDC), Rod Jackson (Leader Summer Crops, NSW DPI) and Lisa Bird (Project Leader). As this was a five year project it was decided that a mid-term review could be useful for providing a detailed update on progress as well as ensuring that the agreed milestones were relevant to the needs of the cotton industry. As part of the review the project leader compiled a summary document (below) outlining the progress against each milestone, identifying key findings and achievements of the project.

Objective 1. Conduct insecticide resistance monitoring annually including delivery of outcomes across the cotton industry			
Milestone	YEAR 1 (2014-15) CRDC & GRDC	YEAR 2 (2015-16) CRDC	YEAR 3 (2016-17) CRDC
1.1 Sampling	Egg collecting by local collection team in the Namoi/Gwydir, Macintyre and Macquarie valleys (Dec. to April), and coordinated in other areas by collaboration with CCA (Sept. to April). An alternative method of sourcing samples from pheromone traps was trialled in a pilot study from October 2014 to March 2015. This was very successful for generating high levels of fertility for establishing isofemale families for F_2 screening.	Egg collecting by local collection team in the Namoi/Gwydir, Macintyre and Macquarie valleys (Dec. to April), and coordinated in other areas by collaboration with CCA (Sept. to April). Pheromone trapping was expanded in the Namoi valley with trapping networks established on the Liverpool Plains and Mullaley areas with small-scale trapping conducted at ACRI. **PROJECT VARIATION.**	Pheromone trapping was introduced as the primary source of samples for resistance testing with trapping networks further expanded and established by local teams in the Namoi/Gwydir and Macquarie valleys and monitored fortnightly (Oct. to April). Linkages with collaborators from QDAF for trapping and transporting live moth samples from the Darling Downs and central QLD to ACRI, and DuPont for monitoring field failures and implementing sampling activities if required.
1.2 Resistance testing	Phenotypic (F_0) testing of broad-spectrum insecticide resistance in *H. armigera* (pyrethroid [fenvalerate and bifenthrin], organophosphate [chlorpyrifos] and carbamate [methomyl]) and *H. punctigera* (fenvalerate and abamectin) **FINAL YEAR OF TESTING.** Genic (F_2) screening of selective insecticide resistance in *H. armigera* (indoxacarb, chlorantraniliprole and emamectin benzoate) using a diet-incorporation bioassay.	Genic (F_2) screening of selective insecticide resistance in H. armigera (indoxacarb, chlorantraniliprole and emamectin benzoate) using a diet-incorporation bioassay.	Genic (F_2) screening of selective insecticide resistance in *H. armigera* (indoxacarb, chlorantraniliprole and emamectin benzoate) using a diet-incorporation bioassay.
1.3 Reporting and involvement with TIMS/NIRM	Mid-Season and End-of-Season Resistance Reports to the CCA (January 2014 and July 2014). Resistance monitoring update at TIMS Insecticide Technical Panel (Brisbane April 2015).	Mid-Season and End-of-Season Resistance Reports to the CCA (January 2015 and July 2015). Resistance monitoring update at TIMS Insecticide Technical Panel (Toowoomba May 2016). Presentations at: 4th Australian Agrichemical Resistance Meeting, Adelaide, 3rd Nov. 2016; CCDM / AHRI 2nd Crop Protection Forum, Wagga Wagga 23rd Nov., 2016.	Resistance monitoring update at TIMS Insecticide Technical Panel (Brisbane May 2017). Project leader as a member of the National Insecticide Resistance Management (NIRM) working group to coordinate the development of an RMS for *H. armigera* in grains. This role includes raising awareness and promoting the benefits of a scientific approach to RM in the grains industry.

Objective 1. Summary of findings and achievements

Broad-spectrum insecticide monitoring (discontinued in 2015) - high frequencies of pyrethroid resistance, moderate frequencies of carbamate resistance and low organophosphate resistance in *H. armigera* in all areas. *H. punctigera* fully susceptible to pyrethroids and abamectin.

Selective insecticide monitoring in *H. armigera* - incipient indoxacarb resistance in all areas with elevated frequencies in Central Qld (9%) by the end of the 2017 season. Low frequency resistance to chlorantraniliprole and emamectin benzoate.

Innovations in sampling methods - extensive pheromone trapping conducted in the 2015-2016 season was shown to be a highly targeted, time- and cost-effective method for sourcing samples of *H. armigera* for resistance testing. There was no significant difference in the frequency of indoxacarb resistance in F_2 lines from pheromone caught moths compared with moths derived from immature stages collected from the field and reared to adult under laboratory conditions. Therefore, the use of pheromone traps for sourcing insects for F_2 screening was implemented to improve efficiencies in the resistance monitoring process and replaced egg and larval sampling in 2016-17.

Increased knowledge of genetic basis of field resistance - analysis of F_2 screening conducted from 2013-16 suggest that resistance is not recessive in the majority of cases where isofemale lines scored positive for indoxacarb resistance and is consistent with previous findings of dominant indoxacarb resistance from quantitative genetic analysis of the GY7-39 strain (see Milestone 2.2 below).

Communication of results to industry - results presented to TIMS and provided to the CCA (in 2014-15 and 2015-16), and used to update the CPMG.

Cross-industry engagement – the lead researcher in the project has taken an active role in coordinating the development of a strategy designed to address the needs of the pulse industry for managing resistance to key Helicoverpa insecticides at high risk as a result of unregulated use. This included a process of industry consultation. A draft RMS proposal has been developed along with detailed technical reports and has been and submitted to CropLife Aust. for ratification.

Award nomination - Project leader nominated for DuPont External Champion Award for an individual or team not employed by DuPont who has demonstrated outstanding contribution regarding stewardship support for DuPont and its products and the markets where those products are used.

Objective 2. Isolation and characterisation of mechanisms that confer resistance to selective chemistries in *H. armigera* detected from the field

Milestone	YEAR 1 (2014-15)	YEAR 2 (2015-16)	YEAR 3 (2016-17)
2.1 Isolation of novel resistance mechanisms	A strain of *H. armigera* with enhanced survival to indoxacarb was isolated in 2013 and previously described in DAN1204/00164. This strain was serially backcrossed to the laboratory susceptible strain New GR to produce a strain (GY7-39OC2) 87.5% isogenic with the parental laboratory strain.	Further serial backcrossing to the laboratory susceptible strain New GR produced a strain (GY7-39OC3) which is >93% isogenic with the parental laboratory strain. Complementation tests for allelism in multiple strains indicate a common mechanism is shared by isofemale families identified as resistant to indoxacarb.	Further serial backcrossing to the laboratory susceptible strain New GR produced a strain (GY7-39OC4) which is >96% isogenic with the parental laboratory strain. This strain was then used in life history trait experiments to determine relative fitness in near-isogenic strains (see *Milestone 2.2* below). In 2017, a strain from Emerald was identified as having enhanced survival to chlorantraniliprole. This strain is currently under directional selection in the laboratory.
2.2 Quantitative genetic analysis of new resistance mechanisms		Analyses, involving a series of backcrosses and bioassays on the near-isogenic 3rd outcross (GY7-39OC3), have been completed. Inhibition studies to test for the presence of a metabolic detoxification mechanism of resistance were initiated in pilot studies.	Inhibition studies to test for the presence of a metabolic detoxification mechanism of resistance completed. Life-history trait analyses to determine relative fitness of indoxacarb resistant and susceptible strains have been conducted and the data is currently being analysed. At this stage there is no indication that large fitness costs are associated with indoxacarb resistance. However, there may be some small fitness penalties in reproductive potential and survival after diapause.
2.3 Cross-resistance in strains with novel resistance to selective chemistries		The third backcross (GY7-39OC3) was bioassayed to determine patterns of cross-resistance to other Helicoverpa insecticides.	

2.4 Molecular basis of new resistance mechanisms	Molecular characterisation of indoxacarb resistance was initiated by collaborations with Grant Herron and Yizhoh Chen (NSW DPI) to undertake linkage mapping analysis of the GY7-39 strain. As a first step in this process three homozygous resistant strains of GY7-39 were established and outcrossed to the New GR susceptible strain to create F1 heterozygotes (equivalent to GY7-39OC3). Samples from the parental strains as well as F_1 and F_2 progeny were provided to EAMI for preliminary linkage mapping analysis.	Recombinant individuals for the full linkage mapping analysis were generated by serially backcrossing resistant males from three homozygous indoxacarb resistant strains to susceptible females sequentially five times to produce F_6 progeny for analysis. Multiple internal replicates from all three families were provided to EMAI for analysis. Two of these families (4 and 9) were subsequently analysed.	Genotype-by-sequencing was performed at EMAI to determine single nucleotide polymorphisms (SNP) associated with the genome of indoxacarb resistant H. armigera. A genome-wide association analysis identified a cluster of markers highly associated with indoxacarb resistance which encode for detoxification enzymes, consistent with predictions from inhibition bioassays. A series of 11 primer pairs were then designed along the haploid block. The combination of these PCR amplifications will confirm sequence alignment and form the basis of a molecular diagnostic tool to discriminate between RR, RS and SS genotypes. Strains that tested positive for resistance in 2016-17 were isolated and stored for use in validation of molecular based methods in resistance diagnostics.

Objective 2. Summary of findings and achievements

Selection of indoxacarb resistance and creation of a near-isogenic strain - the indoxacarb resistant GY7-39 strain of *H. armigera* has >96% genetic similarity to the susceptible parental strain.

Quantitative genetic analysis of indoxacarb resistance completed and published - indoxacarb resistance is autosomal, monogenic and partially dominant; degree of dominance was concentration dependent. Results published in PMS and presented at AACS Conference.

No cross-resistance between indoxacarb and other insecticides registered for H. armigera - pyrethroid, emamectin benzoate, chlorantraniliprole, Cry toxins.

Metabolic detoxification by P450 enzymes identified as the putative mechanism in indoxacarb resistance - we found high-level suppression of resistance in GY7-39 strain by the metabolic inhibitor piperonyl butoxide (PBO). This finding was further supported by results from the molecular analysis which found a cluster of markers highly associated with indoxacarb resistance located in a narrow 2.6 MB region which contain numerous genes that encode detoxification enzymes including the 10 CYP6AE gene cluster.

Development of molecular diagnostics for indoxacarb resistance - primers designed to facilitate the development of a molecular tool for detection of indoxacarb resistance from field samples.

Objective 3. Conduct studies to determine patterns of cross-resistance between Bt-resistant Helicoverpa strains and conventional chemistries

Milestone 3.1	YEAR 1 (2014-15)	YEAR 2 (2015-16)	YEAR 3 (2016-17)
Cross-resistance between VIP3A and conventional insecticides registered for Helicoverpa	In collaboration with CSIRO bioassays were conducted to determine the dose-response of a Vip3A-resistant strain of *H. armigera* (Ha 4.77) to conventional chemistries registered for use against Helicoverpa. These bioassays concluded minimal cross-resistance between synthetic insecticides (fenvalerate, bifenthrin, chlorpyrifos, methomyl, indoxacarb, chlorantraniliprole, emamectin benzoate) and high-level Vip3A resistance conferred in the 4.77 strain of *H. armigera*.	In collaboration with CSIRO bioassays were conducted to determine the dose-response of a Vip3A-resistant strain of *H. punctigera* (Hp 8.48) to conventional chemistries registered for use against Helicoverpa. Results indicated that the 8.48Hp strain of *H. punctigera* was significantly more susceptible to fenvalerate, and methomyl. However, the magnitude of the increase in sensitivity is small (1.6 and 2-fold for fenvalerate and methomyl, respectively). There was no cross-resistance to indoxacarb with small increases in tolerance to chlorantraniliprole and emamectin benzoate (1.9- and 1.6-fold, respectively).	

Objective 3. Summary of findings and achievements

No cross-resistance between Vip3A resistant strains of *H. armigera* or *H. punctigera* and other insecticides registered for use in Helicoverpa control.

Objective 4. Preparing for new product options

Objective 5. Investigation of bio-pesticides for management of Helicoverpa

Objective 6. Three year review of progress against project milestones. Variations requested and enacted on a milestone basis

Scientific Publications:

L.J. Bird and S.J. Downes. 2014. Toxicity and cross-resistance of insecticides to Cry2Ab-resistant and Cry2Ab-susceptible Helicoverpa armigera and Helicoverpa punctigera (Lepidoptera: Noctuidae). Journal of Economic Entomology 107: 1923-1930.

L.J. Bird. 2015. Susceptibility of Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) to indoxacarb, emamectin benzoate and chlorantraniliprole in Australia. Journal of Economic Entomology 108: 294-300.

L.J. Bird. 2016. Susceptibility of Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) to cyantraniliprole in Australia determined from topical and ingestion bioassays. Journal of Economic Entomology 109: 1350-1356. DOI: ECONENT-2016-0027.R1.

L.J. Bird. 2016. Genetics, cross-resistance and synergism of indoxacarb resistance in Helicoverpa armigera (Lepidoptera: Noctuidae). Pest Management Science 73: 575-581. DOI: 10.1002/ps.4334.

Bird, L.J., L.J. Drynan and P.W. Walker. 2017. The use of F2 screening for detection of resistance to emamectin benzoate, chlorantraniliprole and indoxacarb in Australian populations of Helicoverpa armigera (Lepidoptera: Noctuidae). Journal of Economic Entomology 110: 651-659.

Industry Updates & Technical Reports

Bird, L., D. Larsen, S. Downes, L. Wilson, M. Miles & T. Leven. 2014. Insecticide Resistance Management Strategy (IRMS) for 2014/15. In Maas S. (Ed) Cotton Pest Management Guide 2014/15, pages 60-69.

Downes, S. and L. Bird. 2015. Helicoverpa resistance monitoring end of season 2014/15 report to Crop Consultants Australia

Bird, L., D. Larsen, S. Downes, L. Wilson, M. Miles & T. Leven. 2015. Insecticide Resistance Management Strategy (IRMS) for 2015/16. In Maas S. (Ed) Cotton Pest Management Guide 2015/16, pages 57-64.

Downes, S. and L. Bird. 2016. Helicoverpa resistance monitoring end of season 2015/16 report to Crop Consultants Australia.

Bird, L., M. Miles, G. Baker, O. Edwards, S. Powles, A. Hoffmann, G. McDonald and P. Umina. 2017 (in prep). Science behind the resistance management strategy for Helicoverpa armigera in Australia. GRDC National Insecticide Resistance Management working group. Grains Research and Development Corporation.

Umina, P., G. Baker, L. Bird, O. Edwards, M. Miles, S. Powles, C. Preston, C. Lopez-Ruiz, F. Van de Wouw, A. Milgate, G. McDonald and A. Hoffmann. 2017 (in prep). Why do resistance management strategies differ so markedly for insecticides, herbicides and fungicides?

McDonald, G., P. Umina, L. Bird, O. Edwards, S. Powles, M. Miles, G. Baker and A. Hoffmann. 2017 (in prep). Pesticide mixtures and resistance management.

Conferences

Bird, L.J. 2014. Revised Methods for Monitoring Resistance to Conventional Insecticides. 17th Australian Cotton Conference, Brisbane, QLD.

Bird, L.J. 2015. Characteristics of indoxacarb resistance in Australian populations of Helicoverpa armigera. 2nd Australian Cotton Research Conference. Toowoomba, QLD.

Chen, Y., L.K. Woolly, K.L. Langfield, L.J. Bird and G.A. Herron. 2017. Linkage mapping of an indoxacarb resistance gene isolated from a field population of Helicoverpa armigera via genotype-by-sequencing. 3rd Australian Cotton Research Conference. Canberra, ACT.

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Summary of key outcomes and achievements

1. ***Delivery of resistance surveillance outcomes to industry***

   Five years of insecticide resistance surveillance completed. Annual results reported to TIMS technical panel and disseminated to industry.

2. ***Implementation of new and innovative methods of sampling***

   Introduction of pheromone trapping increased operational efficiencies and resulted in substantive improvements in quality and rigour of resistance surveillance outcomes, particularly for improving capacity to interpret regional data.

3. ***Characterisation of indoxacarb resistance in H. armigera***

   Quantitative genetic analysis of indoxacarb resistance found resistance was conferred by an incompletely dominant, autosomal trait. Inhibition studies found the mechanism of resistance was associated with metabolic detoxification and does not confer cross resistance to other registered Helicoverpa insecticides

4. ***Assessment of relative fitness in indoxacarb resistance in H. armigera***

   No major cost was associated with survival, development time or female reproductive potential in indoxacarb resistant *H. armigera*. However, small costs associated with male reproductive capacity and survival in overwintering conditions may contribute to reducing resistance frequency in regions where diapause occurs.

5. ***Molecular basis of indoxacarb resistance in H. armigera identified***

   The indoxacarb resistance gene was mapped to chromosome 16 within a 2.5MB region (HaChr16:6083884- 8558542) with two closely linked genes, *IndoR1* and *IndoR2*, shown to have high association with resistance. This region includes a cluster of nine *CYP6AE* genes which encode for detoxification enzymes. In particular, *CYP6AE17* and *CYP6AE18* are known to metabolise indoxacarb by recombinant P450s and are considered to be the putative detoxifiers.

6. ***Accumulation of baseline dose-response data for selective insecticides in H. punctigera***

   Protocols for resistance surveillance of indoxacarb, chlorantraniliprole and emamectin benzoate were developed and discriminating concentrations for each insecticide were established as a pre-emptive step in resistance management in *H. punctigera*.

7. ***Established cross-resistance patterns between Vip3A and conventional insecticides***

   A lack of cross-resistance between Vip3A resistant phenotypes and conventional insecticides suggests that increasing field frequencies of SP477-type Vip3A resistance in *H. armigera* and Hp4-48-type Vip3A resistance in *H. punctigera* is unlikely to increase the resistance risk for other insecticides.

8. ***Development of H. armigera RMS in grains***

   The development of a resistance management strategy for *H. armigera* in the grains sector was coordinated by the project leader through membership of the National Insecticide Resistance Management (NIRM) working group. The development of a draft strategy was completed in October 2017 and endorsed by CropLife Australia in November 2017. The strategy was subsequently approved for public release by GRDC in April 2018 and formally launched in May 2018

List of Publications

Published articles (see Appendix 5)

1. Chen Y, Bird L, Woolly L, Langfield K, Walsh T, Gordon K and Herron G (2019) Linkage mapping an indoxacarb resistance gene in *Helicoverpa armigera* (Lepidoptera: Noctuidae) by genotype-by sequencing. *Pest Management Science.* doi:10.1002/ps.5557
2. Bird L, Walker P and Drynan L (2019) Insecticide resistance in Helicoverpa armigera: update and implications for management 2018. Northern NSW Research Results. https://www.dpi.nsw.gov.au/data/assets/pdf_file/0011/1022024/NRR-2018-web.pdf
3. Bird LJ and Walker PW (2018) Baseline susceptibility of *Helicoverpa punctigera* (Wallengren) (Lepidoptera: Noctuidae) to indoxacarb, emamectin benzoate and chlorantraniliprole. *Journal of Economic Entomology* doi:10.1093/jee/toy389
4. Walsh TK, Joußen N, Tian KT, McGaughran A, Anderson CJ, Qiu X, Ahn SJ, Bird LJ, Pavlidi N, et al. (2018) Multiple recombination events between two cytochrome P450 loci contribute to global pyrethroid resistance in *Helicoverpa armigera*. *Plos One* 13(11): e0197760
5. Bird LJ (2018) Pyrethroid and carbamate resistance in Australian *Helicoverpa armigera* (Lepidoptera: Noctuidae) 2008-2015: what has changed since the introduction of Bt cotton? *Bulletin of Entomological Research* doi:10.1017/S0007485317001316
6. Bird LJ, Drynan LJ and Walker PW (2017) The use of F_2 screening for detection of resistance to emamectin benzoate, chlorantraniliprole and indoxacarb in Australian populations of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Journal of Economic Entomology* 110:651-659
7. Bird LJ (2017) Genetics, cross-resistance and synergism of indoxacarb resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Pest Management Science* 73:575-581
8. Bird LJ (2016) Susceptibility of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) to cyantraniliprole determined from topical and ingestion bioassay. *Journal of Economic Entomology* 109:1350-1356
9. Bird LJ (2015) Susceptibility of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) to indoxacarb, emamectin benzoate and chlorantraniliprole in Australia. *Journal of Economic Entomology* 108:294-300

Manuscripts submitted for peer-review (see Appendix 5)

1. Bird LJ, Drynan LJ and Walker PW. Fitness and stability of indoxacarb-resistant and susceptible *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Journal of Pest Science*

Conference Papers

1. Chen Y, Woolly LK, Langfield KL, Bird LJ and Herron GA (2017) Linkage mapping of an indoxacarb resistance gene isolated from a field population of *Helicoverpa armigera* via genotype-by-sequencing. 3rd Australian Cotton Research Conference, Canberra ACT
2. Bird LJ (2015) Characteristics of indoxacarb resistance in Australian populations of *Helicoverpa armigera*. In: Proceedings of the 2nd Australian Cotton Research Conference, Toowoomba QLD
3. Bird LJ (2014) Revised Methods for Monitoring Resistance to Conventional Insecticides. In: Proceedings of the 17th Australian Cotton Conference, Brisbane QLD

Part 4 – Final Report Executive Summary

Insecticide resistance results in reduced product efficacy and represents a major cost to agricultural production in terms of economic, environmental and social consequences. The cotton bollworm, *Helicoverpa armigera* is notoriously difficult to manage because of its capacity to develop resistance to a wide range of insecticides. Resistance risk in *H. armigera* is a major concern in mixed production systems that provide a range of hosts for this pest, and is a key stewardship issue shared by the cotton and grains industries. The *Helicoverpa* spp. insecticide resistance surveillance program has implemented highly efficient and sensitive methodologies to deliver scientifically measurable outcomes for supporting industry sustainability by reducing resistance risk associated with insecticide use.

Resistance surveillance and associated research underpins strategic response to emerging resistance issues. Outcomes from this project have been essential for informing a broader process of formulating the Insecticide Resistance Management Strategy (IRMS) used primarily by the cotton industry. This is a key industry pathway for delivering information and recommendations incorporating all pest species and all registered products. However, since the expansion of the pulse industry in eastern Australia in the early 2000's, the grains industry has become a major user of Helicoverpa insecticides, and the cotton IRMS may not be sufficiently effective for managing resistance risk in insecticides utilized to target *H. armigera* across multiple commodities. Moreover, risk is elevated due to ecological factors unique to northern populations which will also favour resistance selection. This could have important implications for long-term sustainability of these products in cotton and highlights the need for a cross-industry approach that promotes stewardship of key Helicoverpa insecticides.

To support resistance management in grains, a resistance management strategy (RMS) for *H. armigera* specifically designed for grains crops was released in April 2018. A key aspect of its development was industry-wide consultation with leading growers and advisors in the cotton and grains industries which highlighted regional concerns about resistance risk due to product overuse in pulse crops. However, there is little value in industry adoption of an RMS without an ongoing monitoring program to support the strategy. Therefore project expansion involving targeted surveillance in northern grains regions provided regionally-specific information for quantifying resistance frequency with benefits to the cotton industry through improved preparedness.

While the cotton and grains RMS's are critical for pre-emptive management of risk factors at the field level, characterisation of the causal factors underlying resistance is also an important predictive tool for future-proofing Helicoverpa insecticides and has been a key focus of this project. For example, the isolation and quantitative genetic analysis of indoxacarb resistance in *H. armigera* has been a key outcome from this project and was also central for developing collaborations resulting in elucidation of a putative molecular mechanism of resistance to this insecticidal class.

Importantly, while molecular technologies in resistance diagnostics will continue to improve, field-based surveillance by bioassay is currently the mainstay of resistance programs because it provides a direct measure of resistance frequency regardless of the molecular basis of resistance. This is important because the limit of detection of new or novel mechanisms from field-based bioassay is high, even if these mechanisms have not been previously identified and/or characterised. Therefore, continued surveillance and research to increase our understanding of emerging field resistance mechanisms will be critical to ensuring that industries retain efficacy of as many rotational options as possible.

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Appendix 1

Five year summary of *Helicoverpa* spp. collections from all regions

Table A.1.1 Annual collections of eggs and larvae

Regional reference	Date Collected	Location	Crop	No. Eggs	No. Larvae	Eggs + Larvae	Total Hatched	Total *Ha*	Total *Hp*	% *Ha*
Resistance Sampling 2014-2015										
UPPER NAMOI										
UN 1	10/09/2014	Carnarvon	Faba beans	0	69	69	4	0	4	0
UN 2	10/09/2014	Nettletops	Chickpeas	0	9	9	5	0	5	0
UN 3	17/09/2014	Breeza Station	Chickpeas	2	0	2	0	0	0	0
UN 5	21/10/2014	Currawidgen	Maize	78	0	78	40	40	0	100
UN 6	21/10/2014	Warilea	Maize	166	0	166	37	37	0	100
UN 7	28/10/2014	Currawidgen	Maize	69	0	69	58	58	0	100
UN 9	12/11/2014	Yeovil	Maize	297	0	297	214	214	0	100
UN 10	12/11/2014	Breeza Station	Maize	247	0	247	161	161	0	100
UN 11	12/11/2014	Gunnadilly	Maize	116	0	116	87	87	0	100
UN 12	27/11/2014	Gunnadilly	Maize	195	0	195	167	167	0	100
UN 13	27/11/2014	Yeovil	Maize	188	0	188	121	121	0	100
UN 14	27/11/2014	Breeza Station	Maize	192	0	192	142	142	0	100
UN 15	27/11/2014	Shangri-la	Maize	420	0	420	0	0	0	0
UN 16	04/12/2014	Tahlea	Maize	116	0	116	45	45	0	100
UN 17	04/12/2014	Telarah	Maize	198	0	198	142	142	0	100
UN 18	04/12/2014	Argyle	Maize	342	0	342	221	221	0	100
UN20	10/12/2014	Killara Feedlot	Maize	315	0	315	247	247	0	100
UN21	10/12/2014	Fairfield	Maize	275	3	278	272	272	0	100
UN22	10/12/2014	Breeza Station	Maize	275	2	277	205	205	0	100
TOTAL				**3491**	**83**	**3574**	**2168**	**2159**	**9**	**99.6**
GWYDIR										
GY 1	24/09/2014	Bulgate	Chickpeas	0	3	3	0	0	0	0
GY 2	24/09/2014	Inverness	Linseed	0	1	1	1	0	1	0
GY 3	08/10/2014	Paul Slack	Chickpeas	0	217	217	0	0	0	0
GY 4	29/10/2014	Bulgate	Maize	270	0	270	190	190	0	100
GY 5	29/10/2014	Koreen	Maize	219	0	219	197	197	0	100
GY 6	18/11/2014	Koreen	Maize	125	0	125	91	91	0	100
GY 7	18/11/2014	Bulgate	Maize	40	3	43	30	30	0	100
GY 8	20/11/2014	Yambin	Sorghum	130	5	135	102	102	0	100
GY 9	20/11/2014	Yambin	Maize	53	0	53	44	44	0	100
GY 10	20/11/2014	Myola Feedlot	Maize	128	6	134	118	118	0	100
GY 11	03/12/2014	Koreen	Maize	1223	19	1242	932	932	0	100
GY 12	03/12/2014	Bulgate	Maize	100	3	103	41	41	0	100
GY 13	10/12/2014	Yambin	Maize	64	1	65	5	5	0	100
GY 14	10/12/2014	Tumba	Sorghum	33	0	33	19	19	0	100
GY 15	15/01/2015	Top Box	Sorghum	9	1	10	1	1	0	100
TOTAL				**2394**	**259**	**2653**	**1771**	**1770**	**1**	**99.9**

Regional reference	Date Collected	Location	Crop	No. Eggs	No. Larvae	Eggs + Larvae	Total Hatched	Total *Ha*	Total *Hp*	% *Ha*
LOWER NAMOI										
LN 1	06/08/2014	Muckabinya	Faba beans	0	8	8	4	2	2	50.0
LN 2	06/08/2014	Muckabinya	Chickpea	0	1	1	1	0	1	0.0
LN 3	13/08/2014	Cockatoo	Faba beans	0	1	1	2	1	1	50.0
LN 4	14/08/2014	Shenstone	Faba beans	0	6	6	0	0	0	0.0
LN 5	14/08/2014	Wolfgang	Chickpeas	0	5	5	5	2	3	40.0
LN 6	14/08/2014	Goroka	Chickpeas	0	20	20	5	1	4	20.0
LN 7	14/08/2014	Auburn	Faba beans	0	5	5	4	1	3	25.0
LN 8	14/08/2014	Weetawaa	Faba beans	0	22	22	14	3	11	21.4
LN 9	14/08/2014	Hazelwood	Faba beans	0	13	13	7	0	7	0.0
LN 10	03/09/2014	Glenwarrie	Chickpeas	0	11	11	6	0	6	0.0
LN 11	03/09/2014	Tarlee	Chickpeas	0	14	14	6	0	6	0.0
LN 12	03/09/2014	Whites	Chickpeas	0	7	7	4	0	4	0.0
LN 13	03/09/2014	ACRI	Canola	0	3	3	2	0	2	0.0
LN 14	20/09/2014	Junefield	Chickpea	0	16	16	9	0	9	0.0
LN 15	24/09/2014	Mt Pleasant	Chickpeas	0	115	115	35	0	35	0.0
LN 16	05/11/2014	Warrangee	Maize	264	0	264	223	223	0	100
LN 17	02/12/2014	Togo	Maize	122	0	122	104	104	0	100
LN 18	02/12/2014	Togo	Maize	15	36	51	13	13	0	100
LN 19	02/12/2014	Taratan	Bollgard	75	0	75	57	2	55	3.5
LN 20	02/12/2014	Willant	Sorghum	115	0	115	90	90	0	100
LN 21	02/12/2014	Glennlee	Sorghum	231	0	231	205	205	0	100
LN 22	09/12/2014	Mirrabooka	Sorghum	45	20	65	0	0	0	0.0
LN 23	09/12/2014	Junefield	Sorghum	34	44	78	0	0	0	0.0
LN 24	09/01/2015	ACRI	Maize	136	44	180	0	0	0	0.0
LN 25	20/01/2015	Leitch Block	Conv. cotton	23	0	23	9	4	5	44.4
LN 26	21/01/2015	Togo	Bollgard	39	0	39	31	15	16	48.4
LN 27	21/01/2015	ACRI	Conv. cotton	39	0	39	28	13	15	46.4
LN 28	04/02/2015	Leitch Block	Conv. cotton	36	0	36	15	10	5	66.7
LN 29	06/02/2015	Mollee	Conv. cotton	64	0	64	49	4	45	8.2
LN 30	06/02/2015	Belah	Pigeon pea	201	0	201	189	27	162	14.3
LN 31	07/02/2015	Thalaba	Pigeon pea	44	1	45	30	11	19	36.7
LN 32	09/02/2015	Leitch Block	Pigeon pea	70	2	72	24	5	19	20.8
LN 33	07/02/2015	Carbeen	Sorghum	45	0	45	11	11	0	100
LN 34	18/02/2015	Trevena Lane	Sorghum	30	1	31	12	12	0	100
LN 35	18/02/2015	Carbeen	Sorghum	134	0	134	25	25	0	100
LN 36	18/02/2015	Thalabar	Pigeon pea	10	11	21	7	0	7	0.0
LN 37	25/02/2015	ACRI	Mungbeans	180	3	183	57	54	3	94.7
LN 38	27/02/2015	Auscott	Sorghum	4	1	5	1	1	0	100
LN 39	27/02/2015	Togo	Sorghum	45	0	45	9	9	0	100
TOTAL				**2001**	**410**	**2411**	**1293**	**848**	**445**	**65.6**

Regional reference	Date Collected	Location	Crop	No. Eggs	No. Larvae	Eggs + Larvae	Total Hatched	Total *Ha*	Total *Hp*	% *Ha*
ST GEORGE										
SG1	25/11/2014	Balonne Plains	Bollgard	43	0	43	27	0	27	0
SG2	25/11/2014	Wagaby	Bollgard	14	0	14	12	0	12	0
SG3	25/11/2014	Bookamerrie	Bollgard	18	0	18	13	0	13	0
SG4	26/11/2014	Home Blocks	Bollgard	55	0	55	17	0	17	0
SG5	26/11/2014	Bookamerrie	Bollgard	51	0	51	34	0	34	0
SG6	26/11/2014	Ashwood	Bollgard	42	0	42	28	0	28	0
SG7	05/12/2014	Moolabah	Bollgard	16	0	16	10	0	10	0
SG8	05/12/2014	Home Blocks	Bollgard	123	0	123	96	8	88	8.3
SG9	05/12/2014	Bundoran	Bollgard	51	0	51	32	3	29	9.4
SG10	05/12/2014	Doondi F2	Bollgard	40	0	40	23	0	23	0
SG11	05/12/2014	Bore Pad F 6	Bollgard	40	0	40	34	4	30	11.8
SG12	05/12/2014	Iona F4	Bollgard	84	0	84	75	11	64	14.7
SG13	05/12/2014	Bookamerrie	Bollgard	127	0	127	96	18	78	18.8
SG14	05/12/2014	Iona F2/3	Bollgard	35	0	35	23	3	20	13.0
SG15	05/12/2014	Warrina F4	Bollgard	75	0	75	72	16	56	22.2
SG16	05/12/2014	Wagaby F1	Bollgard	44	0	44	33	1	32	3.0
SG17	08/12/2014	Ashwood F5	Bollgard	48	0	48	40	10	30	25.0
SG18	08/12/2014	Ashwood F1b	Bollgard	38	0	38	33	2	31	6.1
SG19	08/12/2014	Ashwood F1a	Bollgard	29	0	29	21	1	20	4.8
SG20	08/12/2014	Warrina F2	Bollgard	41	0	41	0	0	0	0
SG21	08/12/2014	Warrina F4	Bollgard	60	0	60	43	3	40	7.0
SG22	08/12/2014	Farm 26/27 F5	Bollgard	30	0	30	24	1	23	4.2
SG23	08/12/2014	Bookamerrie F5	Bollgard	42	0	42	11	1	10	9.1
SG24	08/12/2014	Bundoran F4	Bollgard	95	0	95	64	2	62	3.1
SG25	08/12/2014	Doondi F2	Bollgard	44	0	44	40	0	40	0
SG26	08/12/2014	Wagaby F1a	Bollgard	24	0	24	24	0	24	0
SG27	08/12/2014	Wagaby F5	Bollgard	49	0	49	25	1	24	4.0
SG28	08/12/2014	Kurray F1/ 7	Bollgard	30	0	30	29	0	29	0
SG29	08/12/2014	Balonne Plains	Bollgard	44	0	44	33	3	30	9.1
SG30	08/12/2014	Iona - F2 &3	Bollgard	14	0	14	9	0	9	0
SG31	17/12/2014	Bundoran F2	Bollgard	181	0	181	119	27	92	22.7
SG32	15/12/2014	Wagaby F5	Bollgard	59	0	59	44	5	39	11.4
SF33	15/12/2014	Wagaby F1a	Bollgard	47	0	47	40	6	34	15.0
SG34	16/12/2014	Iona field 2/3	Bollgard	54	0	54	36	7	29	19.4
SG35	15/12/2014	Bookamerrie F5	Bollgard	35	0	35	15	1	14	6.7
SG36	15/12/2014	Balonne Plains	Bollgard	133	0	133	72	3	69	4.2
SG37	15/12/2014	Ashwood	Bollgard	50	0	50	26	3	23	11.5
SG42	06/01/2015	Wagaby	Bollgard	8	0	8	0	0	0	0
SG43	05/01/2015	Moolabah F6/7	Bollgard	37	0	37	29	14	15	48.3
SG44	05/01/2015	Warrina F1/2	Bollgard	31	0	31	18	10	8	55.6
SG45	05/01/2015	Balonne Plains F2	Bollgard	60	0	60	33	17	16	51.5
SG46	05/01/2015	Home Blocks F1	Bollgard	30	0	30	13	10	3	76.9
SG47	05/01/2015	Balonne Plains	Pigeon pea	0	24	24	0	0	0	0
SG48	05/01/2015	Doondi F2	Pigeon pea	0	29	29	7	7	0	100
SG49	13/01/2015	Bullamon Plains	Pigeon pea	0	18	18	6	2	4	33.3

SG50	13/01/2015	Home Blocks	Pigeon pea	0	11	11	0	0	0	0
SG51	13/01/2015	Ashwood	Pigeon pea	0	16	16	10	4	6	40.0
SG52	13/01/2015	Bullamon Plains F2	Pigeon pea	0	9	9	0	0	0	0
SG54	20/01/2015	Farm 26/27	Pigeon pea	0	15	15	9	0	9	0

Regional reference	Date Collected	Location	Crop	No. Eggs	No. Larvae	Eggs + Larvae	Total Hatched	Total *Ha*	Total *Hp*	% *Ha*
SG55	20/01/2015	Bullamon Plains	Pigeon pea	0	20	20	20	1	19	5.0
SG56	19/01/2015	Kurray	Pigeon pea	0	2	2	2	0	2	0
SG57	19/01/2015	Doondi F2	Pigeon pea	0	26	26	0	0	0	0
SG58	19/01/2015	Ashwood	Pigeon pea	0	12	12	4	0	4	0
SG59	27/01/2015	Bundoran	Pigeon pea	0	26	26	1	0	1	0
SG60	27/01/2015	UT Downs	Pigeon pea	0	14	14	5	0	5	0
SG61	27/01/2015	Wagaby	Pigeon pea	0	7	7	2	0	2	0
SG62	02/02/2015	Doondi F2	Pigeon pea	0	21	21	14	14	0	100
SG63	04/02/2015	Kurray	Pigeon pea	0	10	10	7	4	3	57.1
SG64	06/02/2015	Balonne Plains	Pigeon pea	0	71	71	0	0	0	0
SG65	09/02/2015	UT Downs	Pigeon pea	0	28	28	0	0	0	0
TOTAL				**2171**	**359**	**2530**	**1553**	**223**	**1330**	**14.4**
EMERALD										
EM1	10/10/2014	Tyson Blk 6	Bollgard	63	0	63	13	7	6	53.8
EM2	14/10/2014	Farm 149	Bollgard	175	0	175	0			
EM3	23/10/2014	Tiss Farm	Bollgard	83	0	83	32	19	13	59.4
EM4	05/11/2014	Brownie Blk 6	Bollgard	282	0	282	96	71	25	74.0
EM5	14/11/2014	FiFi	Bollgard	127	0	127	0			
EM6	26/11/2014	Blk 5	Bollgard	75	0	75	15	9	6	60.0
EM7	08/12/2014	Killara	Bollgard	389	0	389	240	234	6	97.5
EM8	15/12/2014	Bros F1	Bollgard	50	0	50	26	13	13	50.0
EM9	05/01/2015	Brownie Blk 9	Bollgard	171	0	171	123	119	4	96.7
EM10	12/01/2015	Farm 2	Bollgard	115	0	115	27	26	1	96.3
EM11	16/01/2015	Braemar L5B	Bollgard	76	0	76	9	7	2	77.8
EM13	24/03/2015	Tyson Blk 3	Mungbeans	366	0	366	21	21	0	100.0
EM14	07/04/2015	Sanders	Mungbeans	118	0	118	0			
TOTAL				**2090**	**0**	**2090**	**602**	**526**	**76**	**87.4**
MACINTYRE										
GN1	12/11/2014	Undabri	Maize	75	0	75	56	56	0	100
GN2	12/11/2014	Wondoogle	Sorghum	11	0	11	9	9	0	100
GN3	09/12/2014	Undabri	Maize	270	0	270	25	25	0	100
GN4	10/12/2014	Eumorella	Bollgard	43	0	43	27	3	24	11.1
GN5	16/01/2015	Carrington Farm	Bollgard	50	0	50	36	15	21	41.7
GN6	16/01/2015	Sylvan Plains	Conv. cotton	49	0	49	34	18	16	52.9
GN7	15/01/2015	Tocal	Pigeon pea	21	0	21	15	7	8	46.7
GN8	16/01/2015	Carrington Farm	Pigeon pea	133	7	140	93	64	29	68.8
GN9	11/02/2015	Morella	Pigeon pea	38	25	63	34	23	11	67.6
GN10	11/02/2015	Royston	Pigeon pea	48	52	100	33	25	8	75.8
GN11	10/02/2015	Sth Callondoon	Mungbeans	33	10	43	0	0	0	0
GN12	11/02/2015	Parella	Sorghum	270	0	270	182	182	0	100
GN13	11/02/2015	Undabri	Sorghum	295	2	297	143	143	0	100
GN14	11/02/2015	Eurmorella	Bollgard	24	0	24	18	3	15	16.7

TOTAL				**1360**	**96**	**1456**	**705**	**573**	**132**	**81.3**

Regional reference	Date Collected	Location	Crop	No. Eggs	No. Larvae	Eggs + Larvae	Total Hatched	Total *Ha*	Total *Hp*	% *Ha*
DARLING DOWNS										
DD1	11/11/2014	Tyunga	Maize	69	0	69	13	13	0	100
DD2	25/11/2014	Tyunga	Maize	40	0	40	22	22	0	100
DD3	02/12/2014	Kensington	Bollgard	99	0	99	68	7	61	10.3
DD4	02/12/2014	Tyunga	Bollgard	67	0	67	48	14	34	29.2
DD5	06/01/2015	Condamine Plains	Bollgard	180	0	180	45	43	2	95.6
DD6	06/01/2015	Booboogan	Bollgard	97	0	97	46	45	1	97.8
DD7	20/01/2015	Uralla F7	Bollgard	99	0	99	36	36	0	100
DD8	26/01/2015	Yarramolong	Conv. cotton	24	0	24	2	0	2	0
DD9	02/02/2015	Condamine Plains	Bollgard	50	0	50	1	1	0	100
DD10	03/02/2015	Tarana	Mungbeans	55	0	55	36	36	0	100
DD11	03/02/2015	Walgoona Bank	Bollgard	126	0	126	30	10	20	33.3
DD12	03/02/2015	Broadlea West	Bollgard	90	0	90	13	5	8	38.5
DD13	05/02/2015	Walgoona Dam	Bollgard	95	0	95	30	11	19	36.7
DD14	16/02/2015	QDAF collections	Maize	0	24	24	0	0	0	0
DD15	16/02/2015	QDAF collections	Mungbeans	0	47	47	0	0	0	0
DD16	16/02/2015	QDAF collections	Mungbeans	0	49	49	0	0	0	0
DD17	16/02/2015	QDAF collections	Mungbeans	0	26	26	0	0	0	0
DD18	16/02/2015	QDAF collections	Pigeon pea	0	43	43	0	0	0	0
DD19	25/02/2015	Millmerran	Mungbeans	12	0	12	0	0	0	0
DD20	09/03/2015	Condamine Plains	Maize	0	44	44	0	0	0	0
DD21	09/03/2015	West Prairie Rd	Maize	0	51	51	0	0	0	0
DD22	16/03/2015	Tim Thorn Allora	Maize	0	44	44	0	0	0	0
DD23	11/03/2015	Kingaroy QDAF RS	Maize	0	46	46	0	0	0	0
DD24	16/03/2015	Gatton-Clifton Rd	Maize	0	35	35	0	0	0	0
DD25	16/04/2015	Killarney Rd	Maize	0	45	45	0	0	0	0
DD26	16/04/2015	Kingsthorpe Rd	Maize	0	46	46	0	0	0	0
DD27	16/04/2015	Pittsworth Rd	Maize	0	13	13	0	0	0	0
TOTAL				**1103**	**513**	**1616**	**390**	**243**	**147**	**62.3**
GRIFFITH										
MIA1	04/11/2014	Farm 15	Bollgard	57	0	57	51	0	51	0
MIA2	28/11/2014	Farm 157	Bollgard	24	0	24	17	0	17	0
MIA3	08/12/2014	Huddersfield	Bollgard	161	0	161	60	0	60	0
MIA4	13/01/2015	Eurolibah	Bollgard	0	0	0	0	0	0	0
MIA6	10/02/2015	Mundora	Pigeon pea	0	0	0	0	0	0	0

MIA7	28/01/2015	Kulki	Pigeon pea	0	0	0	0	0	0	0
MIA8	02/02/2015	Wingbadge	Pigeon pea	0	5	5	5	5	0	100
MIA9	23/02/2015	Kera	Pigeon pea	0	6	6	6	6	0	100
MIA10	24/02/2015	Weir	Pigeon pea	0	40	40	1	0	1	0
TOTAL				**242**	**51**	**293**	140	11	129	**7.9**

Regional reference	Date Collected	Location	Crop	No. Eggs	No. Larvae	Eggs + Larvae	Total Hatched	Total *Ha*	Total *Hp*	% *Ha*
MUNGINDI										
MUN1	11/11/2014	Yarramildi	Maize	53	0	53	43	43	0	100
MUN2	11/11/2014	Riverside	Maize	32	0	32	28	28	0	100
MUN3	10/12/2014	Tarrawatta	Conv. cotton	143	0	143	59	8	51	13.6
MUN4	09/12/2014	Yarramildi	Maize	360	3	363	34	33	1	97.1
MUN5	09/12/2014	Riverside	Maize	45	0	45	10	10	0	100
MUN6	12/12/2014	Riverside	Maize	152	10	162	43	43	0	100
MUN7	06/01/2015	Tarrawatta	Conv. cotton	525	0	525	325	180	145	55.4
MUN8	06/01/2015	Worral Creek	Bollgard	180	0	180	121	43	78	35.5
MUN9	06/01/2015	Yarramildi	Bollgard	112	0	112	57	24	33	42.1
MUN10	07/01/2015	Strathgyle	Bollgard	125	0	125	97	26	71	26.8
MUN11	15/01/2015	Sth Bunarba	Conv. cotton	8	0	8	6	1	5	16.7
MUN12	15/01/2015	Wyadrigah	Sorghum	5	10	15	5	5	0	100
MUN13	15/01/2015	Wyadrigah	Pigeon pea	98	7	105	17	12	5	70.6
MUN14	27/01/2015	Tarrawatta	Conv. cotton	90	0	90	23	19	4	82.6
MUN15	27/01/2015	Worral Creek	Bollgard	150	0	150	107	61	46	57.0
MUN16	27/01/2015	Strathgyle	Bollgard	450	0	450	280	172	108	61.4
MUN17	10/02/2015	Boston	Pigeon pea	84	2	86	66	14	52	21.2
MUN18	10/02/2015	Yarramildi	Pigeon pea	62	17	79	25	12	13	48.0
MUN19	16/01/2015	Strathgyle	Bollgard	83	0	83	52	32	20	61.5
TOTAL				**2757**	**49**	**2806**	**1398**	**766**	**632**	**54.8**
MACQUARIE										
MQ 1	26/11/2014	Mallawa	Maize	6	0	6	4	4	0	100
MQ 2	25/11/2014	Cowal Park	Sweet corn	104	0	104	67	67	0	100
MQ 3	16/12/2014	Auscott	Bollgard	17	0	17	15	3	12	20.0
MQ 4	16/12/2014	Mallawa	Maize	92	0	92	59	59	0	100
MQ 5	16/12/2014	Nundah	Bollgard	7	0	7	4	0	4	0
MQ 6	17/12/2014	Lombard Farm	Bollgard	93	0	93	75	16	59	21.3
MQ 7	17/12/2014	Lombard Farm	Pigeon pea	34	0	34	30	3	27	10.0
MQ 8	17/12/2014	Agriland	Bollgard	190	0	190	148	15	133	10.1
MQ 9	29/01/2015	Pippagitta	Pigeon pea	150	0	150	108	2	106	1.9
MQ 10	29/01/2015	Karamea	Maize	52	0	52	41	41	0	100
MQ 11	29/01/2015	Nundah	Bollgard	133	0	133	93	36	57	38.7
MQ 12	28/01/2015	Boomanulla	Bollgard	12	0	12	0	0	0	0
MQ 13	28/01/2015	Boomanulla	Pigeon pea	54	3	57	0	0	0	0
MQ 14	28/01/2015	Wilawa	Pigeon pea	125	0	125	0	0	0	0
MQ 15	29/01/2015	Pippagitta	Bollgard	10	0	10	0	0	0	0
MQ 16	24/02/2015	Kiewa	Maize	307	3	310	36	36	0	100
MQ 17	25/02/2015	Auscott	Pigeon pea	31	6	37	28	7	21	25.0
MQ 18	24/02/2015	Cowal Park	Sweet corn	10	1	11	5	5	0	100
MQ 19	25/02/2015	Auscott	Bollgard	10	0	10	7	2	5	28.6

TOTAL				**1437**	**13**	**1450**	**720**	**296**	**424**	**41.1**
KUNUNURRA										
KUN1	14/7/14	NR	Chia		63	63	63	5	58	7.9
KUN2	28/7/14	NR	Chickpeas		53	53	17	17	0	100
KUN3	28/7/14	NR	Chickpeas		90	90	46	46	0	100
KUN4	14/8/14	NR	Bollgard	49		49	18	7	11	38.9
KUN5	14/8/14	NR	Tomatoes	19		19	8	7	1	87.5
TOTAL				**68**	**206**	**274**	**152**	**82**	**70**	**53.9**

Regional reference	Date Collected	Location	Crop	No. Eggs	No. Larvae	Eggs + Larvae	Total Hatched	Total *Ha*	Total *Hp*	*% Ha*
Resistance Sampling 2015-2016										
UPPER NAMOI										
UN 1	13/10/2015	Warilea	Chickpea	0	35	35	11	0	11	0.0
UN 2	13/10/2015	Warilea	Maize	87	0	87	75	75	0	100.0
UN 3	13/10/2015	Currawidgen	Maize	25	0	25	20	20	0	100.0
UN 4	14/10/2015	Fairfield	Chickpea	0	25	25	15	2	13	13.3
UN 5-6	14/10/2015	Breeza Station	Chickpea/fabas	0	15	15	13	2	11	15.4
UN 7	14/10/2015	Nullabean	Chickpea	0	29	29	20	13	7	65.0
UN 8	20/10/2015	Tallara	Linseed	0	237	237	224	0	224	0.0
UN 9	20/10/2015	Nuckledoon	Chickpea	0	7	7	5	2	3	40.0
UN 10	28/10/2015	Currawidgen	Maize	285	0	285	219	219	0	100.0
UN 11	12/11/2015	Warilda	Maize	373	0	373	230	230	0	100.0
UN 12	12/11/2015	Breeza Station	Maize	423	0	423	321	321	0	100.0
UN 13	12/11/2015	Shangri-la	Maize	289	0	289	238	238	0	100.0
UN 14	12/11/2015	Baan Baa Roadside	Twiggy	468	35	503	165	204	3	123.6
UN 15	17/11/2015	Fairfield	Maize	163	0	163	139	139	0	100.0
UN 16	17/11/2015	Waverley	Maize	81	1	82	65	65	0	100.0
UN 17	17/11/2015	Glen Ayr	Maize	174	0	174	156	156	0	100.0
UN 18	17/11/2015	Glenkeith	Sorghum	110	0	110	68	68	0	100.0
UN 19-20	25/11/2015	Currawidgen	Maize	299	0	299	141	141	0	100.0
UN21	25/11/2015	Collie Blue	Maize	176	0	176	107	107	0	100
UN22	25/11/2015	Premier Road	Twiggy	49	50	99	67	67	0	100
UN23	02/12/2015	Shangri-la	Maize	180	0	180	153	153	0	100
UN24	02/12/2015	Breeza Station	Maize	65	0	65	40	40	0	100
UN25	02/12/2015	Shangri-la	Sorghum	110	0	110	74	74	0	100
UN26	02/12/2015	Tremayne	Sorghum	29	3	32	7	7	0	100
UN27	16/12/2015	Glen Ayr	Maize	162	0	162	127	127	0	100
UN28	16/12/2015	Waverley	Maize	535	0	535	153	153	0	100
UN29	16/12/2015	Fairfield	Maize	230	0	230	83	83	0	100
UN30	16/12/2015	Breeza Station	Maize	123	0	123	82	82	0	100
UN31	21/12/2015	Marlo	Sorghum	205	3	208	108	108	0	100
UN32	21/12/2015	Tallara	Maize	175	0	175	144	144	0	100
UN33	21/12/2015	Tahlea	Maize	108	0	108	82	82	0	100
UN34	05/01/2016	Undoolya	Pigeon pea	32	0	32	17	0	17	0
UN35	05/01/2016	Brigadoon	Bollgard	197	0	197	77	16	61	20.8
UN36	05/01/2016	The Willows	Bollgard	310	0	310	162	24	138	14.8
UN37	05/01/2016	The Willows	Pigeon pea	304	0	304	207	10	197	4.8
UN38	12/01/2016	Shangri-la	Pigeon pea	53	2	55	33	12	21	36.4
UN39	12/01/2016	DPI Breeza	Maize	15	4	19	4	4	0	100
UN40	12/01/2016	Spring View	Conv. cotton	88	0	88	35	4	31	11.4

UN41	12/01/2016	Breeza Station	Sorghum	45	0	45	11	11	0	100
UN42	12/01/2016	Gabo	Sorghum	40	0	40	26	26	0	100
UN43	12/01/2016	Warilda	Sorghum	98	0	98	2	2	0	100
UN44	20/01/2016	Battery Hill	Pigeon pea	178	0	178	127	59	68	46.5
UN45	20/01/2016	Breeza Station	Sorghum	100	0	100	22	22	0	100
UN46	20/01/2016	Glengowrie	Sorghum	4	2	6	1	1	0	100
UN47	20/01/2016	Battery Hill	Sorghum	65	0	65	23	23	0	100
UN48	02/02/2016	Argyle	Pigeon pea	56	11	67	52	13	29	25.0
UN49	02/02/2016	Argyle	Bollgard	40	0	40	3	0	3	0
UN50	02/02/2016	Kooiyong	Sorghum	390	0	390	208	208	0	100
UN51	02/02/2016	Carnarvon	Sorghum	261	0	261	98	98	0	100

Regional reference	Date Collected	Location	Crop	No. Eggs	No. Larvae	Eggs + Larvae	Total Hatched	Total *Ha*	Total *Hp*	% *Ha*
UN52	02/02/2016	Tallara	Sorghum	92	0	92	10	10	0	100
UN53	02/02/2016	Gli-Don	Sorghum	360	0	360	123	123	0	100
UN54	11/02/2016	Shangri-la	Pigeon pea	0	38	38	38	30	8	78.9
UN55	11/02/2016	Denistone	Pigeon pea	72	2	74	35	17	18	48.6
UN56	11/02/2016	Breeza Station	Sorghum	135	0	135	22	22	0	100
UN57	11/02/2016	Denistone	Sorghum	245	0	245	16	16	0	100
UN58	11/02/2016	Gowangardie	Sorghum	176	3	179	83	83	0	100
UN63	10/03/2016	Red Bobs	Sorghum	0	0	0	0	0	0	0
UN64	10/03/2016	Fairwinds	Sorghum	0	0	0	0	0	0	0
UN65	10/03/2016	Drummonds	Sorghum	0	0	0	0	0	0	0
UN66	10/03/2016	Tahlea	Maize	67	0	67	4	4	0	100
UN70	27/01/2016	TAI	Sorghum	9	78	87	24	24	0	100
UN71	27/01/2016	Draytons	Sorghum	0	23	23	22	22	0	100
UN72	27/01/2016	Breeza Station	Sorghum	7	26	33	21	21	0	100
TOTAL				**8363**	**629**	**8992**	**4858**	**4027**	**863**	**83.1**
GWYDIR										
GY 1	21/10/2015	Wonga	Maize	123	0	123	94	92	2	97.9
GY 2	21/10/2015	Coffin Hill	Maize	150	0	150	108	106	2	98.1
GY 3	21/10/2015	Koreen	Maize	135	0	135	79	79	0	100
GY 4	11/11/2015	Beefwood	Maize	164	0	164	109	109	0	100
GY 5	15/11/2015	Tullin Tulla	Maize	316	0	316	120	120	0	100
GY 6	15/11/2015	Myola Feedlot	Maize	53	0	53	31	31	0	100
GY 7	01/12/2015	Wonga	Maize	76	10	86	31	31	0	100
GY 8	01/12/2015	Coffin Hill	Maize	6	1	7	3	3	0	100
GY 9	01/12/2015	Koreen	Maize	10	1	11	4	4	0	100
GY 10	01/12/2015	Wonga	Sorghum	10	0	10	8	8	0	100
GY 11	01/12/2015	Gurley Station	Sorghum	13	1	14	2	2	0	100
GY 12	01/12/2015	Bulgate	Sorghum	99	0	99	26	26	0	100
GY 13	21/01/2016	Merrinbula	Conv. Cotton	63	0	63	21	0	21	0
GY 14	21/01/2016	Red Mill	Pigeon Pea	43	0	43	20	3	17	15.0
GY 15	21/01/2016	Glen Prairie	Pigeon Pea	55	0	55	33	7	26	21.2
TOTAL				**1316**	**13**	**1329**	**689**	**621**	**68**	**90.1**
LOWER NAMOI										
LN 1	29/09/2015	Yallambee	Chickpeas	0	22	22	15	1	14	6.7
LN 2	29/09/2015	Walma	Chickpeas	0	58	58	18	16	2	88.9
LN 3	29/09/2015	Merrylands	Chickpeas	0	185	185	54	17	37	31.5
LN 4	09/10/2015	Trevena Lane	Chickpeas	0	21	21	5	4	1	80.0

LN 5	09/10/2015	Stoltenberg Lane	Chickpeas	0	65	65	22	6	16	27.3
LN 6	09/10/2015	Junefield	Chickpeas	0	73	73	19	5	14	26.3
LN 7	30/10/2015	Warrangee	Maize	293	0	293	200	200	0	100
LN 8	18/11/2015	The Wilgas	Sorghum	384	1	385	181	181	0	100
LN 9	23/11/2015	Mirrabooka	Sorghum	75	1	76	27	27	0	100
LN 10	23/11/2015	Togo	Sorghum	42	1	43	57	57	0	100
LN 11	25/11/2015	Warrangee	Maize	175	0	175	123	123	0	100
LN 12	25/11/2015	Warrangee	Maize	180	0	180	115	115	0	100
LN 13	25/11/2015	Old Weetawah	Sorghum	178	0	178	123	123	0	100
LN 14	01/12/2015	The Wilgas	Sorghum	4	0	4	2	2	0	100
LN 15	15/12/2015	Federation Farm	Conv. cotton	23	68	91	37	5	32	13.5
Regional reference	**Date Collected**	**Location**	**Crop**	**No. Eggs**	**No. Larvae**	**Eggs + Larvae**	**Total Hatched**	**Total *Ha***	**Total *Hp***	**% *Ha***
LN 16	15/12/2015	Leitch Block	Sorghum	333	0	333	18	18	0	100
LN 18	08/01/2016	Taratan	Pigeon pea	270	0	270	217	113	104	52.1
LN 19	08/01/2016	Myalla	Soya beans	20	0	20	13	10	3	76.9
LN 20	08/01/2016	Auburn	Bollgard	144	0	144	83	14	69	16.9
LN 21	08/01/2016	Mirrambeena	Pigeon pea	545	0	545	372	8	364	2.2
LN 22	08/01/2016	Taratan	Bollgard	58	0	58	28	3	25	10.7
LN 23	19/01/2016	Cumberdeen	Pigeon pea	48	0	48	36	17	19	47.2
LN 24	19/01/2016	Little Mollee	Pigeon pea	107	0	107	69	21	48	30.4
LN 25	19/01/2016	Athelstone	Bollgard	270	0	270	93	5	88	5.4
LN 26	19/01/2016	Cockatoo	Sorghum	22	0	22	5	5	0	100
LN 27	19/01/2016	Eskdale	Sorghum	9	0	9	3	3	0	100
LN 29	02/02/2016	ACRI	Maize	530	0	530	65	65	0	100
LN 30	02/02/2016	ACRI	Pigeon pea	75	2	77	27	9	18	33.3
LN 31	03/02/2016	Auscott	Pigeon pea	41	4	45	30	14	16	46.7
LN 33	10/02/2016	Wolfgang	Pigeon pea	270	0	270	166	43	123	25.9
LN 34	10/02/2016	Wentworth	Sorghum	107	1	108	28	28	0	100
LN 35	10/02/2016	Beechworth	Pigeon pea	67	5	72	44	17	26	38.6
LN 36	16/02/2016	Apple Trees	Pigeon pea	261	6	267	129	1	128	0.8
LN 38	19/02/2016	Wentworth	Sorghum	132	0	132	37	37	0	100
LN42	23/02/2016	Leitch Block	Conv. cotton	135	4	139	43	15	28	34.9
LN43	24/02/2016	Wentworth	Sorghum	180	0	180	22	22	0	100
LN48	09/03/2016	Glencoe	Mungbeans	44	0	44	26	26	0	100
LN49	09/03/2016	Auscott	Mungbeans	113	0	113	40	40	1	100
TOTAL				**5135**	**517**	**5652**	**2592**	**1416**	**1176**	**54.6**
ST GEORGE										
SG1	07/09/2015	Balonne Plains	Faba beans	0	16	16	14	2	12	14.3
SG2	07/09/2015	Farm 26/27	Chickpeas	0	19	19	18	7	11	38.9
SG3	07/09/2015	Doondi	Chickpeas	0	16	16	15	4	11	26.7
SG4	25-29/08/2015	Balonne/Kurray	Faba beans	0	4	4	4	4	0	100
SG5	25-29/08/2015	Bundaoran/Doondi	Chickpeas	0	23	23	23	23	0	100
SG6	23/11/2015	Farm 26/27 F3	Bollgard	79	0	79	52	0	52	0
SG7	24/11/2015	Brooklyn F135-2	Bollgard	114	0	114	77	1	76	1.3
SG8	24/11/2015	Iona	Bollgard	76	0	76	53	2	51	3.8
SG9	24/11/2015	UT Downs F7	Bollgard	26	0	26	21	0	21	0
SG10	24/11/2015	UT Downs F4	Bollgard	48	0	48	37	2	35	5.4
SG11	24/11/2015	UT Downs F5	Bollgard	66	0	66	56	1	55	1.8
SG12	24/11/2015	UT Downs F6	Bollgard	61	0	61	51	3	48	5.9
SG13	30/11/2015	Farm 26/27 F3	Bollgard	62	0	62	25	0	25	0

SG14	01/12/2015	Iona F2	Bollgard	62	0	62	43	0	43	0
SG15	01/12/2015	Bundoran F1	Bollgard	55	0	55	46	0	46	0
SG16	01/12/2015	Bundoran F 5	Bollgard	46	0	46	39	0	39	0
SG17	30/11/2015	Brookglen 135/2	Bollgard	67	0	67	33	0	33	0
SG18	01/12/2015	Gin Blocks F1	Bollgard	51	0	51	26	1	25	3.8
SG19	01/12/2015	Ashwood	Bollgard	50	0	50	21	0	21	0
SG20	30/11/2015	Kurray F2	Bollgard	36	0	36	16	0	16	0
SG21	01/12/2015	UT Downs F4	Bollgard	46	0	46	29	0	29	0
SG22	30/11/2015	Ashwood	Bollgard	47	0	47	42	0	42	0
SG23	30/11/2015	Clyde	Bollgard	24	0	24	21	0	21	0

Regional reference	Date Collected	Location	Crop	No. Eggs	No. Larvae	Eggs + Larvae	Total Hatched	Total *Ha*	Total *Hp*	% *Ha*
SG24	30/11/2015	Bookamerrie	Bollgard	50	0	50	30	1	29	3.3
SG25	08/12/2015	Ashwood	Bollgard	76	0	76	25	0	25	0
SG26	08/12/2015	Balonne Plains	Bollgard	88	0	88	48	8	40	16.7
SG27	08/12/2015	UT Downs	Bollgard	46	0	46	7	0	7	0
SG28	14/12/2015	Brookglen 135/2	Bollgard	26	0	26	19	3	16	15.8
SG29	14/12/2015	Warrina	Bollgard	17	0	17	10	0	10	0
SG30	15/12/2015	Kurray F2	Bollgard	22	0	22	15	3	12	20.0
SG31	15/12/2015	Bundoran	Bollgard	24	0	24	16	1	15	6.3
SG32	12/01/2016	Ashwood	Pigeon pea	0	26	26	8	4	4	50.0
SG33	12/01/2016	UT Downs	Pigeon pea	0	21	21	13	3	10	23.1
SG34	11/01/2016	Farm 129/12	Bollgard	35	0	35	18	3	15	16.7
SG35	11/01/2016	Bullamon Plains	Bollgard	21	0	21	17	1	16	5.9
SG36	01/02/2016	Kia Ora	Pigeon pea	0	20	20	7	7	0	100
SG37	29/01/2016	Kurray F2	Pigeon pea	0	3	3	0	0	0	0
SG38	09/02/2016	Gin Blocks F1	Pigeon pea	0	13	13	6	5	1	83.3
SG39	09/02/2016	Iona	Pigeon pea	0	25	25	9	8	1	88.9
SG40	15/02/2016	Ashwood	Pigeon pea	0	22	22	4	4	0	100
SG41	15/02/2016	UT Downs	Pigeon pea	0	15	15	12	9	3	75.0
SG42	16/02/2016	Iona	Pigeon pea	0	30	30	23	15	8	65.2
SG43	16/02/2016	Gin Blocks	Pigeon pea	0	25	25	0	0	0	0
SG44	23/02/2016	UT Downs	Pigeon pea	0	14	14	1	1	0	100
SG45	22/02/2016	Bookamerrie	Pigeon pea	0	27	27	12	4	8	33.3
SG46	19/02/2016	Iona	Pigeon pea	0	29	29	9	7	2	77.8
SG47	27/02/2016	Clyde/Dirranbandi	Pigeon pea	0	10	10	6	0	6	0
SG48	29/02/2016	Bookamerrie	Mungbeans	0	8	8	2	1	1	50.0
TOTAL				**1421**	**366**	**1787**	**1079**	**138**	**941**	**12.8**
EMERALD										
EM1	05/10/2015	Farm 129	Bollgard	135	0	135	72	4	68	5.6
EM2	19/10/2015	Deepfields Blk 3	Bollgard	195	0	195	160	46	114	28.8
EM3	09/11/2015	Hampton Blk 4	Bollgard	289	0	289	162	34	128	21.0
EM4	16/11/2015	Farm 149 Blk 1	Bollgard	126	0	126	71	25	46	35.2
EM5	22/11/2015	Hampton Bkl 5	Bollgard	410	0	410	168	11	157	6.5
EM6	29/11/2015	Farm 148 Blk 5	Bollgard	110	0	110	34	5	29	14.7
EM7	07/12/2015	Tyson Blk 1	Bollgard	36	0	36	9	4	5	44.4
EM8	15/12/2015	Sanders Blk 6	Bollgard	181	0	181	80	39	41	48.8
EM9	05/01/2016	Tyson Blk 1	Bollgard	54	0	54	4	4	0	100
EM10	11/01/2016	Killara Blk 3	Bollgard	140	0	140	72	42	30	58.3
EM11	17/02/2016	Sanders Blk 6	Pigeon pea	125	0	125	68	12	56	17.6

TOTAL				**1801**	**0**	**1801**	**900**	**226**	**674**	**25.1**
MACINTYRE										
GN1	08/12/2015	Mundine	Bollgard	9	0	9	7	0	7	0
GN2	09/12/2015	Fairfield	Bollgard	140	0	140	86	25	61	29.1
GN3	09/12/2015	Parella	Bollgard	128	0	128	97	29	68	29.9
GN4	12/01/2016	Turkey Lagoon	Pigeon pea	270	0	270	196	25	171	12.8
GN5	12/01/2016	Morella	Bollgard	38	0	38	19	2	17	10.5
GN6	12/01/2016	Warendi	Bollgard	154	0	154	103	67	36	65.0
GN7	12/01/2016	Kildoonan	Bollgard	84	0	84	32	12	20	37.5
Regional reference	**Date Collected**	**Location**	**Crop**	**No. Eggs**	**No. Larvae**	**Eggs + Larvae**	**Total Hatched**	**Total *Ha***	**Total *Hp***	**% *Ha***
GN8	10/02/2016	Springfield	Bollgard	87	0	87	44	15	29	34.1
GN9	10/02/2016	Alcheringa	Bollgard	68	0	68	37	17	20	45.9
GN10	10/02/2016	Alcheringa	Pigeon pea	43	2	45	21	17	4	81.0
GN11	10/02/2016	Springfield	Pigeon pea	165	1	166	88	48	40	54.5
GN12	10/02/2016	Sylvan Plains	Sorghum	16	0	16	1	1	0	100
TOTAL				**1202**	**3**	**1205**	**731**	**258**	**473**	**35.3**
DARLING DOWNS										
DD1	18/11/2015	Tyunga	Maize	90	0	90	62	62	0	100
DD2	25/11/2015	Tyunga	Maize	86	0	86	55	55	0	100
DD3	01/12/2015	Kewood K2	Bollgard	118	0	118	62	0	62	0
DD4	02/12/2015	Amaroo	Bollgard	70	0	70	52	5	47	9.6
DD5	08/12/2015	Tosari F8	Bollgard	46	0	46	37	8	29	21.6
DD6	10/12/2015	Warrabrook	Conv. cotton	56	0	56	25	0	25	0
DD7	15/12/2015	Lleumeah Grazing	Bollgard	48	0	48	28	0	28	0
DD8	07/01/2016	Oban Field 1	Mungbeans	49	0	49	15	13	2	86.7
DD9	07/01/2016	Tarana Field 1	Bollgard	57	0	57	9	8	1	88.9
DD10	12/01/2016	Tyunga	Bollgard	68	0	68	7	4	3	57.1
DD11	12/01/2016	Duandine M6	Bollgard	81	0	81	19	9	10	47.4
DD12	13/01/2016	Tyunga C8	Bollgard	50	0	50	8	3	5	37.5
DD13	14/01/2016	Uralla Past. Field 5	Bollgard	50	0	50	13	0	13	0
DD14	20/01/2016	Courtney Bills	Bollgard	59	0	59	2	0	2	0
DD15	21/01/2016	Arnmour	Pigeon pea	102	0	102	66	5	61	7.6
DD16	20/01/2016	Kensington Park	Bollgard	43	0	43	3	0	3	0
DD17	28/01/2016	Tarcoola Park	Bollgard	74	0	74	32	10	22	31.3
DD18	02/02/2016	Tarcoola Park	Bollgard	122	0	122	43	17	26	39.5
DD19	02/02/2016	Smiths/Pampas	Bollgard	59	0	59	8	5	3	62.5
DD20	17/02/2016	Bemang F4C	Bollgard	48	0	48	6	3	3	50.0
DD21	23/02/2016	Tyunga	Bollgard	45	0	45	4	0	4	0
Kingaroy DAF 1	25/01/2016	Kingaroy	Chickpeas	0	74	74	60	60	0	100
Kingaroy DAF 2	21/12/2016	West Wooroolin	Peanuts	0	78	78	78	52	26	66.7
Kingaroy DAF 3	13/01/2016	Kingaroy DAF RS	Sorghum	0	120	120	109	109	0	100
Kingaroy DAF 4	08/01/2016	Hervey Bay	Mungbeans	0	60	60	53	53	0	100
TOTAL				**1421**	**332**	**1753**	**856**	**481**	**375**	**56.2**
GRIFFITH										
MIA1	13/10/2015	Coonara	Faba beans	0	40	40	27	1	26	3.7
MIA2	20/10/2015	Gundaline	Faba beans	0	28	28	26	0	26	0

MIA3	31/10/2015	Kycantha	Lucerne	0	12	12	8	0	8	0
MIA4	24/11/2015	Violi	Bollgard	60	0	60	34	0	34	0
MIA5	24/11/2015	Garoonga	Bollgard	22	0	22	15	0	15	0
MIA6	24/11/2015	Carrathool	Bollgard	19	0	19	14	0	14	0
MIA7	01/12/2015	Tubbo	Bollgard	134	0	134	31	0	31	0
MIA8	03/12/2015	Kiagarthur	Bollgard	43	0	43	15	0	15	0
MIA9	16/12/2015	Coonara F5006	Bollgard	30	0	30	17	0	17	0
MIA10	15/12/2015	Carrathool	Bollgard	92	0	92	35	0	35	0
MIA11	09/02/2016	Coleambally F2020	Pigeon pea	0	31	31	1	1	0	100
MIA12	17/02/2016	Farm 36 B	Pigeon pea	0	104	104	9	8	1	88.9
TOTAL				**400**	**215**	**615**	**232**	**10**	**222**	**4.3**

Regional reference	Date Collected	Location	Crop	No. Eggs	No. Larvae	Eggs + Larvae	Total Hatched	Total *Ha*	*Total Hp*	*% Ha*
MUNGINDI										
MUN1	10/11/2015	Burren Downs	Bollgard	60	0	60	47	1	46	2.1
MUN2	08/12/2015	Boston	Bollgard	135	0	135	48	1	47	2.1
MUN3	08/12/2015	Wyadrigah	Bollgard	155	0	155	57	2	55	3.5
MUN4	08/12/2015	Tarrawatta	Bollgard	100	0	100	66	5	61	7.6
MUN5	05/01/2016	Riverside	Bollgard	106	0	106	53	12	41	22.6
MUN6	11/01/2016	Reardon Farms	Bollgard	317	0	317	211	24	187	11.4
MUN7	11/01/2016	Strathgyle	Bollgard	83	0	83	54	13	41	24.1
MUN8	11/01/2016	Wongatoo	Bollgard	180	0	180	128	2	126	1.6
MUN9	11/01/2016	Wongatoo	Pigeon pea	163	0	163	143	1	142	0.7
MUN10	11/01/2016	Cleveland	Bollgard	301	0	301	231	31	200	13.4
MUN11	09/02/2016	Eagle Farm	Bollgard	86	0	86	49	8	41	16.3
MUN12	09/02/2016	Burren Downs	Pigeon pea	138	3	141	75	8	67	10.7
MUN13	09/02/2016	Riverside	Bollgard	72	0	72	49	6	43	12.2
MUN14	15/03/2016	Yarramildi	Maize	206	0	206	97	97	0	100
TOTAL				**2102**	**3**	**2105**	**1308**	**211**	**1097**	**16.1**
MACQUARIE										
MQ 1	13/10/2015	Pretoria	Lupins	0	37	37	0	0	0	0
MQ 2	28/10/2015	Auscott	Bollgard	31	0	31	21	2	19	9.5
MQ 3	11/11/2015	Auscott	Bollgard	20	0	20	1	1	0	100
MQ 4	18/11/2015	Willawa	Bollgard	218	0	218	147	9	138	6.1
MQ 5	03/12/2015	Auscott	Bollgard	225	0	225	170	1	169	0.6
MQ 6	08/12/2015	Auscott	Bollgard	106	0	106	55	1	54	1.8
MQ 7	13/01/2016	Auscott	Bollgard	95	0	95	42	0	42	0
MQ 8	20/01/2016	Megunyah	Bollgard	225	0	225	157	2	155	1.3
MQ 9	09/02/2016	Auscott	Bollgard	38	0	38	13	2	11	15.4
MQ 10	15/02/2016	Auscott	Bollgard	42	0	42	14	3	11	21.4
MQ 11	15/02/2016	Auscott	Pigeon pea	18	15	33	11	2	9	18.2
MQ 12	15/02/2016	Coldstream	Sorghum	83	17	100	32	31	1	96.9
MQ 13	26/02/2016	Megunyah	Pigeon pea	10	2	12	0	0	0	0
MQ 14	26/02/2016	Auscott	Pigeon pea	0	26	26	6	6	0	100
MQ 15	26/02/2016	Auscott	Bollgard	5	0	5	5	5	0	100
MQ 16	01/03/2016	Cowal Park	Maize	9	296	305	257	257	0	100
MQ 17	01/03/2016	Ningawalla	Maize	0	66	66	51	51	0	100
MQ 18	10/03/2016	Denistons	Pigeon pea	23	32	55	0	0	0	0
MQ 19	15/03/2016	Auscott	Pigeon pea	0	6	6	0	0	0	0

TOTAL	**1148**	**497**	**1645**	**982**	**373**	**609**	**38.0**

Table A.1.2 Annual collections of moths – all regions

Regional reference	Date Collected	Location	Crop	Number of F_0 pairs
Resistance Sampling 2014-2015				
UPPER NAMOI				
UN4	13/10/2014	Breeza Station	Chickpeas	82
UN8	27/10/2014	Breeza Station	Chickpeas	65
UN19	8/12/2014	Kilmarnock	Bollgard	46
UN23	7/01/2015	Ruvigne	Bollgard	20
UN24	13/01/2015	Bellevue	Bollgard	70
UN25	9/02/2015	NR		40
UN26	4/03/2015	Draytons	Mungbeans	30
UN27	18/03/2015	Draytons	Mungbeans	18
Resistance Sampling 2015-16				
UPPER NAMOI				
B1	21/01/2016	Breeza Station	Sorghum	53
C1	21/01/2016	Craigs	Sorghum	29
D1	21/01/2016	Draytons	Sorghum	39
B2	04/02/2016	Breeza Station	Sorghum	101
C2	04/02/2016	Craigs	Sorghum	90
D3	10/02/2016	Draytons	Sorghum	88
B3	10/02/2016	Breeza Station	Sorghum	38
C3	10/02/2016	Craigs	Sorghum	19
D4	25/02/2016	Draytons	Sorghum	16
B4	25/02/2016	Breeza Station	Sorghum	47
C4	25/02/2016	Craigs	Sorghum	8
RM	25/02/2016	DPI RS Breeza	Mungbeans	21
RS	25/02/2016	DPI RS Breeza	Sorghum	29
RB	25/02/2016	DPI RS Breeza	Soya beans	81
UN59	03/03/2016	Mullaley	Maize	23
UN60	03/03/2016	Mullaley	Mungbeans	38
UN61	03/03/2016	DPI RS Breeza	Soya beans	53
UN62	03/03/2016	DPI RS Breeza	Sorghum	78
UN 67	17/03/2016	Mullaley	Maize/pigeon pea	91
UN68	17/03/2016	Gabo Breeza	Pigeon pea	86
UN69	17/03/2016	DPI RS Breeza	Sorghum/soya beans	56
UN73	07/04/2016	DPI RS Breeza	Mungbeans	22
UN74	07/04/2016	Mullaley	Sorghum	9
TAI 1	05/01/2016	DPI RS Tamworth	Sorghum	18
TAI 2	06/01/2016	DPI RS Tamworth	Sorghum	9

TAI 3	07/01/2016	DPI RS Tamworth	Sorghum	40
TAI 4	12/01/2016	DPI RS Tamworth	Sorghum	38
TAI 5	13/01/2016	DPI RS Tamworth	Sorghum	22
TAI 6	14/01/2016	DPI RS Tamworth	Sorghum	9
TAI 7	19/01/2016	DPI RS Tamworth	Sorghum	12
TAI 8	27/01/2016	DPI RS Tamworth	Sorghum	18
TAI 9	03/02/2016	DPI RS Tamworth	Sorghum	31
TAI 10	04/02/2016	DPI RS Tamworth	Sorghum	19
TAI 11	10/02/2016	DPI RS Tamworth	Sorghum	15
TAI 12	11/02/2016	DPI RS Tamworth	Sorghum	17
TAI 13	26/02/2016	DPI RS Tamworth	Sorghum	24

Regional reference	Date Collected	Location	Crop	Number of F_0 pairs
LOWER NAMOI				
LN 17	08/01/2016	ACRI	Bollgard /sorghum	70
LN28	21/01/2016	ACRI	Bollgard /sorghum	3
LN32	09/02/2016	ACRI	Bollgard /sorghum	6
LN37	17/02/2016	ACRI	Bollgard /sorghum	3
LN39	23/02/2016	ACRI	Bollgard /sorghum	17
LN40	23/02/2016	ACRI	Bollgard /sorghum	8
LN41	24/02/2016	Wentworth	Bollgard	46
LN44	25/02/2016	Wentworth	Bollgard	18
LN45	08/03/2016	Wentworth	Bollgard	20
LN47	09/03/2016	Auscott	Bollgard	16
LN50	30/03/2016	Auscott	Bollgard	6
Resistance Sampling 2016-2017				
UPPER NAMOI				
TAI 1	21/10/2016	DPI RS Tamworth	Sorghum	40
B1	27/10/2016	Breeza Station	Chickpeas	35
C1	27/10/2016	Craigs	Chickpeas	35
D1	27/10/2016	Draytons	Chickpeas	35
B2	30/11/2016	Breeza Station	Chickpeas	62
C2	30/11/2016	Craigs	Chickpeas	2
D2	30/11/2016	Draytons	Chickpeas	33
B3	25/01/2017	Breeza Station	Maize	5
DPI RS 1	25/01/2017	DPI RS Breeza	Sorghum	50
B4P	28/02/2017	Breeza Station	Pigeon pea	50
B4C	28/02/2017	Breeza Station	Bollgard	50
D4	28/02/2017	Draytons	Pigeon pea	50
DPI RS 2	28/02/2017	DPI RS Breeza	Sorghum/maize	50
BM 1‡	28/02/2017	Breeza Station	Maize	41
LOWER NAMOI				
LN1	07/10/2016	Aitkins Edgeroi	Chickpeas	17
LN2	12/10/2016	Leitch Block	Chickpeas	20
LN3	10/10/2016	Leitch Block	Chickpeas	50
LN4	03/11/2016	Peppertree Bellata	Chickpeas	62
LN5	17/11/2016	Beechworth F3 Burren Jn	Chickpeas	26
LN6	30/11/2016	Killara F3	Chickpeas	43
LN7	11/12/2016	Peppertree Bellata	Chickpeas	9
LN8	31/01/2017	Tremayne	Sorghum	3
LN9	01/02/2017	Yarral	Mungbeans	4
GWYDIR				

GY1	09/11/2016	Wonga/Bulgate	Chickpeas	100
GY2	12/12/2016	Bulgate	Maize/chickpeas	100
GY3	17/01/2017	Bulgate	Maize	33
GY4	22/02/2017	Burundah	Mungbeans	6
GY5	22/02/2017	Cutlers Lane	Soya beans	14
MAQUARIE				
MQ1	15/02/2017	Auscott	Bollgard /pigeon pea	31
MQ2	15/02/2017	Edithville	Mungbeans	20
MQ3	15/02/2017	Edithville	Bollgard /pigeon pea	31
MQ4	15/02/2017	Ceeney's	Bollgard /pigeon pea	34

Regional reference	Date Collected	Location	Crop	Number of F_0 pairs
MQ5	23/03/2017	Auscott	Bollgard /pigeon pea	77
MQ6	23/03/2017	Ceeney's	Bollgard /pigeon pea	15
MQ7	23/03/2017	Woodleigh	Bollgard /pigeon pea	11
DARLING DOWNS				
DD1	31/10/2016	Kingaroy DAF RS	Chickpeas/wheat	35
DD2	15/11/2016	Irongate	Chickpeas	80
DD3	05/12/2016	Irongate	Chickpeas	64
EMERALD				
EM1	12/07/2016	NR	Chickpeas	91
EM2	25/08/2016	Cowal Ag	Chickpeas	110
EM3	25/08/2016	Cowal Ag	Chickpeas	110
EM4	04/10/2016	House Field	Chickpeas	80
EM5	04/10/2016	Road 1	Chickpeas	86
EM6	26/10/2016	Ryans	Chickpeas	50
EM7	26/10/2016	Emerald Ag College	Chickpeas	50
EM8	15/11/2016	Emerald Ag College	Chickpeas	50
EM9	15/11/2016	Research field	Chickpeas	50
EM10	28/11/2016	Dingo paddock	Chickpeas	50
EM11	28/11/2016	Trial paddock	Chickpeas	50
EM12	04/05/2017	Emerald Ag College	Chickpeas	60
Resistance Sampling 2017-18				
UPPER NAMOI				
UN1	18/10/2017	Breeza Station	Chickpeas	160
UN2	18/10/2017	Breeza Station	Chickpeas	0
UN3	28/11/2017	Breeza Station	Chickpeas/maize	90
UN4	28/11/2017	Draytons	Bollgard/pigeon pea	10
UN5	16/01/2018	Breeza Station	Bollgard /Maize	41
UN6	16/01/2018	Breeza Station	Bollgard /Maize	47
UN7	16/01/2018	DPI RS Breeza	Maize/beans/sorghum	5
UN8	16/01/2018	DPI RS Breeza	Sunflowers	25
UN9	16/01/2018	Draytons	Bollgard/pigeon pea	20
LOWER NAMOI				
LN1	20/09/2017	Leitch Block	Chickpeas	18
LN2	20/09/2017	Avondale	Chickpeas	9
LN3	10/10/2017	Leitch Block	Chickpeas	50
LN4	06/12/2017	Athelstone	Bollgard	50
LN5	09/01/2018	Mt Pleasant	Bollgard	60
LN6	20/03/2018	Mayfield	Mungbeans	55
GWYDIR				
GY1	19/10/2017	Wonga	Chickpeas	40

GY2	19/10/2017	Koreen	Chickpeas	40
GY3	19/10/2017	Koreen	Maize	40
GY4	20/10/2017	Myola Feedlot	Chickpeas	21
GY5	27/11/2017	Koreen	Chickpeas	3
GY6	27/11/2017	Koreen	Maize	32
DARLING DOWNS				
DD1	09/10/2017	Toowoomba	NR	50
DD2	15/11/2018	Kingaroy	NR	51
DD3	17/04/2018	Jimbour	Mungbeans	54

Regional reference	Date Collected	Location	Crop	Number of F_0 pairs
EMERALD				
EM1	02/08/2017	Andersons	Chickpeas	50
EM2	05/09/2017	NR	Chickpeas	50
EM3	10/09/2017	NR	Chickpeas	50
EM4	25/10/2017	Deneliza	Chickpeas	24
EM5	25/10/2017	Mathew B	Chickpeas	25
EM6	09/01/2018	NR	Bollgard	48
EM7	06/02/2018	Andersons	Bollgard/maize	57
MAQUARIE				
MQ1	09/01/2018	Iona Trangie	Bollgard	44
MQ2	24/01/2018	Auscott Warren	Bollgard/pigeon pea	30
MQ3	24/01/2018	Edithville	Unsprayed Bollgard	30
MQ4	24/01/2018	Brownings Narromine	Bollgard/pigeon pea	30
MQ5	06/02/2018	Glenrowan	Bollgard	74
MQ6	28/03/2018	Auscott/Edithville/Browinings	Bollgard/pigeon pea	15
MACINTYRE				
GN1	09/11/2017	Sapphire	Chickpeas	15
GN2	09/11/2017	Morella	Sorghum	14
GN3	09/11/2017	Allandale	Chickpeas	50
GN4	04/01/2018	Mundine	Bollgard	70
GN5	30/01/2018	Turkey Lagoon	Sorghum	45
GN6	30/01/2018	Vonrick	Pigeon pea	31
BURDEKIN				
BUR1	10/04/2018	Aye	Soya beans	5
BUR2	11/04/2018	Queens Beach	Tomatoes	17
Resistance Sampling 2018-19				
UPPER NAMOI				
UN1	30/10/2018	DPI RS Breeza	Faba beans	24
UN2	30/10/2018	DPI RS Breeza	Chickpeas	73
UN3	05/02/2019	Glenkeith	Sorghum	12
LOWER NAMOI				
LN1	25-28/10/2018	ACRI	Wheat	40
LN2	02/11/2018	ACRI	Wheat	51
LN3	05/11/2018	Auscott	Chickpeas	50
LN4	05/11/2018	Togo	Chickpeas	47
LN5	05/01/2018	South Bobbiwaa	Sorghum	37
GWYDIR				
GY1	15/08/2018	Bristol	Chickpeas/canola	115
GY2	14/11/2018	Berlin	Sorghum/maize	70
GY3	14/11/2018	Berlin	Sorghum	26

GY4	03/01/2019	Yamboon	Sorghum	30
GY5	03/01/2019	Denna/Manobe	Mungbeans	30
GY6	03/01/2019	Cocomo	Sorghum	31
EMERALD				
EM1	16/08/2018	Deneliza	Chickpeas	43
EM2	05/09/2018	Therea Downs	Chickpeas	80
EM3	02/10/2018	Deneliza	Chickpeas	40
EM4	15/11/2018	Emerald Ag College	Sorghum	12

Regional reference	Date Collected	Location	Crop	Number of F_0 pairs
DARLING DOWNS				
DD1	05/12/2018	Ian Spereal's	Sorghum	50
DD2	05/12/2018	Homewood Vale	Sorghum	50
DD3	05/12/2018	Russell Grundy's	Sorghum	50
DD4	15/01/2019	Homewood Vale	Sorghum	14
DD5	15/01/2019	Broadleaves	Sorghum	50
DD6	15/01/2019	Russell Grundy's	Sorghum	51
CLERMONT				
CL1	10/07/2018	Walton Downs	Chickpeas	7
CL2	10/07/2018	Brendan Swaffer's	Chickpeas	26
CL3	14/07/2018	Brendan Swaffer's	Chickpeas	80
CL4	09/09/2018	Walton Downs	Chickpeas	19
CL5	09/09/2018	Bungara	Chickpeas	28
DAWSON/CALLIDE				
DC1	18/09/2018	Lauriston	Chickpeas	4
DC2	18/09/2018	Cab-berra	Chickpeas	1
DC3	18/09/2018	Waynoc	Chickpeas	9
DC4	18/09/2018	Avonmore	Chickpeas	4
DC5	15/10/2018	Saleyards	Chickpeas	51
DC6	15/10/2018	Lochleigh	Chickpeas	60
DC7	22/01/2019	Hodgett's Biloela	Bollgard	6
DC8	22/01/2019	Dawson Vale	Bollgard	15
DC9	22/01/2019	Lauriston	Mungbeans	60
BURDEKIN				
BUR1	11/09/2018	Dry Creek	Tomatoes	2
BUR2	11/09/2018	Mugowie Farms	Green beans	3
BUR3	11/09/2018	Mugowie Farms	Sweet corn	3
BUR4	11/09/2018	Koorelah Farms	Tomatoes	11
BUR5	03/12/2018	Walsh Rd, Clare	Mungbeans	23
BUR6	03/12/2018	Mulgrove Rd, Clare	Soya beans	6
BUR7	21/01/2019	Walsh Rd, Clare	Soya beans	3
BUR8	21/01/2019	Ayr Rd, Mona Vale	Soya beans	72

‡Collected as larvae

Appendix 2

Regional testing results for indoxacarb, chlorantraniliprole and emamectin benzoate from 2014-15 to 2018-19

Table A.2.1 Indoxacarb testing results all regions 2014-15 to 2016-17

	Indoxacarb			
	Total tests	Total positives	Proportion resistant	%R
2014-15 Samples collected as egg/larvae unless otherwise stated				
Lower Namoi	77	2	0.026	2.6
Upper Namoi	78	0	0.000	0.0
Upper Namoi traps	229	10	0.044	4.4
Gwydir	82	2	0.024	2.4
Macquarie	16	2	0.125	12.5
Mungindi	38	1	0.026	2.6
Darling Downs	12	0	0.000	0.0
St George	38	4	0.105	10.5
Emerald	50	4	0.080	8.0
Macintyre	23	0	0.000	0.0
MIA	5	0	0.000	0.0
Kununurra	18	0	0.000	0.0
Total	666	25	0.038	3.8
2015-16 Samples collected as egg/larvae unless otherwise stated				
Lower Namoi	8	0	0.000	0.0
Lower Namoi traps	64	0	0.000	0.0
Upper Namoi	59	4	0.068	6.8
Upper Namoi traps	338	10	0.030	3.0
TAI	6	0	0.000	0.0
TAI traps	108	1	0.009	0.9
Gwydir	9	0	0.000	0.0
Macquarie	1	0	0.000	0.0
Mungindi	3	0	0.000	0.0
Darling Downs	32	0	0.000	0.0
St George	16	1	0.063	6.3
Emerald	4	0	0.000	0.0
Macintyre	2	0	0.000	0.0
Total	650	16	0.025	2.5

2016-17 Samples collected as moths unless otherwise stated				
Lower Namoi	143	7	0.049	4.9
Upper Namoi	220	7	0.032	3.2
Gwydir	70	3	0.043	4.3
Macquarie	112	4	0.036	3.6
Darling Downs	55	2	0.036	3.6
Emerald	385	37	0.036	3.6
Emerald larvae	41	2	0.092	9.2
Total	1026	62	0.060	6.0

Table A.2.2 Indoxacarb testing results all regions 2017-18 to 2018-19

	Indoxacarb			
	Total tests	Total positives	Proportion resistant	%R
2017-18 Samples collected as moths				
Lower Namoi	157	14	0.089	8.9
Upper Namoi	213	10	0.047	4.7
Gwydir	109	5	0.046	4.6
Macquarie	116	2	0.017	1.7
Darling Downs	97	6	0.062	6.2
Emerald	156	17	0.109	10.9
Macintyre	134	9	0.067	6.7
Burdekin	6	1	0.167	16.7
Total	988	64	0.065	6.5
2018-19 Samples collected as moths				
Lower Namoi	140	8	0.057	5.7
Upper Namoi	60	5	0.083	7.3
Gwydir	209	11	0.053	5.3
Macintyre	69	2	0.029	2.9
Darling Downs	174	10	0.057	5.7
Emerald	120	15	0.125	12.5
Clermont	94	11	0.117	11.7
Burdekin	66	11	0.167	16.7
Dawson/Callide	138	20	0.145	14.5
Total	1070	93	0.087	8.7

Table A.2.3 Chlorantraniliprole testing results all regions 2014-15 to 2016-17

	Chlorantraniliprole			
	Total tests	Total positives	Proportion resistant	%R
2014-15 Samples collected as egg/larvae unless otherwise stated				
Lower Namoi	76	0	0.000	0.0
Upper Namoi	77	0	0.000	0.0
Upper Namoi traps	228	1	0.004	0.4
Gwydir	80	0	0.000	0.0
Macquarie	16	0	0.000	0.0
Mungindi	35	0	0.000	0.0
Darling Downs	12	0	0.000	0.0
St George	38	0	0.000	0.0
Emerald	49	0	0.000	0.0
Macintyre	23	0	0.000	0.0
MIA	4	0	0.000	0.0
Kununurra	18	0	0.000	0.0
Total	656	1	0.002	0.2
2015-16 Samples collected as egg/larvae unless otherwise stated				
Lower Namoi	7	0	0.000	0.0
Lower Namoi traps	62	0	0.000	0.0
Upper Namoi	57	0	0.000	0.0
Upper Namoi traps	329	1	0.003	0.3
TAI	6	1	0.009	0.9
TAI traps	110	0	0.000	0.0
Gwydir	9	1	0.111	11.1
Macquarie	1	0	0.000	0.0
Mungindi	3	0	0.000	0.0
Darling Downs	32	0	0.000	0.0
St George	16	1	0.063	6.3
Emerald	2	0	0.000	0.0
Macintyre	2	0	0.000	0.0
Total	636	4	0.006	0.6
2016-17 Samples collected as moths unless otherwise stated				
Lower Namoi	141	0	0.000	0.0
Upper Namoi	214	1	0.005	0.5
Gwydir	63	0	0.000	0.0
Macquarie	95	0	0.000	0.0
Darling Downs	55	0	0.000	0.0
Emerald	379	2	0.007	0.7
Emerald larvae	41	1	0.024	2.4
Total	988	4	0.004	0.4

Table A.2.4 Chlorantraniliprole testing results all regions 2017-18 to 2018-19

	Chlorantraniliprole			
	Total tests	**Total positives**	**Proportion resistant**	**%R**
2017-18 Samples collected as moths				
Lower Namoi	157	0	0.000	0.0
Upper Namoi	211	2	0.009	0.9
Gwydir	103	1	0.010	1.0
Macquarie	115	0	0.000	0.0
Darling Downs	97	2	0.021	2.1
Emerald	153	2	0.013	1.3
Macintyre	134	1	0.007	0.7
Burdekin	6	0	0.000	0.0
Total	976	8	0.008	0.8
2018-19 Samples collected as moths				
Lower Namoi	138	0	0.000	0.0
Upper Namoi	59	0	0.000	0.0
Gwydir	204	0	0.000	0.0
Macintyre	66	0	0.000	0.0
Darling Downs	173	1	0.006	0.6
Emerald	119	0	0.000	0.0
Clermont	93	1	0.011	1.1
Burdekin	66	0	0.000	0.0
Dawson/Callide	133	3	0.024	2.3
Total	1051	5	0.005	0.5

Table A.2.5 Emamectin benzoate testing results all regions 2014-15 to 2016-17

	Emamectin Benzoate			
	Total tests	Total positives	Proportion resistant	%R
2014-15 Samples collected as egg/larvae unless otherwise stated				
Lower Namoi	73	0	0.000	0.0
Upper Namoi	76	0	0.000	0.0
Upper Namoi traps	225	0	0.000	0.0
Gwydir	81	0	0.000	0.0
Macquarie	16	0	0.000	0.0
Mungindi	35	0	0.000	0.0
Darling Downs	14	0	0.000	0.0
St George	38	0	0.000	0.0
Emerald	47	0	0.000	0.0
Macintyre	24	0	0.000	0.0
MIA	5	0	0.000	0.0
Kununurra	18	0	0.000	0.0
Total	652	0	0.000	0.0
2015-16 Samples collected as egg/larvae unless otherwise stated				
Lower Namoi	8	0	0.000	0.0
Lower Namoi traps	60	0	0.000	0.0
Upper Namoi	53	0	0.000	0.0
Upper Namoi traps	330	0	0.000	0.0
TAI	6	0	0.000	0.0
TAI traps	104	0	0.000	0.0
Gwydir	9	0	0.000	0.0
Macquarie	1	0	0.000	0.0
Mungindi	3	0	0.000	0.0
Darling Downs	32	0	0.000	0.0
St George	16	0	0.000	0.0
Emerald	4	0	0.000	0.0
Macintyre	2	0	0.000	0.0
Total	628	0	0.000	0.0
2016-17 Samples collected as moths unless otherwise stated				
Lower Namoi	136	0	0.000	0.0
Upper Namoi	200	0	0.000	0.0
Gwydir	61	0	0.000	0.0
Macquarie	78	0	0.000	0.0
Darling Downs	44	0	0.000	0.0
Emerald	373	0	0.000	0.0
Emerald larvae	41	0	0.000	0.0
Total	933	0	0.000	0.0

Table A.2.4 Emamectin benzoate testing results all regions 2017-18 to 2018-19

	Emamectin benzoate			
	Total tests	Total positives	Proportion resistant	%R
2017-18 Samples collected as moths				
Lower Namoi	143	0	0.000	0.0
Upper Namoi	209	0	0.000	0.0
Gwydir	102	0	0.000	0.0
Macquarie	111	0	0.000	0.0
Darling Downs	94	0	0.000	0.0
Emerald	151	0	0.000	0.0
Macintyre	129	0	0.000	0.0
Burdekin	6	0	0.000	0.0
Total	945	0	0.000	0.0
2018-19 Samples collected as moths				
Lower Namoi	133	0	0.000	0.0
Upper Namoi	59	0	0.000	0.0
Gwydir	187	0	0.000	0.0
Macintyre	57	0	0.000	0.0
Darling Downs	166	0	0.000	0.0
Emerald	119	0	0.000	0.0
Clermont	91	0	0.000	0.0
Burdekin	63	0	0.000	0.0
Dawson/Callide	129	0	0.000	0.0
Total	1004	0	0.000	0.0

Appendix 3

Resistance Management Strategy for *Helicoverpa armigera* in Australian grains

Key points

Helicoverpa armigera is a pest of many commodity crops including cotton, pulses, oilseeds, coarse grains and winter cereals as well as some vegetable crops. In grain crops it is most significant in summer and winter pulses, maize, sorghum, canola, linseed, safflower and sunflowers.

There are over 200 insecticide products registered in Australia against *H. armigera* for grains, cotton and vegetable crops. The majority are from 3 chemical sub-groups with broad spectrum activity: carbamates (Group 1A); organophosphates (Group 1B); and synthetic pyrethroids (Group 3A).

Selective insecticides from Groups 6 (emamectin benzoate), 22A (indoxacarb) and 28 (chlorantraniliprole) are now widely used in pulses.

Chemical control of *H. armigera* is complicated due to historical field resistance to broad-spectrum insecticides and increased selection pressure to important selective insecticides.

Within this Resistance Management Strategy (RMS), growers are encouraged to use integrated pest management tactics, including biological and cultural options and the appropriate use of selective insecticides to maximise control and maintain efficacy of currently available chemistries.

***Helicoverpa armigera* and insecticide resistance**

Helicoverpa armigera is a major pest of grain crops and represents a significant challenge for the grains industry given the ongoing reliance on chemical control methods. *H. armigera* reduces yield of pulses, oilseeds, coarse grains and occasionally winter cereals. Economic losses result from larvae feeding directly on the reproductive structures of crops (seeds and grain). Grain quality may also be downgraded through unacceptable levels of damage. Although widely distributed and recorded in all states and territories within Australia, *H. armigera* is more common in the northern and coastal regions of eastern states, particularly in warmer areas.

There are over 200 insecticide products registered in Australia against *H. armigera* for grain, cotton and vegetable crops. The majority are from 3 chemical sub-groups with broad-spectrum activity: carbamates (Group 1A); organophosphates (Group 1B); and synthetic pyrethroids (Group 3A). Organophosphates are not registered against *H. armigera* in Australian grain crops. Insecticides from Group 6 (emamectin benzoate), Group 22A (indoxacarb) and Group 28 (chlorantraniliprole) have become more widely used in pulses due to high efficacy and low impact on natural enemies.

Control is complicated because field populations are resistant to numerous insecticide groups. Due to these factors, timing of chemical applications and coverage are critical issues, and growers need to understand how to minimise yield loss without increasing resistance levels. The following RMS should guide growers' selection of control options and provides best practice product windows and use restrictions to manage resistance in *H. armigera.*

Resistance management & minimisation strategy

The aim of the strategy is to minimise the selection pressure for resistance to the same chemical groups across consecutive generations of *H. armigera*. The design of the strategy is centred on chickpea and mungbean. Currently, and for the foreseeable future, insecticide use in these crops is most likely to have the greatest impact on the management of resistance in *H. armigera* populations. However, the RMS should be applied to all broadleaf crops including adzuki bean, faba bean and soybean.

The strategy is primarily built around product windows for chlorantraniliprole and indoxacarb because:

1. Chlorantraniliprole (Altacor®) is at risk from high levels of over-reliance in pulses, but resistance frequencies are currently low.

2. Indoxacarb (e.g. Steward®) is at risk due to genetic predisposition (high level genetic dominance and metabolic mechanism) and pre-existing levels of resistance in NSW and QLD. In addition, indoxacarb is now off-patent in Australia which provides the opportunity for lower priced products to enter the market.

There are two RMS regions:

1. Northern grains region: Belyando, Central Highlands, Dawson & Callide (See Table 1)

2. Central grains region: Balonne, Bourke, Burnett, Darling Downs, Gwydir, Lachlan, Macintyre, Macquarie & Namoi (See Table 2)

There is no RMS for the Southern and Western grain regions of Australia

There has been little formal monitoring of *H. armigera* in the Southern and Western regions (Victoria, Tasmania, South Australia and Western Australia). These regions have very little broad-acre summer grain crop production and biological indicators suggest that the risk of *H. armigera* occurring at densities that may result in control failures is low. If required, the Central grains region RMS may be used for *H. armigera* management in summer crops in the Southern and Western regions.

Use of broad-spectrum insecticides

The early use of synthetic pyrethroids (SPs) in winter pulses (August – early September) in this RMS assumes that early infestations of *Helicoverpa* will be predominantly *H. punctigera.* This species is susceptible to SPs. If adopting this strategy, be aware of the following risks:

Recent monitoring with pheromone traps has shown that *H. armigera* can be present in all parts of the Northern grains region from early August.

Reduced efficacy of SPs when *H. armigera* is present can be masked when treating very low population densities (< 2/sqm).

Even low-level populations of *H. armigera,* when treated with SPs, will select for further resistance.

Integrated pest management is a central feature of the RMS

The use of integrated pest management (IPM) tactics for *H. armigera* management is integral to achieving a reduction in insecticide use and consequently helping to minimise resistance selection pressures. Examples of IPM tactics for Helicoverpa management include minimising the risk of pest build up on in-crop weeds, pupae-busting stubbles where pupae are overwintering (especially corn/maize, late sorghum, summer mungbean), spraying only when pest populations exceed economic threshold, and using the softest insecticides available to preserve beneficial insects.

When using insecticide, monitoring is the key to better targeted spraying and effective management. The use of pheromone traps (which attract male moths) can provide an early warning of moth immigration into an area or their emergence from local winter diapause. Traps should be set up in late winter-early spring (July-August). The beatsheet is the most commonly used sampling tool in the northern region. Obtain an estimate of pest and beneficial insect numbers in a crop by taking at least 5-10 beatsheet samples from across the field.

Sweep netting is a quick and easy method to sample some crops but is more subjective. Take a minimum of 5 sets of 10 sweeps and calculate the average number of larvae per 10 sweeps.

Other general recommendations

Avoid repeated use of insecticides from the same chemical group against *H. armigera* or other pests, as this will increase selection pressure for resistance development, not only in *H. armigera,* but, also in other species;

Comply with all directions for use on product labels including applying the recommended label rate. **DO NOT cut rates and DO NOT exceed the maximum number of allowable applications per crop per season**;

Do not re-spray a crop in the same season where a known spray failure has occurred using the same product or another product from the same chemical group, or if a spray failure has occurred where the cause has not been identified;

Where possible, avoid use of SPs and organophosphates (OPs) for control of other crop pests, and instead use target-specific “soft chemicals” such as pirimicarb for aphids and Bt or virus for caterpillars;

Ensure the target pest is identified correctly to ensure the most effective insecticide and rate is used. Misidentification and incorrect insecticide selection results in poor control and contributes to selection for resistance;

Assess *H. armigera* and beneficial populations by regular monitoring to determine if chemical control is warranted;

Consider the impact on target and non-target pests and beneficial invertebrates when applying insecticide sprays;

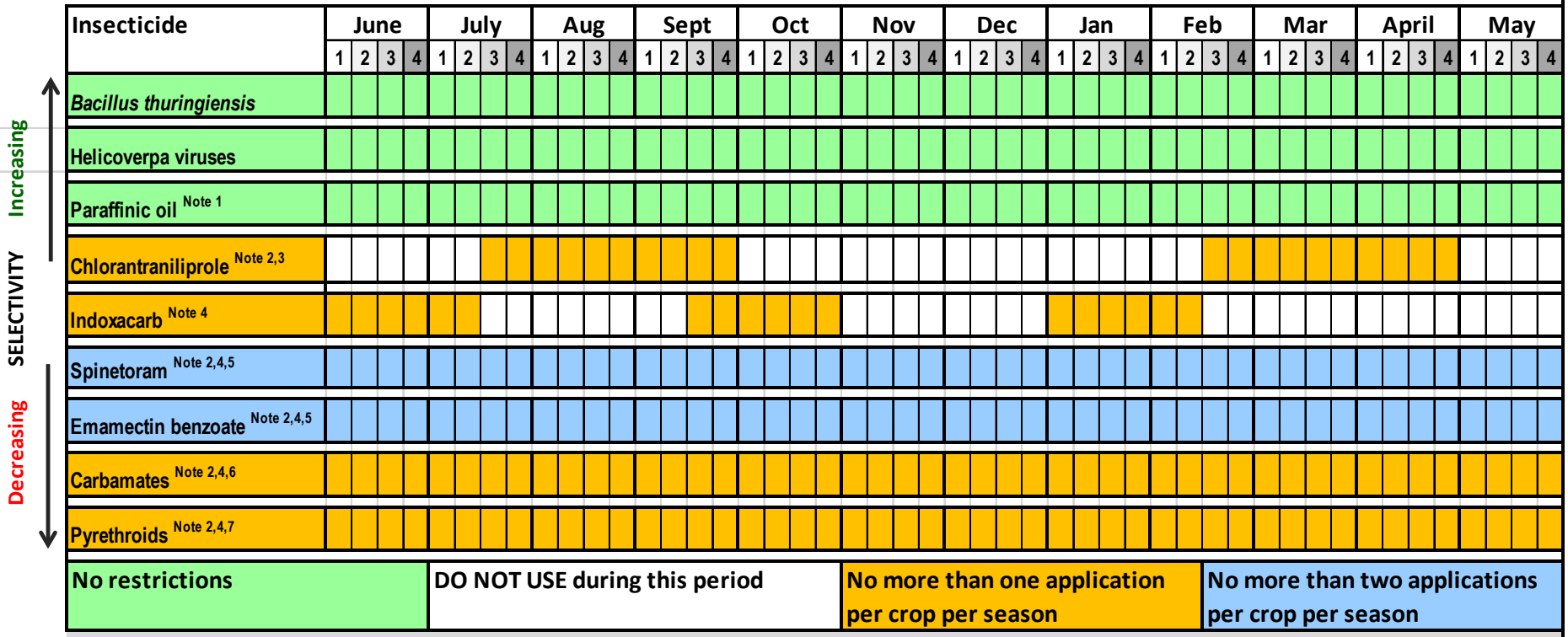
Ensure spray rigs (both ground and aerial) are calibrated properly and sprays achieve good coverage, particularly in crops with a bulky canopy;

Monitor post-treatment pest populations for evidence of loss of field efficacy and report field failures.

Table 1. Grains resistance management strategy for *Helicoverpa armigera* across Australia

Best practice product windows and use restrictions to manage insecticide resistance in *H. armigera*

Northern Region: Belyando, Central Highlands, Dawson & Callide



ADDITIONAL INFORMATION

Note 1: Can be used to suppress Helicoverpa populations and are best used as part of an IPM program

Note 2 : Observe withholding periods. Products in this group have WHP 14 days or longer.

Note 3: Maximum one spray of chlorantraniliprole alone or in mixtures per crop per season.

Note 4: Refer to label for warning of insecticide risk to bee populations.

Note 5: Maximum two consecutive sprays alone or in mixtures per crop per season.

Note 6: MODERATE RESISTANCE IS PRESENT IN *H. ARMIGERA* POPULATIONS – FIELD FAILURES LIKELY.

Note 7: HIGH RESISTANCE IS PRESENT IN *H. ARMIGERA* POPULATIONS – FIELD FAILURES EXPECTED!

Note 8: Refer to IRMS for cotton. Selection in conventional cotton is considered low due to high use of Bt cotton.

Table 2. Grains resistance management strategy for *Helicoverpa armigera* across Australia

Best practice product windows and use restrictions to manage insecticide resistance in *H. armigera*

Southern QLD, Central & Northern NSW Regions: Balonne, Bourke, Burnett, Darling Downs, Gwydir, Lachlan, Macintyre, Macquarie & Namoi

SELECTIVITY — Increasing ↑ / Decreasing ↓

Insecticide	June				July				Aug				Sept				Oct				Nov				Dec				Jan				Feb				Mar				April				May			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Bacillus thuringiensis																																															
Helicoverpa viruses																																															
Paraffinic oil Note 1																																															
Chlorantraniliprole Note 2,3																																															
Indoxacarb Note 4																																															
Spinetoram Note 2,4,5																																															
Emamectin benzoate Note 2,4,5																																															
Carbamates Note 2,4,6																																															
Pyrethroids Note 2,4,7																																															

No restrictions	DO NOT USE during this period	No more than one application per crop per season	No more than two applications per crop per season

ADDITIONAL INFORMATION

Note 1: Can be used to suppress Helicoverpa populations and are best used as part of an IPM program

Note 2 : Observe withholding periods. Products in this group have WHP 14 days or longer.

Note 3 : Maximum one spray of chlorantraniliprole alone or in mixtures per crop per season.

Note 4 : Refer to label for warning of insecticide risk to bee populations.

Note 5 : Maximum two consecutive sprays alone or in mixtures per crop per season.

Note 6: MODERATE RESISTANCE IS PRESENT IN *H. ARMIGERA* POPULATIONS – FIELD FAILURES LIKELY.

Note 7: HIGH RESISTANCE IS PRESENT IN *H. ARMIGERA* POPULATIONS – FIELD FAILURES EXPECTED!

Note 8: Refer to IRMS for cotton. Selection in conventional cotton is considered low due to high use of Bt cotton.

Table 3. Explanatory notes for product windows in all regions

Insecticide	Number of insecticide windows	Duration of insecticide windows	Maximum number of applications/crop/season
Chlorantraniliprole (Altacor®)	2	10 weeks	1
10 week windows restrict selection to a maximum of 2 consecutive generations of *H. armigera* (includes 2-3 weeks residual beyond the end of each window i.e. 12-13 weeks total exposure). Start date of first window correlates well with historical data relating to average daily temperatures that result in early pod-set. Exposure of 2 consecutive generations is off-set by long non-use periods (8 weeks in southern/central region and 18 weeks in northern region). Use is not recommended in spring mung beans as there is less likelihood of both *H. armigera* and bean pod borer being present.			
Indoxacarb (e.g. Steward®)	Northern - 3 Central - 2	6 weeks	1
6 week windows restrict selection to a single generation of *H. armigera*. Each window is followed by a non-use period of a minimum of 6 weeks. Indoxacarb is an important early season rotation option for chickpeas and faba beans, and provides a robust selective alternative to Altacor® when Helicoverpa pressure is high.			
Bacillus thuringiensis Helicoverpa viruses Spinetoram (e.g. Success Neo®)*	1	Season long	No restrictions No restrictions 2
Low resistance risk and not widely used.			
Emamectin benzoate (e.g. Affirm®)*	1	Season long	2
Very low resistance frequency and not used widely. However, emamectin benzoate is a good option for rotation to spread resistance risk away from Altacor®. BUT industry needs to become more confident with using this product for it to be of value in resistance management.			
Carbamates Synthetic pyrethroids	1	Season long	1
H. armigera resistance is present at moderate to high levels, but one strategic application per season in regions where *H. punctigera* predominates in early spring may be effective. Carbamates are a rotation tool for indoxacarb and Altacor® either early season in chickpeas or late season in mungbean.			

*Resistance monitoring for selective products is a key component of the RMS and changes in resistance frequencies may result in the introduction of product windows for those insecticides not currently windowed.

Frequently asked questions

Why doesn't this grains RMS have the same windows as the cotton IRMS?

The RMS for grain crops is not intended to 'sync' with the cotton IRMS. Recommended windows for use in grains and cotton do not align, and the level of insecticide used for Helicoverpa control in cotton is relatively small in comparison with the areas of winter and summer grain crops potentially treated each year. It is considered that insecticide use patterns in cotton pose little risk to the ongoing management of resistance, relative to the risk posed by year-round, high level use in grains.

For further information on the cotton IRMS go to: http://www.cottoninfo.com.au/publications/cotton-pest-management-guide.

Will resistance develop to emamectin benzoate and spinetoram if their use is not windowed?

Insecticides that are not windowed, such as emamectin benzoate and spinetoram, are currently not considered to be at high risk of resistance development based on low frequencies of resistance in field populations of *H. armigera*. However, if usage patterns of these insecticides increases then selection for resistance may also increase. In the event that field susceptibility of *H. armigera* to these products is reduced, the strategy may be modified to mitigate further loss of product efficacy.

What natural enemies should I be looking out for and can they control *Helicoverpa armigera?*

There are a large number of natural enemies that will attack the eggs, larvae and pupae of *H. armigera* and contribute to suppression of this pest (and other crop pests). These include predators (e.g. damsel bugs, red and blue beetle, ants, lacewings) and parasitoids (e.g. *Trichogramma*, *Microplitis*, Ichneumon wasps and Tachinid flies). Growers are encouraged to monitor trends in beneficial populations and avoid the use of unnecessary insecticide applications.

Why are product windows common to all areas in Southern QLD and central and northern NSW?

H. armigera moths are highly mobile and have the capacity to move between all areas in the central region, potentially increasing the risk of further exposing cohorts of insects previously selected for resistance.

What is the likelihood that I will have a spray failure?

The risk of spray failures is high for SPs and carbamates due to historical resistance to these products. The risk of spray failures due to resistance is currently low for the selective products indoxacarb, chlorantraniliprole or emamectin benzoate. If spray failures of these products occur, first check the application was made correctly. These products are primarily active by ingestion, which means the larva has to eat plant material treated with the insecticide. To achieve a high level of efficacy, the insecticide must be applied with excellent coverage. This means that attention to water volume, nozzle selection, speed in relation to crop canopy and weather conditions is critical. In addition, Helicoverpa larvae will not feed if temperatures are below 12°C. Application under these cooler conditions may result in reduced or slower rates of larval mortality. If application is ruled out as a reason for unsatisfactory control with selective products and you suspect resistance, contact your local entomologist.

How do I prevent spray failures into the future?

The RMS is designed to minimise selection pressure for resistance to the same chemical group across consecutive generations of *H. armigera*. The use of a broad range of IPM options will reduce over-reliance on any one chemical group. Growers should also use recommended economic thresholds and avoid prophylactic sprays. Following these recommendations and complying with label instructions will minimise the risk of spray failures occurring as the result of insecticide resistance and maintain effective insecticide control of *H. armigera* into the future.

NIRM contributors:

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Special thanks to Iain MacPherson (MacPherson Agricultural Consultants), Paul Castor (MCA), Sally Ceeney (Cotton Industry Development & Delivery Team), Susan Mass (CRDC), Prof Stephen Powles (UWA), Frances Morell (**cesar**) & the Grains Research & Development Corporation. Resistance monitoring data for *H. armigera* is provided by NSW Department of Primary Industries with support from the Cotton Research and Development Corporation (CRDC) and the Grains Research and Development Corporation (GRDC).

Useful resources:

Science behind the Resistance Management Strategy for *Helicoverpa armigera* in Australian grains, NIRM, http://ipmguidelinesforgrains.com.au/ipm-information/resistance-management-strategies/

Helicoverpa, QDAF, http://ipmguidelinesforgrains.com.au/pests/helicoverpa/

This strategy was developed by the National Insecticide Resistance Management (NIRM) working group of the Grains Pest Advisory Committee (GPAC), and endorsed by CropLife Australia. GPAC is a GRDC-funded project which provides strategic advice to GRDC on pest issues. NIRM, chaired by Dr Paul Umina, is responsible for developing insecticide resistance management strategies for a number of grains pests. The group's representative membership ensures engagement of agro-chemical industries, researchers, advisers and CropLife Australia.

Appendix 4

Science behind the Resistance Management Strategy for Helicoverpa armigera in Australian grains

Developed by the grains National Insecticide Resistance Management (NIRM) working group of the Grains Pest Advisory Committee (GPAC)

Authors: Dr Lisa Bird (NSWDPI), Dr Melina Miles (QDAF), Mr Geoff Cornwell (DuPont Crop Protection), Dr Paul Umina (cesar), Dr Garry McDonald (University of Melbourne), Prof Ary Hoffmann (University of Melbourne), Mr Greg Baker (SARDI), and Dr Owain Edwards (CSIRO).

Special thanks to Iain MacPherson (MacPherson Agricultural Consultants), Paul Castor (MCA), Sally Ceeney (Cotton Industry Development & Delivery Team), Susan Mass (CRDC), Prof Stephen Powles (UWA) & and the Grains Research & Development Corporation.

March 2018

Table 1. Background information on *Helicoverpa armigera*

Attribute	What is known for pest?	References	Knowledge gaps
Economic importance to grains	• Direct feeding by *Helicoverpa* spp. reduces yield of pulses, oilseeds, coarse grains and, to a lesser extent, winter cereals. • Ranked as the second most important invertebrate grains pest based on estimated losses from grains crops and the third most costly pest to control with pesticides.	Brier et al. 2008; Murray et al. 2013	Prevalence and impact in southern and western regions of Australia remains unclear.
Mode of reproduction	• Sexual reproduction, nocturnal. • Males respond to sex pheromones released by receptive females. • Sex pheromone commercially available routinely used in monitoring species composition and insecticide resistance. • High fecundity, 90% of eggs laid in dark cycle.	Rothschild 1978 Baker et al. 2013; Bird et al. 2017 Singh and Rembold 1989	
Life cycle (incl. # generations)	• Australian tropical and sub-tropical climates (central QLD and areas further north) support *H. armigera* populations year-round. • In temperate and cool climate zones (southern QLD and areas further south) the majority of *H. armigera* undergo a facultative pupal diapause from mid-March onwards. • Emergence from diapause in late September onwards initiates the first of 3-5 generations per season in central and southern regions. • The lifecycle (egg to adult) is typically 42 days at 25°C. In summer, it takes 5-6 weeks to complete a single generation and over 8 weeks in spring and autumn. • Discrete *H. armigera* populations occur in spring, peaking in late summer by which time there can be overlapping cohorts and multiple life-stages in host crops.	Sequeira and Playford 2001 Fitt and Daly 1990; Murray and Zalucki 1994; Lloyd et al. 2008. Room 1983 Room 1983 Baker et al. 2011	
Crop hosts	• Polyphagous, including broadleaf and grass-related species. • Commodity crops include cotton, pulses, oilseeds, Solanaceous vegetables, coarse grains and winter cereals. • Most significant grains crops are chickpea, soybean, mung bean adzuki bean, faba bean, maize, sorghum, canola, lupins, linseed, and sunflowers.	Zalucki et al. 1986	
Non-crop hosts	• Common weed hosts include Pattersons curse, *Verbenaceae* spp., *Malvaceae* spp., and the scrophulariaceous weed host, *Verbascum virgatum*. • Cultivated pigeon pea as a refuge crop for Bt cotton.	Zalucki et al. 1986 Baker and Tann 2014	
Distribution	• Introduced species with origins in the Old World. • Closely associated with agricultural areas which supply a sequence of suitable hosts across a wide area. • Most prevalent across all northern grain growing regions of eastern Australia with usually lower abundance in the south. • Rarely reported in inland Australia.	Zalucki et al. 1986 Fitt et al. 1995	Do pest populations arise due to increased abundance of local cohorts because of a change in carrying capacity, or does it expand its range by migration into agricultural

	• Reports of populations in some southern mainland districts. • Immigrants occasionally reported in northern TAS but not known to establish beyond one or two generations.	Gregg et al. 1989; Zalucki et al. 1994 N. Meyers, R. Fox (Pers. Comm.) L. Hill (Pers. Comm.)	areas driven by ephemeral nature of host crops?
Dispersal/movement	• Facultative migrant. • Known to be capable of long-range, wind assisted migration. • Local dispersal is more typical of *H. armigera* and is strongly influenced by local cropping and climatic conditions.	Daly and Gregg 1985; Fitt 1989 Feng et al. 2005 Fitt et al. 1989	What is the relative amount of immigration and local emergence and how does this contribute to population dynamics and gene flow?
Feeding behaviour	• Chewing pest; larvae can feed and survive on leaves but are most damaging when feeding directly on growing terminals, and reproductive plant parts (buds or squares, flowers, pods, seed and/or fruit). • Economic thresholds are well established in cotton. • Economic thresholds in grains are largely nominal, best guesses. Northern region has empirically derived, dynamic thresholds for chickpeas, mungbean, soybean, sorghum.	Fitt 1989 Williams 2016 Brier et al. 2008 GPAC/NIPI/Miles threshold review 2014-16	
Chemical controls	• Chemical insecticides remain the key to control within grains and as well as other industries. • There are over 200 insecticide products registered in Australia. • The majority are from 3 chemical groups with broad spectrum activity (organophosphates, carbamates and pyrethroids) and to which *H. armigera* has evolved some level of resistance. • There are an additional 3 insecticides with unique MOA which are selective for *Helicoverpa* spp. (indoxacarb, avermectin and diamides) and to which there is low or no resistance. • Spinetoram is also registered for *Helicoverpa* spp. in cotton, chickpeas, summer pulses and fruit/vegetable crops.	Murray et al. 2013 APVMA McCaffery 1998 Wing et al. 2004; Cordova et al. 2006; Ishaaya et al. 2002 Bird 2015; Bird et al. 2017	
Biological control options	• There are many effective natural enemies of *H. armigera*. These include: predatory beetles such as red and blue beetles, and the larvae of various species of lady beetles; predatory bugs such as damsel bug, big eyed bug, assassin bug, and various species of shield bugs; lacewing larvae; spiders; hoverfly larvae; larval parasitoids including various species of ichnumonid and braconid wasps, and tachinid flies; the egg parasitoids including trichogramma and telenomis wasps. • Pathogens available commercially as biopesticides include formulated products of Nuclear Polyhedrosis Virus (NPV) and the bacterial toxins from *Bacillus thuringiensis* (Bt). • Plant extract from *C. ternatea* is commercially available as Sero-X.	Williams 2016	

	• Entomopathogenic fungi are also being investigated for future commercialization.		

Table 2. Products with label claims for *Helicoverpa armigera* (and *Helicoverpa* spp. generally) in Australia

IRAC MoA Group	Insecticide category	Active ingredient	Example trade name	Registered crops for *Helicoverpa armigera* (Helicoverpa spp.)
1A	Carbamates	methomyl	Lannate, Marlin, Nudrin, Electra	Maize, sorghum, canola, summer and winter pulses (incl. mung beans [seed production only], chickpeas, adzuki beans, soybeans), lupins, lentils, linseed, sunflowers, winter cereals (wheat, oats, barley), apples, citrus, blueberries, capsicum, cotton, ginger, lettuce, poppies, peanuts, sesame, strawberries, stone fruit, sweet corn, tobacco, tomatoes, brassica and legume vegetables.
1A	Carbamates	thiodicarb	Larvin, Showdown, Confront	Maize, summer and winter pulses (incl. mung beans, chickpeas, soybeans, navy beans), cotton, tomatoes, potatoes, sweetcorn, tobacco.
1B	Organophosphates	chlorpyrifos	Chlorban, Chlorpos, Lorsban, Chlorpyrifos (various products)	tomatoes, brassica vegetables
3A	Pyrethroids	alpha-cypermethrin	Alpha-Scud, Astound, Dominex Duo	Maize, sorghum, canola, field peas, chickpeas, faba beans, mung beans, navy beans, soybeans, lupins, linseed, sunflowers, cotton, vegetable crops (incl. lettuce, sweetcorn, tomatoes)
3A	Pyrethroids	beta-cypermethrin	Chix, Banshee	Maize, sorghum, canola, field peas, mung beans, navy beans, lupins, linseed, sunflowers, cotton, vegetable crops (incl. tomatoes, brassicas)
3A	Pyrethroids	cypermethrin	Cyrux 250, Cyper Plus	Maize, sorghum, field peas, faba beans, mung beans, navy beans, soybeans, lupins, linseed, sunflowers, cotton, tobacco, vegetable crops (incl. tomatoes, sweetcorn)
3A	Pyrethroids	deltamethrin	Decis Options, Ballistic Elite, Deltashield	Maize, sorghum, canola, cereals, field peas, chick peas, faba beans, mung beans, navy beans, soybeans, lupins, lentils, linseed, sunflowers, safflower, cotton, tobacco, vegetable crops (incl. tomatoes, sweetcorn, berries)
3A	Pyrethroids	gamma-cyhalothrin	Trojan	Sorghum, canola, field peas, chickpeas, faba beans, mung beans, navy beans, soybeans, lupins, sunflower, cotton, tomatoes
3A	Pyrethroids	lambda-cyhalothrin	Karate, Cyhella, Matador	Sorghum, canola, field peas, chickpeas, faba beans, mung beans, navy beans, soybeans, lupins, lentils, sunflowers, cotton, tomatoes
3A	Pyrethroids	esfenvalerate	Sumi-Alpha Flex	Maize, sorghum, canola, cereals, field peas, chick peas, pigeon peas, faba beans, mung beans, navy beans, soybeans, lupins, lentils, linseed, sunflowers, safflower, cotton, tobacco, brassica and legume vegetables, sweetcorn, tomatoes
3A	Pyrethroids	permethrin	Ambush, Pounce, Axe	Field peas, linseed (TAS and WA only), legume vegetables (incl. green beans and peas), sweetcorn, tomatoes, tobacco, nursery plants and ornamentals
3A	Pyrethroids	bifenthrin	Talstar, Compel, Astral	Navy beans, cotton, tomatoes, cucurbit vegetables
3A	Pyrethroids	tau-fluvalinate	Klartan, Mavrik	Cauliflowers, tomatoes, ornamentals, cotton (Mavrik concentrate restricted to use on cotton only)
Synergists		piperonyl butoxide	PBO 800, Puppet	Cotton

			Synergy	Also for use on cucurbits and tomatoes
5	Spinosyns	spinetoram	Success Neo	Cotton Chickpeas Pulses (all summer and winter) Canola & forage brassicas Vegetable crops: Brassica, cucurbit (incl. cucumbers, melons, squash zucchinis), leafy (incl. lettuce, endive, spinach, silverbeet), fruiting (incl. capsicums/peppers, eggplants, okra, tomatoes), legume (incl. beans and peas; snow and sugar snap), root/tuber, stalk/stem, berry fruit. Fruit trees: Citrus (incl. grapefruits, mandarins, lemons, limes, oranges).
6	Avermectins	Emamectin benzoate	Affirm Warlock Proclaim	Canola, summer and winter pulses (incl. chickpeas, faba beans, adzuki beans, mung beans, soybeans), cotton Cotton, capsicums, lettuce, tomatoes, sweetcorn Capsicums, lettuce, tomatoes, sweetcorn
6	Avermectins	Abamectin	Agrimec, Stealth, Abachem	Cotton
11C	*Bacillus thuringiensis*	B.t. subsp. kurstaki	DiPel, Delfin, Costar	Cereal grains, oilseeds, cotton, vegetables, fruits, nuts, vines, herbs, tobacco, ornamentals
11C	*Bacillus thuringiensis*	B.t. subsp. kurstaki	Bollgard II & III	Cotton
11C	*Bacillus thuringiensis*	B.t. subsp. aizawai	Bacchus	Cereal grains, oilseeds, cotton, vegetables, fruits, nuts, vines, herbs, tobacco, ornamentals
22A	Oxadiazines	Indoxacarb	Steward, King doxa, Commissioner Avatar	Summer and winter pulses but limited to chickpeas, faba beans, adzuki beans, mung beans, soybeans, cotton Vegetable crops: Brassica, leafy (incl. cress, kale, lettuce, spinach, silverbeet, Chinese veg), fruiting (incl. capsicums/peppers, eggplants, tomatoes) Fruit trees: pome fruit (incl. apples, pears, Nashi pears), stone fruit (incl. apricots, nectarines, peaches, plums)
28	Diamides	Chlorantraniliprole	Altacor Coragen	Chickpeas, mung beans, soybeans, sunflower (permit only), cotton Vegetable crops: Brassica, stalk/stem, leafy, legume, tuberous, cucurbit (cucumber, melons, pumpkins, squash, zucchinis) and fruiting (capsicums/peppers, eggplants, tomatoes, sweetcorn)
28	Diamides	Cyantraniliprole	Benevia Exirel	Vegetables crops: Cucurbit (cucumber, melons, pumpkins, squash, zucchinis) and fruiting (capsicums/peppers, eggplants, tomatoes) Cotton
28	Diamides in combination with other insecticides	Chlorantraniliprole + thiamethoxam (4A) Chlorantraniliprole + abamectin (6)	Voliam Flexi Voliam Targo	Cotton Pome fruit

No Group	Attract and Kill Formulations	(Eucalyptol, D-Limonene, Phenylacetaldehyde, Anisyl Alcohol, Butyl Salicylate, Alpha-Pinene) + mixing partner (Group 1A or 5)	Magnet	Cotton, green beans, sweetcorn
No Group	Nuclear polyhedrosis virus	Nuclear polyhedrosis virus	Gemstar, Vivus Max/Gold, Heliocide, Helicovex, Armigen	Cereal grains, oilseeds, pulses, sorghum, cotton Vegetable crops: Brassica, stalk/stem, leafy, legume, tuberous, cucurbit (cucumber, melons, pumpkins, squash, zucchinis) and fruiting (capsicums/peppers, eggplants, tomatoes, sweetcorn) Fruit crops: berry fruit, pome fruit
No Group	Diatomaceous earth	Amorphous silica	Abrade	Cotton Brassica vegetables
No Group	Paraffinic spray oils	Paraffinic oil	Canopy Bioclear, Biopest	Pulse and oilseed crops Cotton Fruit and vegetable crops (numerous)

Source: APVMA-Public Chemical Registration Information System Search (PubCRIS), Australian Pesticides & Veterinary Medicines Authority; accessed March 2017.
Note: crops in red are grain crops.

Table 3 Industry chemical use and secondary exposure

Crop	Target pest	Active(s)
Canola	DBM	Spinetoram, emamectin benzoate
Summer pulses	loopers	Bt, indoxacarb, emamectin benzoate, chlorantraniliprole
Summer pulses	Podsucking bugs	SP
Mungbean, soybean	beanpodborer	chlorantraniliprole
Munbean, soybean	mirids	Indoxacarb, dimethoate
Sorghum	Sorghum midge	SP
	Rutherglen bug	SP, OP
Winter cereals	armyworm	SP, OP
Sunflower	Rutherglen bug	SP, OP
Sunflower	Loopers	Bt, SP, OP
Chickpea	*H. punctigera*	SP
Linseed (permit to 2021)	Helicoverpa	chlorantraniliprole
Sunflower (permit to Oct. 31st 2018)	Helicoverpa	chlorantraniliprole

Table 4 Current status of *Helicoverpa armigera* resistance in Australia

Insecticide	Attribute	What is known for *Helicoverpa armigera?*	References
1A Organophosphates	Mode of Action	ACHE inhibition	McCaffery 1998
	Resistance status	Low (<1%) to chlorpyrifos Moderate (10%) to profenofos and methyl parathion No cross-resistance between chlopyrifos and profenofos/methyl parathion	Rossiter et al. 2008; Appendix 1 Gunning 2002 Gunning et al. 1998
	Mechanism of resistance	Target site (insensitive ACHE) Metabolic (sequestration by carboxylesterase)	Gunning et al. 1998 Teese et al. 2010; Farnsworth et al. 2010
	Fitness costs	Slower larval growth of resistant genotypes	Gunning et al. 1998
	Genetic basis for resistance	Not evaluated in Australian populations	
1B Carbamates	Mode of Action	ACHE inhibition	McCaffery 1998
	Resistance status	Moderate – high (30-50%) Cross resistance between methomyl and thiodicarb No cross resistance between carbamates and OPs	Rossiter et al. 2008; Bird 2018; Appendix 1 Gunning et al. 1996a Farnsworth et al. 2010
	Mechanism of resistance	Target site (insensitive ACHE - different to OPs) Metabolic (P450/esterase)	Gunning et al. 1996a Gunning et al. 1992
	Fitness costs	Not evaluated in Australian populations	
	Genetic basis for resistance	Incompletely dominant	Gunning et al. 1996a
3A Pyrethroids	Mode of Action	Voltage-gated Na channel	McCaffery 1998
	Resistance status	Metabolic resistance is high (50-100%) Target site resistance is low (<5%) Broad cross-resistance to all SPs	Rossiter et al. 2008; Bird 2018; Appendix 1 Gunning et al. 1996b; L.J.B Unpublished Forrester et al. 1993
	Mechanism of resistance	Primarily metabolic (P450 [*CYP337B3*] /esterase) Target site (*kdr* and *super-kdr*) present under some circumstances and in some locations	Joußen et al. 2012; Teese et al. 2013 Gunning et al. 1991; 1996b
	Fitness costs	Reduced survival in resistance genotypes after diapause	Daly and Fisk 1995
	Genetic basis for resistance	Metabolic resistance inherited as a completely dominant trait *Target site resistance inherited as an incompletely recessive trait*	Daly and Fisk 1992 *Ru et al. 1998**
5 Spinosyns	Mode of Action	Nicotinic ACH receptor	Orr et al. 2009
	Resistance status	Low to moderate before transgenics (10-15%) Very low following introduction of Bollgard (<2%) Low or no cross-resistance to products from other MoA groups	Gunning 2002 Rossiter et al. 2008 Sparks et al. 2012
	Mechanism of resistance	*Metabolic (P450)*	**Wang et al. 2009*

	Fitness costs	*Reduced survival, reduced larval and pupa weights, slower larval development*	**Wang et al. 2010*
	Genetic basis for resistance	Unknown in *H. armigera*	
6 **Avermectins**	Mode of Action	Glutamate-gated chloride channel	Ishaaya et al. 2002
	Resistance status	Low or no resistance detected Cross-resistance status unknown in *H. armigera* *Cross resistance between emamectin benzoate and abamectin in Spodoptera exigua*	Rossiter et al. 2008; Bird et al. 2017; Appendix 2 **Che et al. 2015*
	Mechanism of resistance	Unknown in *H. armigera*	
	Fitness costs	Unknown in *H. armigera*	
	Genetic basis for resistance	Unknown in *H. armigera* *Incompletely dominant in Spodoptera* spp.	 **Shad et al. 2010; *Che et al. 2015*
11C ***Bacillus thuringiensis***	Mode of Action	Midgut receptor	Van Rie et al. 1990
	Resistance status	Low (<5%)	Mahon et al. 2007a
	Mechanism of resistance	Target site resistance Metabolic resistance	Akhurst et al. 2003 Gunning et al. 2005
	Fitness costs	Recessive fitness cost - delayed development in homozygous resistant genotypes	Bird and Akhurst 2004; **Cao et al.*
	Genetic basis for resistance	Target site resistance inherited as a completely or incompletely recessive trait	Bird and Akhurst 2004; Mahon et al. 2007b
22A **Indoxacarb**	Mode of Action	Voltage-gated Na channel (different receptor for SPs)	Wing et al. 2004
	Resistance status	Low (<5%) But increased frequencies observed in CQ in 2016-17	Bird et al. 2017; Appendix 2 L.J.B. Unpublished
	Mechanism of resistance	Metabolic	Bird 2016
	Fitness costs	No evidence of fitness cost associated with larval survival or development under lab conditions.	L.J.B. Unpublished
	Genetic basis for resistance	Partially dominant (*D*LC = 0.8)	Bird 2016
28 **Diamides**	Mode of Action	Ryanodine receptor	Cordova et al. 2006
	Resistance status	Very low (<1%). Strains with enhanced survival are unstable under laboratory conditions	Bird et al. 2017; Appendix 2
	Mechanism of resistance	Unknown in *H. armigera*	
	Fitness costs	Unknown in *H. armigera*	
	Genetic basis for resistance	Unknown in *H. armigera*	

* Indicates studies were performed on Chinese strains of *H. armigera*

Resistance management & minimisation strategy

General rationale for the design of the strategy

Chickpeas and mung beans are currently, and for the foreseeable future, the most valuable grains crops influenced by the RMS. Therefore, the RMS is primarily focused on insecticide MoA rotation in these systems and is built around product windows for Altacor and Steward because:

1. Altacor at risk from dangerously high levels of over-reliance in pulses, but resistance frequencies are currently low.
2. Steward at risk due to genetic predisposition (high level genetic dominance and metabolic mechanism) and pre-existing levels of resistance in NSW and QLD (with elevated levels in CQ during 2016-17). In addition, the use of indoxacarb in pulses is expected to increase.

There are two RMS regions:

1. Northern Grains Region: Belyando, Callide Central Highlands & Dawson (Table 1)
2. Central Grains Region: Balonne, Bourke, Burnett, Darling Downs, Gwydir, Lachlan, Macintyre, Macquarie & Namoi (Table 2)

- The RMS provides windows-based recommendations common to Southern QLD, Central & Northern NSW because *H. armigera* moths are highly mobile and have the capacity to move between these regions, potentially increasing the risk of further exposing cohorts of insects previously selected for resistance.
- We have limited knowledge of the likely risk of *H. armigera* occurrence in winter crops in the Southern and Western Grains regions (Victoria, South Australia and Western Australia) because there has been little formal monitoring for this species in these regions. However, there is some historical data, and anecdotal records of *H. armigera* outbreaks in the Southern region, which suggests that in some years and regions there is a risk of control failure and/or selection of resistance in the Helicoverpa population because of the presence of *H. armigera*.
- No RMS is currently proposed for these regions. Biological indicators are that the risk of *H. armigera* occurring in winter crops, at densities where control failures may occur, is presently considered low. Helicoverpa control in summer crops in these regions to use the Central Grains region RMS.

Use of broad-spectrums

The early use of SPs in winter pulses (August – early September) is a strategy adopted where the assumption is made that early infestations of Helicoverpa will be predominantly *H. punctigera* which are susceptible to SPs. If adopting this strategy, be aware of the following risks:

- Recent monitoring with pheromone traps has shown *H. armigera* to be present in all parts of the Northern Grains region from early August.
- Reduced efficacy of SPs when *H. armigera* is present can be masked when treating very low population densities (< 3/sqm).
- If *H. armigera* are present, even at low levels in a population treated with SPs, the treatment will select for further resistance.

Does the RMS impact on recommendations for insecticide use in cotton?

The RMS is not intended to compromise the ability of the cotton industry to utilise any products registered for *Helicoverpa* spp. in Bollgard. This is because selection for insecticide resistance is considered low due to the high likelihood that survivors of conventional sprays used in Bollgard would be killed by Bt toxins expressed in plants. For further information go to: http://www.cottoninfo.com.au/publications/cotton-pest-management-guide

General principles to avoid or minimise resistance development:

- Avoid repeated use of insecticides from the same chemical group against *H. armigera* or other pests, as this will increase selection pressure for resistance development, not only in *H. armigera,* but, also in other species;
- Comply with all directions for use on product labels including applying the recommended label rate. **DO NOT cut rates and DO NOT exceed the maximum number of allowable applications per crop per season**;
- Do not re-spray a crop in the same season where a known spray failure has occurred using the same product or another product from the same chemical group, or if a spray failure has occurred where the cause has not been identified;
- Where possible, avoid use of SPs and organophosphates (OPs) for control of other crop pests, and instead use target-specific “soft chemicals” such as pirimicarb for aphids and Bt or virus for caterpillars;
- Ensure the target pest is identified correctly to ensure the most effective insecticide and rate is used. Misidentification and incorrect insecticide selection results in poor control and contributes to selection for resistance;
- Assess *H. armigera* and beneficial populations by regular monitoring to determine if chemical control is warranted;

- Consider the impact on target and non-target pests and beneficial invertebrates when applying insecticide sprays;
- Ensure spray rigs (both ground and aerial) are calibrated properly and sprays achieve good coverage, particularly in crops with a bulky canopy;
- Monitor post-treatment pest populations for evidence of loss of field efficacy and report field failures.

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Resistance Tables

Table 1. Frequency of resistance in *Helicoverpa armigera* to broad-spectrum insecticides pyrethroid, carbamate and organophosphate in populations sampled from NSW and QLD.

Insecticide	Year	Number tested	Frequency of R	Binomial CI	%R
Fenvalerate	2008-09	253	0.704	0.6446-0.7564	70.4
	2009-10	194	0.680	0.6065-0.7372	68.0
	2010-11	243	0.617	0.5548-0.6761	61.7
	2011-12	902	0.905	0.8837-0.9221	90.5
	2012-13	2446	0.906	0.8933-0.9156	90.6
	2013-14	542	0.906	0.8784-0.9277	90.6
	2014-15	1053	0.933	0.9158-0.9462	93.3
Bifenthrin	2008-09	456	0	0.0000-0.0084	0.0
	2009-10	276	0.065	0.0416-0.1007	6.5
	2010-11	402	0.075	0.0528-0.1045	7.5
	2011-12	1197	0.396	0.3687-0.4240	39.6
	2012-13	2215	0.389	0.3691-0.4096	38.9
	2013-14	596	0.438	0.3986-0.4780	43.8
	2014-15	1047	0.553	0.5228-0.5829	55.3
Methomyl	2008-09	666	0.333	0.2957-0.3669	33.3
	2009-10	229	0.266	0.2133-0.3271	26.6
	2010-11	598	0.171	0.1425-0.2028	17.1
	2011-12	1049	0.340	0.3123-0.3695	34.0
	2012-13	1965	0.231	0.2124-0.2497	23.1
	2013-14	559	0.283	0.2469-0.3214	28.3
	2014-15	951	0.493	0.4615-0.5249	49.3
Chlorpyrifos	2008-09	645	0.002	0.0000-0.0087	0.2
	2009-10	158	0.000	0.0000-0.0237	0.0
	2010-11	659	0.005	0.0015-0.0133	0.5
	2011-12	1051	0.010	0.0059-0.0186	1.0
	2012-13	2037	0.013	0.0087-0.0186	1.3
	2013-14	562	0.018	0.0097-0.0324	1.8
	2014-15	979	0.019	0.0125-0.0301	1.9

This monitoring program was conducted by NSW Department of Primary Industries with support from the Cotton Research and Development Corporation (CRDC) and the Grains Research and Development Corporation (GRDC).

Table 2. Frequency of resistance in *Helicoverpa armigera* to selective insecticides indoxacarb, chlorantraniliprole and emamectin benzoate in populations sampled from NSW and QLD.

Insecticide	Year	Total tests	Total alleles	Total positives	Frequency of R	%R
Indoxacarb	2013-14	548	1096	9	0.016	1.6
	2014-15	665	1330	25	0.038	3.8
	2015-16	650	1300	16	0.025	2.5
	2016-17	1026	2052	62	0.060	6.0
Chlorantraniliprole	2013-14	525	1050	0	0.000	0.0
	2014-15	656	1312	1	0.002	0.2
	2015-16	636	1272	4	0.006	0.6
	2016-17	988	1976	4	0.004	0.4
Emamectin Benzoate	2013-14	500	1000	0	0.000	0
	2014-15	652	1304	0	0.000	0
	2015-16	628	1256	0	0.000	0
	2016-17	932	1864	0	0.000	0

This monitoring program was conducted by NSW Department of Primary Industries with support from the Cotton Research and Development Corporation (CRDC) and the Grains Research and Development Corporation (GRDC)

Appendix 5

Publications

Baseline Susceptibility of *Helicoverpa armigera* (Lepidoptera: Noctuidae) to Indoxacarb, Emamectin Benzoate, and Chlorantraniliprole in Australia

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ABSTRACT Baseline susceptibility of *Helicoverpa armigera* (Hübner) to emamectin benzoate, chlorantraniliprole, and indoxacarb was determined in feeding assays on insecticide-incorporated artificial diet in the laboratory. The intraspecific variation of *H. armigera* was established from field populations collected between September 2012 and March 2013, primarily from commercial farms across eastern Australia. Emamectin benzoate had the highest toxicity with a median lethal concentration (LC_{50}) of 0.01 µg/ml diet ($n = 20$ strains). The LC_{50} for chlorantraniliprole was 0.03 µg/ml diet ($n = 21$ strains), while indoxacarb had the lowest relative toxicity with an average LC_{50} of 0.3 µg/ml diet ($n = 22$ strains). Variation in susceptibility amongst field strains was 2.3-fold for emamectin benzoate and 2.9-fold for chlorantraniliprole and indoxacarb. Discriminating concentrations of 0.2, 1, and 12 µg of insecticide per milliliter of diet for emamectin benzoate, chlorantraniliprole, and indoxacarb, respectively, were calculated from toxicological data from field *H. armigera* strains as a first step in resistance management of these classes of insecticide in Australia. The low intraspecific tolerance, high slope values, and goodness-of-fit to a probit binomial model obtained in this study suggest that a feeding assay using diet incorporated insecticide is an effective laboratory method for measuring the dose–responses of these classes of insecticides in *H. armigera*.

KEY WORDS insecticide, *Helicoverpa armigera*, resistance monitoring, emamectin benzoate, chlorantraniliprole

Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner), is major pest of field crops in Australasia, Asia, Africa, and South America and has developed resistance to many conventional insecticides including synthetic pyrethroids (Forrester et al. 1993), organophosphates (Gunning et al. 1998), carbamates (Gunning et al. 1996a), and spinosad (Wang et al. 2009). This capacity to develop resistance is partly due to its distribution, which is one the widest for any insect pest (Fitt 1989). This species is also highly polyphagous, with 72 known host species distributed in 29 families in Australia (Zalucki 1986). In addition, *H. armigera* is a significant pest of cotton with ∼30% of pesticide applications made worldwide used to target this pest (Ahmad 2007).

Since the early 1980s, a resistance monitoring program has been in place for detecting changes in resistance frequencies in Australian field populations of *H. armigera* (Forrester et al. 1993; Gunning et al. 1996b, 1998). The resistance monitoring program is currently part of a broader preemptive insecticide resistance management strategy used primarily by the Australian cotton industry to formulate responses to emerging *H. armigera* resistance issues (Bird et al. 2013). Monitoring methods for the insecticide resistance management strategy were originally developed for broad-spectrum contact insecticides such as synthetic pyrethroids, organophosphates, and endosulfan. However, management of *Helicoverpa* spp., particularly *H. armigera*, is becoming increasingly reliant on newer selective insecticides such as emamectin benzoate, chlorantraniliprole, and indoxacarb because of their low toxicity to beneficial insects (Chukwudebe et al. 1997, Hewa-Kapuge et al. 2003, Bostanian et al. 2004, Gonzalez-Zamora et al. 2004, Dinter et al. 2009) and, therefore, high compatibility with integrated pest management programs.

Techniques for monitoring insecticide resistance in *Helicoverpa* spp. in Australia were originally developed for contact insecticides using topical application of a discriminating concentration of insecticide (Forrester et al. 1993). Resistance to selective chemistries was also monitored in Australia by topical application since they were first commercialized; emamectin benzoate in 2000, indoxacarb in 2001, and chlorantraniliprole in 2008 (Rossiter et al. 2008). However, emamectin benzoate, chlorantraniliprole, and indoxacarb intoxicate insects via both contact and ingestion, with the latter considered the primary route whereby insects accumulate a lethal dose of insecticide (Lasota and Dybas 1991, Wing et al. 2004, Temple et al. 2009).

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Emamectin benzoate is a semisynthetic insecticide derived from the avermectin family of compounds and belongs to the Insecticide Resistance Action Committee (IRAC) mode of action class 6 (IRAC 2012). Avermectins are naturally occurring macrocyclic lactones isolated from fermentation products of the soil microorganism *Streptomyces avermitilis*. Emamectin benzoate acts as an agonist for gamma-aminobutyric acid-gated chloride channels (Kass et al. 1980). The resulting irreversible activation of chloride channels causes disruption of nerve impulses and rapid paralysis in a range of lepidopteran species (Jansson et al. 1997, Argentine et al. 2002, Ishaaya et al. 2002).

Chlorantraniliprole is an anthranilic diamide belonging to the IRAC mode of action class 28 (IRAC 2012), providing a broad range of activity across a range of lepidopteran species (Temple et al. 2009). As a selective agonist for ryanodine receptors in the muscle cells of insects, it stimulates the release and depletion of calcium from the internal stores in the sarcoplasmic reticulum, causing impaired regulation of muscle contraction leading to feeding cessation (Cordova et al. 2006).

Indoxacarb is a pyrazoline-type sodium channel blocker with activity against a range of lepidopteran, coleopteran, and sucking insect pests (Wing et al. 1998). It belongs to the IRAC mode of action class 22 (IRAC 2012). Metabolism studies in Lepidoptera have shown that indoxacarb is bioactivated to the active *N*-decarbomethoxyllated metabolite, which blocks voltage-dependent sodium channels, preventing the influx of sodium into neurons (Wing et al. 2004).

The objectives of this study were to 1) develop a feeding bioassay method to determine toxicity of the selective insecticides to *H. armigera*, and evaluate the use of this method as an appropriate alternative to the traditional topical method of bioassay; 2) accumulate baseline susceptibility data to determine the full range of intraspecific tolerance in field populations of *H. armigera*; and 3) utilize the baseline data generated to determine discriminating concentrations for emamectin benzoate, chlorantraniliprole, and indoxacarb for use in resistance monitoring programs.

Materials and Methods

Insect Strains. A laboratory-susceptible strain was used to check for consistency of bioassay at 4- to 6-wk intervals throughout the duration of the study. The laboratory strain New GR was established from a cohort of a general laboratory strain GR. Progenitor strains of New GR were sourced during the mid-1980s from a series of collections from cotton fields in the Namoi Valley, northern New South Wales (NSW).

Field strains were primarily sourced from major cropping areas located across NSW and in southern and central Queensland (QLD) in Australia. Populations were established from insects ranging from egg to pupa collected between September 2012 and March 2013 from cotton, sorghum, pigeon pea, maize, pulses, and a scrophulariaceous weed host, *Verbascum virgatum* Stokes. All strains were tested within three generations of establishment in the laboratory. A minimum of 50 field-collected individuals constituted any one geographically distinct strain.

Insect Rearing. Rearing methods used to maintain both field and laboratory strains were the procedures described by Teakle and Jensen (1985) except that formalin was omitted and soybean flour was heat-treated in a microwave for 10 min to remove enzyme inhibitors. Rearing trays (Tacca Plastics, Sydney, Australia) were covered and heat-sealed with perforated lids (Oliver Products, Grand Rapids, MI). Moths were provided with a 4% honey/sugar solution fed through a cotton wick and housed in containers open at the top and covered with cloth liners secured around the lip of the containers.

In the larval stage, insect strains were maintained under a laboratory environment of 25 ± 2°C, a photoperiod of 14:10 (L: D) h, and 45–55% relative humidity. Adults were maintained in a separate facility under the same conditions of light and temperature with relative humidity maintained at 70–80% for the duration of the dark cycle using a steam humidifier.

Insect Bioassays. The dose–responses of laboratory and field strains were measured by performing bioassays on artificial diet into which formulated insecticide was incorporated. The ratio of diet to toxin determined the concentration calculated as micrograms of insecticide per milliliter of diet. Formulated insecticides were diluted in distilled water producing twofold serial insecticide dilutions spanning six or seven insecticide concentrations that were expected to induce from 1 to 99% mortality. Serial dilutions were added to 200 ml of diet and incorporated by vigorous shaking by hand for 30 s to produce a homogenous mixture. Insecticide-incorporated diet was then dispensed into 45-well bioassay trays (Tacca Plastics, Sydney, Australia), each with approximately 1.5 ml of diet per well.

Insects for use in bioassays were reared on untreated diet to the late second or third larval instar and then introduced to trays containing bioassay diet (one larva per well) and covered with heat-sealed, perforated lids. The use of late second- or third-instar larvae ensured that only strains with sufficient fitness were utilized as bioassay subjects. Each bioassay was performed in triplicate with individual treatments (insecticide concentrations) in replicates consisting of a minimum of 20 individuals; untreated diet was used as the control.

Bioassays were performed on a minimum of four nonsynchronous cohorts of New GR between November 2012 and May 2013. The results were pooled in the final analysis because there were no significant differences between cohorts for any of the insecticides tested. The average LC_{50} generated from these bioassays was used as the estimate of baseline susceptibility for the New GR strain.

Bioassays were maintained for 7 d under the same conditions described above for larval rearing. Larvae were considered dead if one or more of the following criteria were demonstrated: larvae unable to perform coordinated movement when prodded; paralysis of prolegs; larvae very slow to right themselves (time exceeding 3 s).

Insecticides. Commercial insecticide formulations were used in all bioassays: indoxacarb (Steward EC [15% active ingredient], Du Pont Australia Ltd., Macquarie Park, Australia), emamectin benzoate (Affirm [1.9% active ingredient], Syngenta Crop Protection), and chlorantraniliprole (Altacor [35% active ingredient], Du Pont Australia Ltd., Macquarie Park, Australia).

Data Analysis. The dose–responses of larvae to insecticides were corrected for control mortality using the formula of Abbott (1925). Slope, LC_{50} and $LC_{99.9}$ estimates, and associated 95% fiducial limits (FLs) were calculated by probit analysis using the POLO-PC software (LeOra Software, Berkeley, CA). The toxicity ratio of emamectin benzoate, indoxacarb, and chlorantraniliprole was calculated by dividing the LC_{50} value of each population by the LC_{50} value (average of four nonsynchronous cohorts) of the laboratory strain.

Results

Toxicity of Insecticides to Laboratory Strain. The relative toxicity of insecticides was highest for the susceptible strain. Emamectin benzoate was the most toxic of the three insecticides tested ($LC_{50} = 0.007$ μg/ml; Table 1). Chlorantraniliprole was twofold less toxic to the laboratory strain ($LC_{50} = 0.014$ μg/ml; Table 2), with indoxacarb the least toxic insecticide tested ($LC_{50} = 0.147$ μg/ml; Table 3).

Toxicity of Insecticides to Field Strains. The response of field populations of *H. armigera* to emamectin benzoate is shown in Table 1. There was a narrow range of variation between field strains with LC_{50} values ranging from 0.007 to 0.017 μg/ml. The dose–responses were accompanied by high slopes (3.5 from the pooled data of 20 field strains). High susceptibility was demonstrated by low toxicity ratios ranging between 1.0 and 2.3. A population collected from chickpea in St. George southern QLD had the highest tolerance, with an $LC_{99.9}$ value of 0.154 μg/ml.

The response of field populations of *H. armigera* to chlorantraniliprole is shown in Table 2. The range of intraspecific variation between field strains to chlorantraniliprole was narrow, with LC_{50} values ranging from 0.016 to 0.048 μg/ml. The pooled slope value from chlorantraniliprole bioassays of 21 strains was 2.5. Low toxicity ratios ranging between 1.1 and 3.4 indicate that susceptibility of field populations was relatively high. A population originating from a soya bean field at Wee Waa in northern NSW had the highest tolerance with an $LC_{99.9}$ value of 1.238 μg/ml.

The response of field populations of *H. armigera* to indoxacarb is shown in Table 3. High susceptibility of field *H. armigera* to indoxacarb was demonstrated by low toxicity ratios ranging between 1.2 and 3.5. There was a narrow range of intraspecific variation in indoxacarb toxicity between field strains, with LC_{50} values ranging from 0.175 to 0.518 μg/ml. The slope value from pooled indoxacarb bioassays of 22 strains was 2.3. The most tolerant strains had $LC_{99.9}$ values >11 μg/ml. In one case (North Star maize and sorghum) the $LC_{99.9}$ exceeded 14 μg/ml. However, this result was accompanied by a low slope value of 1.9.

Determination of Discriminating Concentrations of Insecticide. Discriminating concentrations for use in monitoring resistance to emamectin benzoate, chlorantraniliprole, and indoxacarb in *H. armigera* were based on the $LC_{99.9}$ response of the most tolerant

Table 1. Bioassay on 20 field strains and 1 laboratory-susceptible strain of *H. armigera* tested as early third instars on diet-incorporated emamectin benzoate (19 g/liter) and assessed for mortality at 7 d

Collection data			LC_{50} (μg/ml diet; 95% FL)	$LC_{99.9}$ (μg/ml diet)	Slope ± SE	Toxicity ratio[b]	% Mortality at 0.095 μg/ml diet[c]
Origin of field strains (G[a])	Host crop	Collection date					
Pampas QLD (F_1)	Pigeon pea	Jan. 2013	0.007 (0.006–0.009)	0.047	3.9 ± 0.54	1.0	100 (60)
Nandi QLD (F_1)	Cotton/pigeon pea	Jan. 2013	0.008 (0.006–0.009)	0.027	5.5 ± 1.10	1.0	100 (60)
Wee Waa NSW (F_2)	Sorghum	Dec. 2012	0.008 (0.006–0.009)	0.067	3.3 ± 0.45	1.0	100 (60)
Narromine NSW (F_2)	Maize	Nov. 2012	0.008 (0.005–0.011)	0.046	4.0 ± 0.97	1.0	100 (56)
Wee Waa NSW (F_1)	Soya bean	Feb. 2013	0.009 (0.006–0.013)	0.058	3.8 ± 0.90	1.2	100 (60)
Emerald QLD (F_1)	Chickpea	Sept. 2012	0.009 (0.008–0.010)	0.052	4.1 ± 0.41	1.2	100 (60)
Breeza NSW (F_1)	Pigeon pea	Feb. 2013	0.009 (0.007–0.012)	0.040	4.9 ± 1.09	1.3	100 (60)
Griffith NSW (F_2)	Cotton/pigeon pea	Mar. 2013	0.009 (0.007–0.012)	0.031	5.9 ± 1.40	1.3	100 (59)
Wee Waa NSW (F_2)	Maize	Dec. 2012	0.010 (0.007–0.012)	0.040	4.9 ± 1.08	1.3	100 (56)
Mullaley NSW (F_2)	Maize	Dec. 2012	0.010 (0.009–0.011)	0.060	4.0 ± 0.39	1.4	100 (60)
North Star NSW (F_1)	Maize/sorghum	Mar. 2013	0.010 (0.008–0.013)	0.084	3.4 ± 0.56	1.4	100 (60)
Cecil Plains QLD (F_2)	Cotton	Jan. 2013	0.010 (0.009–0.012)	0.045	4.9 ± 0.52	1.4	100 (60)
Wee Waa NSW (F_1)	Pigeon pea	Jan. 2013	0.011 (0.008–0.014)	0.055	4.3 ± 0.88	1.4	100 (60)
St George QLD (F_1)	Pigeon pea	Jan. 2013	0.011 (0.009–0.014)	0.073	3.8 ± 0.58	1.5	100 (60)
Wee Waa NSW (F_1)	Mung bean	Feb. 2013	0.011 (0.008–0.015)	0.100	3.2 ± 0.61	1.5	100 (60)
St George QLD (F_1)	Pigeon pea	Feb. 2013	0.012 (0.009–0.015)	0.085	3.6 ± 0.53	1.6	100 (60)
ACRI NSW (F_1)	Sorghum	Feb. 2013	0.012 (0.011–0.014)	0.127	3.0 ± 0.28	1.6	98.3 (60)
Felton QLD (F_2)	Canola	Oct. 2012	0.012 (0.010–0.015)	0.146	2.9 ± 0.33	1.7	100 (100)
Loxton QLD (F_1)	Cotton	Jan. 2013	0.013 (0.010–0.017)	0.092	3.7 ± 0.61	1.8	100 (60)
St George QLD (F_2)	Chickpea	Sept. 2012	0.017 (0.013–0.022)	0.155	3.2 ± 0.52	2.3	98.8 (80)
Pooled			0.010 (0.009–0.011)	0.079	3.5 ± 0.19		
Laboratory strain (New GR)			0.007 (0.006–0.009)	0.030	5.1 ± 1.00		100 (60)

[a] Generation tested.
[b] Toxicity ratio = LC_{50} of population/LC_{50} of New GR strain (average of four nonsynchronous New GR cohorts).
[c] Mortality at highest concentration tested.

Table 2. Bioassay on 21 field strains and 1 laboratory-susceptible strain of *H. armigera* tested as early third instars on diet-incorporated chlorantraniliprole (350 g/kg) and assessed for mortality at 7 d

Collection data			LC_{50} (μg/ml diet; 95% FL)	$LC_{99.9}$ (μg/ml diet)	Slope ± SE	Toxicity ratio[b]	% Mortality at 0.5 μg/ml diet[c]
Origin of field strains (G[a])	Host crop	Collection date					
Nandi QLD (F_1)	Cotton/pigeon pea	Jan. 2013	0.016 (0.014-0.019)	0.161	3.1 ± 0.32	1.1	100 (60)
Emerald QLD (F_1)	Chickpea	Sept. 2012	0.018 (0.015-0.021)	0.223	2.8 ± 0.24	1.2	100 (60)
Loxton QLD (F_2)	Cotton	Jan. 2013	0.018 (0.012–0.024)	0.815	1.9 ± 0.28	1.3	100 (59)
ACRI NSW (F_1)	Sorghum	Feb. 2013	0.019 (0.014–0.025)	0.193	3.1 ± 0.54	1.3	100 (60)
Wee Waa NSW (F_1)	Mung bean	Feb. 2013	0.019 (0.016–0.023)	0.304	2.6 ± 0.26	1.4	100 (60)
Griffith NSW (F_2)	Cotton/pigeon pea	Mar. 2013	0.020 (0.015–0.025)	0.148	3.6 ± 0.55	1.4	100 (60)
Walgett NSW (F_2)	Chickpea	Sept. 2012	0.020 (0.015–0.027)	0.590	2.1 ± 0.29	1.4	100 (60)
Narromine NSW (F_2)	Sweet corn	Mar. 2013	0.023 (0.020–0.026)	0.192	3.3 ± 0.33	1.6	100 (60)
St. George QLD (F_1)	Pigeon pea	Feb. 2013	0.023 (0.020–0.026)	0.222	3.1 ± 0.32	1.6	100 (60)
Mullaley NSW (F_2)	Maize	Dec. 2012	0.023 (0.021–0.026)	0.139	4.0 ± 0.40	1.6	100 (60)
Wee Waa NSW (F_1)	Pigeon pea	Jan. 2013	0.024 (0.018–0.032)	0.137	4.1 ± 0.76	1.7	100 (60)
Wee Waa NSW (F_2)	Maize	Dec. 2012	0.025 (0.018–0.033)	0.305	2.8 ± 0.47	1.7	100 (60)
Felton QLD (F_2)	Canola	Oct. 2012	0.027 (0.023–0.032)	0.669	2.2 ± 0.20	1.9	98.3 (60)
St George QLD (F_2)	Chickpea	Sept. 2012	0.027 (0.024–0.031)	0.244	3.2 ± 0.27	1.9	100 (60)
Narromine NSW (F_2)	Maize	Nov. 2012	0.028 (0.022–0.036)	0.403	2.6 ± 0.35	1.9	100 (60)
Pampas QLD (F_2)	Pigeon pea	Jan. 2013	0.030 (0.025–0.036)	0.991	2.0 ± 0.18	2.1	100 (60)
Warren NSW (F_1)	Sunflower	Mar. 2013	0.030 (0.026–0.034)	0.237	3.4 ± 0.32	2.1	100 (60)
Wee Waa NSW (F_2)	Sorghum	Dec. 2012	0.031 (0.026–0.036)	0.505	2.5 ± 0.24	2.1	100 (60)
Breeza NSW (F_1)	Pigeon pea	Feb. 2013	0.035 (0.030–0.041)	0.585	2.5 ± 0.22	2.4	100 (58)
Cecil Plains QLD (F_1)	Cotton	Jan. 2013	0.041 (0.033–0.052)	0.698	2.5 ± 0.29	2.9	100 (80)
Wee Waa NSW (F_2)	Soya bean	Feb. 2013	0.048 (0.037–0.062)	1.238	2.2 ± 0.26	3.4	100 (58)
Pooled			0.025 (0.023–0.027)	0.454	2.5 ± 0.10		
Laboratory strain (New GR)			0.014 (0.013–0.016)	0.106	3.5 ± 0.30		

[a] Generation tested.
[b] Toxicity ratio = LC_{50} of population/LC_{50} of New GR strain (average of four nonsynchronous New GR cohorts).
[c] Mortality at highest concentration tested.

Table 3. Bioassay on 22 field strains and 1 laboratory-susceptible strain of *H. armigera* tested as early third instars on diet-incorporated indoxacarb (150 g/liter) and assessed for mortality at 7 d

Collection data			LC_{50} (μg/ml diet; 95% FL)	$LC_{99.9}$ (μg/ml diet)	Slope ± SE	Toxicity ratio[b]	% Mortality at 6 μg/ml diet[c]
Origin of field strains (G[a])	Host crop	Collection date					
Walgett NSW (F_2)	Chickpea	Sept. 2012	0.175 (0.132–0.229)	2.950	2.5 ± 0.35	1.2	100 (60)
Emerald QLD (F_1)	Chickpea	Sept. 2012	0.196 (0.154–0.248)	1.019	4.3 ± 0.78	1.3	100 (60)
Narromine NSW (F_2)	Sweet corn	Mar. 2013	0.215 (0.169–0.273)	3.881	2.5 ± 0.31	1.5	100 (60)
Griffith NSW (F_2)	Cotton/pigeon pea	Mar. 2013	0.217 (0.160–0.294)	3.843	2.5 ± 0.37	1.5	100 (60)
St George QLD (F_1)	Pigeon pea	Feb. 2013	0.229 (0.158–0.310)	5.285	2.3 ± 0.37	1.6	100 (60)
Wee Waa NSW (F_2)	Maize	Dec. 2012	0.231 (0.188–0.281)	1.665	3.6 ± 0.49	1.6	100 (60)
Warren NSW (F_1)	Sunflower	Mar. 2013	0.245 (0.188–0.312)	2.691	3.0 ± 0.44	1.7	100 (60)
Wee Waa NSW (F_2)	Sorghum	Dec. 2012	0.254 (0.222–0.290)	2.077	3.4 ± 0.33	1.7	100 (60)
ACRI NSW (F_1)	Sorghum	Feb. 2013	0.280 (0.213–0.357)	6.283	2.3 ± 0.29	1.9	100 (60)
Nandi QLD (F_1)	Cotton/pigeon pea	Jan. 2013	0.286 (0.247–0.329)	3.039	3.0 ± 0.28	1.9	100 (60)
St George QLD (F_2)	Chickpea	Sept. 2012	0.286 (0.240–0.342)	9.686	2.0 ± 0.18	1.9	100 (60)
Wee Waa NSW (F_1)	Soya bean	Feb. 2013	0.298 (0.243–0.358)	11.213	2.0 ± 0.19	2.0	100 (60)
Loxton QLD (F_1)	Cotton	Jan. 2013	0.305 (0.223–0.406)	6.731	2.3 ± 0.34	2.1	100 (60)
St George QLD (F_1)	Pigeon pea	Jan. 2013	0.315 (0.241–0.409)	3.702	2.9 ± 0.42	2.1	100 (60)
Pampas QLD (F_1)	Pigeon pea	Jan. 2013	0.327 (0.241–0.439)	4.898	2.6 ± 0.42	2.2	100 (60)
North Star NSW (F_1)	Maize/sorghum	Mar. 2013	0.346 (0.288–0.415)	14.803	1.9 ± 0.15	2.3	100 (60)
Breeza NSW (F_1)	Pigeon pea	Feb. 2013	0.348 (0.287–0.416)	12.372	2.0 ± 0.18	2.4	100 (60)
Mullaley NSW (F_2)	Maize	Dec. 2012	0.357 (0.287–0.441)	4.579	2.8 ± 0.33	2.4	100 (60)
Wee Waa NSW (F_2)	Pigeon pea	Jan. 2013	0.359 (0.303–0.422)	7.592	2.3 ± 0.21	2.4	100 (60)
Cecil Plains QLD (F_1)	Cotton	Jan. 2013	0.440 (0.339–0.579)	11.190	2.2 ± 0.30	3.0	100 (60)
Narromine NSW (F_2)	Maize	Nov. 2012	0.447 (0.350–0.563)	11.917	2.2 ± 0.23	3.0	100 (60)
Wee Waa NSW (F_1)	Mung bean	Feb. 2013	0.518 (0.405–0.659)	11.864	2.3 ± 0.25	3.5	98.3 (60)
Pooled			0.291 (0.270–0.314)	6.812	2.3 ± 0.10		
Laboratory strain (New GR)			0.147 (0.137–0.158)	1.172	3.4 ± 0.19		

[a] Generation tested.
[b] Toxicity ratio = LC_{50} of population/LC_{50} of New GR strain (average of four nonsynchronous New GR cohorts).
[c] Mortality at highest concentration tested.

strains, and the empirical response to the highest concentration of the insecticide tested. In each case, a very low rate of survival was observed at the highest concentration of the serial dose response. To ensure that the discriminating concentration was rigorous enough to remove false positives, the concentration was set at double the dose at which this low rate of survival was observed.

Table 4. Comparison of toxicity of insecticides on a laboratory-susceptible strain using topical and feeding methods of bioassay

Insecticide	LD_{50} (μg/ml; 95% FL)	Slope ± SE	Reference
Emamectin benzoate	7.4 (5.9–9.0)	1.7 ± 0.10	Bird and Downes (2014)
Chlorantraniliprole	3.1 (2.4–3.9)	1.9 ± 0.12	Bird and Downes (2014)
Indoxacarb	25.5 (22.8–28.4)	4.0 ± 0.26	Bird and Downes (2014)

Empirical mortality observed at the highest concentration of the serial dose–response for emamectin benzoate (0.095 μg/ml), which produced between 98.3 and 100% mortality, is shown in Table 1. On the basis of the observed response to the highest concentration of emamectin benzoate tested, and taking into account the $LC_{99.9}$ value of the most tolerant strains, the discriminating concentration for emamectin benzoate is recommended as 0.2 μg/ml. Using observed data from the highest concentration of chlorantraniliprole tested (0.5 μg/ml; Table 2), and the $LC_{99.9}$ estimates of mortality in the most tolerant strain, a discriminating concentration for chlorantraniliprole of 1 μg/ml is recommended. To test the suitability of a discriminating concentration, strains were assessed for mortality at a dose of 6 μg/ml and mortality ranged from 98.3 to 100% (Table 3). Using these data and the $LC_{99.9}$ estimates of mortality in the most tolerant strains, the recommended discriminating concentrations for indoxacarb is 12 μg/ml.

Comparison of Toxicity of Insecticides Using Topical and Feeding Bioassay Methods. In the laboratory-susceptible stain, emamectin benzoate, chlorantraniliprole, and indoxacarb were more toxic when administered orally in a feeding bioassay as compared with contact activity when administered topically (Table 4). The slope values associated with the dose–response from the feeding bioassay were also higher than that for the topical bioassay (Table 4). In particular, the slopes were markedly higher in feeding bioassays for both emamectin benzoate and chlorantraniliprole with values of 5.1 and 3.5, respectively, as compared with slopes of 1.7 and 1.9 for emamectin benzoate and chlorantraniliprole, respectively, in topical tests.

Discussion

The use of a discriminating concentration technique in resistance monitoring programs is considered to be a highly efficient method for monitoring resistance in insect populations (Roush and Miller 1986). A discriminating concentration technique based on topical application of insecticide has been used in *Helicoverpa* spp. resistance monitoring in Australia since the mid-1980s. This technique was originally developed for broad-spectrum insecticides whereby toxicity is mediated primarily through contact mode of entry (Forrester et al. 1993). Although topical bioassays produce usable dose–response curves, they can be associated with low slope values.

More recently, selective chemistries have been introduced for which intoxication occurs primarily by ingestion (Lasota and Dybas 1991, Wing et al. 2004, Temple et al. 2009). Results from the present study demonstrate that emamectin benzoate, chlorantraniliprole, and indoxacarb are more toxic when administered by ingestion than orally in *H. armigera*. This study also demonstrates that high slope values are associated with the dose–response regressions from diet-incorporation bioassays. This suggests that delivery by ingestion using a diet-incorporation method of bioassay is highly effective for assessing the toxicity of these chemistries and would be an appropriate alternative method for use in resistance monitoring programs.

Ideally, baseline susceptibility should be assessed before product commercialization, particularly for products with narrow spectra of activity, or where activity is concentrated against pests with historical problems of insecticide resistance (ffrench-Constant and Roush 1990, Jutsum et al. 1998). After more than a decade of commercial use of indoxacarb and emamectin benzoate, and several years of commercial use of chlorantraniliprole in Australia, increased resistance frequencies to these chemistries have yet to be reported from direct field screening of Australian populations of *H. armigera* using established topical bioassay methods (L.J.B. unpublished data). Results from the present study demonstrate high susceptibility in Australian populations of *H. armigera* to emamectin benzoate, chlorantraniliprole, and indoxacarb along with a narrow range (less than threefold) of intraspecific variation between geographically diverse populations. Notwithstanding the long-term registration of these products in Australia, it is unlikely that these small differences are the result of resistance selection. Rather they are likely to be the result of natural variability reflecting the true range of intraspecific tolerance inherent in this species to these chemistries.

Low intraspecific variation in baseline sensitivity to emamectin benzoate and indoxacarb has also been reported among populations of *H. armigera* from Pakistan (Ahmad et al. 2003), with low variation in baseline chlorantraniliprole toxicity reported for field populations of other noctuid species including *Spodoptera litura* (F.) (Su et al. 2012) and *Helicoverpa zea* (Boddie) (Temple et al. 2009). However, a narrow range of susceptibility among intraspecific populations may not preclude the potential to respond to selection pressure (Tabashnik 1994). For example, despite a low (less than fivefold) range of variation observed in baseline studies of chlorantraniliprole toxicity to *Plutella xylostella* (L.) (Wang et al. 2010), subsequent reports suggest rapid resistance development in this species (Wang and Wu 2012).

Increased detoxification enzyme activity in pest populations is considered one of the most important factors

for development of insecticide resistance (Denholm and Roland 1992, Li et al. 2007). Enhanced activities of mixed function oxidase and esterase enzymes are associated with pyrethroid resistance in Australian populations of *H. armigera* (Gunning et al. 1996b, Joußen et al. 2012, Teese et al. 2013). Increased metabolic capability has also been implicated as a mechanism for resistance in *H. armigera* to selective insecticidal chemistries and insecticidal toxins from the bacterium *Bacillus thuringiensis* (Gunning et al 2005, Alvi et al. 2012). High susceptibility to emamectin benzoate, chlorantraniliprole, and indoxacarb in Australian *H. armigera* with existing high frequencies of resistance to fenvalerate (L.J.B. unpublished data) suggest a lack of cross-resistance to pyrethroid. Further studies involving the use of enzyme inhibitors are required to confirm this. A lack of cross-resistance to indoxacarb was also found in pyrethroid-resistant populations of *H. armigera* from Pakistan (Ahmad et al. 2003) and Central Africa (Achaleke et al. 2009), and in pyrethroid-resistant strains of *P. xylostella* (Lasota et al. 1996, Yu and McCord 2007). On the other hand, pyrethroid-selected strains of *H. armigera* from India and Australia were shown to be negatively cross-resistant to indoxacarb (Gunning and Devonshire 2002, Ramasubramanian and Regupathy 2004).

Determination of a discriminating concentration of insecticide for resistance monitoring is an empirical compromise based, firstly, on the limits of tolerance, and secondly on a concentration of insecticide that kills most (99.9%) of the susceptible individuals. Various methods can be used to assign a discriminating concentration for use in resistance monitoring (Busvine 1971). But regardless of the method, it is important to identify a concentration that is sufficiently high so as to effectively discriminate between resistant and susceptible phenotypes without detecting false positives. On the other hand, the concentration should not be so high so as to mask resistance.

In the present study, determination of discriminating concentration is based on a theoretical estimate of the highest $LC_{99.9}$ value, while taking into account the empirical mortality observed at the upper limits of the dose–response curve (Robertson et al. 2007). Based on these criteria, we propose a discriminating concentration of 0.2, 1, and 12 μg/ml of diet for emamectin benzoate, chlorantraniliprole, and indoxacarb, respectively.

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Research article

Insecticide Resistance and Resistance Management

Susceptibility of *Helicoverpa armigera* (Lepidoptera: Noctuidae) to Cyantraniliprole Determined From Topical and Ingestion Bioassays

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Abstract

The use of insect bioassay to establish baseline susceptibility and monitor changes in sensitivity to insecticides over time has been a key component of resistance management of *Helicoverpa armigera* Hübner in Australia for over 30 yr. Cyantraniliprole is a recently introduced insecticide, where toxicity is mediated at the ryanodine receptor. Baseline susceptibility of *H. armigera* to cyantraniliprole was determined in both topical and ingestion assays performed on field populations collected primarily from commercial farms across eastern Australia. Intraspecific variation in cyantraniliprole susceptibility amongst field strains was 9.3-fold in topical bioassays ($n = 23$ strains) and 2.6-fold in ingestion bioassays ($n = 31$ strains). The median lethal concentration in field strains was 28 mg/liter in topical bioassays and 0.065 mg/liter in ingestion bioassays, demonstrating that cyantraniliprole was >400-fold more toxic when administered orally than by contact. The narrow range of intraspecific tolerance, high slope values, goodness-of-fit to the probit binomial model, and enhanced toxicity in diet incorporation bioassays compared with topical bioassays suggest that delivery by ingestion is an effective laboratory method for measuring the dose-response of cyantraniliprole in *H. armigera*. A discriminating dose of 1.5 mg of cyantraniliprole per liter of diet was calculated from diet incorporation bioassays, as a first step in resistance management of cyantraniliprole in Australia.

Key words: insecticide resistance monitoring, anthranilic diamide, Exirel, discriminating dose

The cotton bollworm, *Helicoverpa armigera* (Hübner), is a key pest of agriculture in many regions of the world including Australia, Asia, Africa, and South America. *Helicoverpa armigera* is well adapted to exploiting a range of cropping systems and has demonstrated a global historical trend for developing resistance to a wide range of conventional insecticides (McCaffrey 1998). The capacity of *H. armigera* to develop resistance is due to several unique ecological characteristics including a wide range of distribution, high fecundity, multiple generations per year, migratory behavior, and the ability to undergo a pupal diapause (Fitt 1989).

Since the development of high-level resistance to broad-spectrum insecticides, management of *H. armigera* has become increasingly reliant on selective products such as anthranilic diamides, classified as Group 28 insecticides under the IRAC mode of action system of classification (Insecticide Resistance Action Committee [IRAC] 2012). This chemical class is a potent and selective activator of insect ryanodine receptors that affects calcium metabolism by stimulating release and depletion of calcium from internal stores in the sarcoplasmic reticulum, causing impaired regulation of muscle contraction leading to feeding cessation and death (Cordova et al. 2006). Anthranilic diamides show low mammalian toxicity (Lahm et al. 2009) with selectivity due to the structural differences between insect and mammalian ryanodine receptors (Sattelle et al. 2008). Favorable acute toxicological profiles extend to nontarget organisms (Dinter et al. 2009) and, therefore, make diamide insecticides highly compatible for use within integrated pest management systems.

Cyantraniliprole is a substituted anthranilic diamide and the second of its type to be introduced for control of *H. armigera* in cotton in Australia, following the earlier introduction of chlorantraniliprole in 2008 (Australian Pesticides and Veterinary Medicines Authority [APVMA] 2015). While chlorantraniliprole activity is specific for lepidopteran species, cyantraniliprole has a wider insecticidal spectrum, with additional activity on hemipteran pests such as *Bemisia tabaci* (Gennadius) (Caballero et al. 2013, Grávalos et al. 2014), *Myzus persicae* (Sulzer) (Barry et al. 2014), *Diaphorina citri* Kuwayama (Tiwari and Stelinski 2013), as well as dipteran, coleopteran, and thysanopteran pests (Jacobson and Kennedy 2011, Herron et al. 2014, Zhang et al. 2014, Caballero et al. 2015).The unique mode of action, high potency, and residual activity make anthranilic diamides a key management tool in a range of cropping systems that play host to *H. armigera*. But resistance risk for this group of insecticides is high, based on the demonstrated capacity of

H. armigera to develop resistance to other insecticides (McCaffrey 1998). Because of the expected increase in the use of anthranilic diamide insecticides in Australia and the possible issues of cross-resistance between different Group 28 products, it is necessary to establish baseline susceptibility of *H. armigera* to this insecticide, and to use these data as reference values to diagnose early changes in susceptibility of field populations (Jutsum et al. 1998).

Methods for measuring dose-response of synthetic insecticides in *H. armigera* were originally developed for broad-spectrum contact insecticides, whereby toxicity is mediated primarily through contact by topical application of insecticide (Forrester et al. 1993). In contrast, anthranilic diamides intoxicate insects by both contact and ingestion, with the later considered the primary route, whereby insects accumulate a lethal dose of insecticide (Temple et al. 2009).

The objectives of this study were to 1) investigate the relative toxicity of cyantraniliprole measured by a traditional topical bioassay method compared with a diet incorporation bioassay to determine the optimal method for evaluating dose-response in *H. armigera*, 2) establish baseline susceptibility data to determine a representative range of intraspecific tolerance in field populations of *H. armigera* to cyantraniliprole, and 3) utilize those baseline data to calculate a discriminating dose for cyantraniliprole against *H. armigera* for use in resistance monitoring.

Materials and Methods

Insect Strains

Field strains were established from insect life stages ranging from egg to pupa collected between September 2012 and March 2013 from cotton, sorghum, pigeon pea, maize, pulses, and the scrophulariaceous weed host, *Verbascum virgatum* Stokes. A minimum of 50 field collected individuals constituted any one geographically distinct strain. Insects were primarily sourced from major cropping areas located across New South Wales (NSW) and in southern and central Queensland. All strains were tested within two generations of laboratory establishment.

A laboratory strain, New GR, was used to check for consistency of response to cyantraniliprole at 4- to 6- wk intervals throughout the duration of the study. This strain was established from a cohort of a general laboratory strain GR. Progenitor strains of GR were sourced during the mid-1980s from a series of collections from cotton fields in the Namoi Valley, northern New South Wales.

Insect Rearing

Rearing methods used were as described by Teakle and Jensen (1985) except that formalin was omitted and soybean flour was heat treated in a microwave for 10 min to remove enzyme inhibitors. Rearing trays (Tacca Plastics, Sydney, Australia) were covered with heat-sealed perforated lids (Oliver Products, Grand Rapids, MI). Pupae were washed in 1% bleach solution and transferred to 5-liter round containers open at the top and covered with cloth liners secured around the lip of the containers. Moths were provided with a 4% honey–sugar solution fed through a cotton wick. Eggs were harvested by washing cloth liners in 1% bleach and collecting them on a Whatman number 54 filter paper by vacuum filtration. Filter papers were allowed to air dry and were then placed in sealed plastic bags until emergence of neonates.

In the larval stage, insects were maintained at 25 ± 2°C, 45–55% relative humidity (RH), and a photoperiod of 14:10 (L:D) h. Adults were maintained in a separate facility under the same conditions of light and temperature but with RH maintained at 70–80% for the duration of the dark cycle using a steam humidifier.

Insecticides

Insecticide solutions used in topical bioassays were prepared from technical cyantraniliprole (97.1% active ingredient [ai]) provided by DuPont Australia Ltd. (Macquarie Park, Australia) and dissolved in analytical grade acetone. Insecticide solutions used in diet incorporation bioassays were prepared from a commercial formulation, Exirel (100 g ai/liter) provided by DuPont Australia Ltd., and diluted in distilled water. Stock solutions were serially diluted to produce a series of six or seven concentrations expected to induce one to 99% mortality.

Bioassays

Topical bioassays were performed according to the methods described by Forrester et al. (1993). Third- or fourth-instar larvae within a weight range of 30–40 mg were treated with 1 µl aliquots of acetone–insecticide solution on the dorsal thorax using a 50 µl microsyringe in a repeating dispenser (Hamilton Company, Reno, NV). Trays containing larvae were then covered with heat-sealed perforated lids. Each bioassay was performed on three cohorts of insects with individual treatments (insecticide concentrations) of each cohort consisting of a minimum of 20 individuals; acetone alone was used as the control.

Insects used in diet incorporation assays were reared to the late second or third larval instar. Serial dilutions of formulated insecticide were added to 200 ml of diet at a ratio of 20:1 and incorporated by vigorous shaking by hand for 30 s to produce a homogenous mixture. Diet was dispensed into 45-well bioassay trays (Tacca Plastics, Sydney, Australia) with each well containing ~1.5 ml of diet. Larvae were introduced to trays (one larva per well) and covered, as above. Each bioassay was performed on three cohorts of insects, as above; untreated diet was used as the control.

Bioassays were performed on nonsynchronous cohorts of New GR twice using the topical method, and four times using the ingestion method. The results for each method were pooled in the final analysis because there were no significant differences between cohorts tested. The pooled LC_{50} generated from both types of bioassays was used as the estimate of baseline susceptibility for the New GR strain.

Topical bioassays were maintained for 4 d, and diet incorporation bioassays were maintained for 7 d under the same conditions described earlier for larval rearing. Larvae were considered dead if one or more of the following criteria were demonstrated: larvae unable to perform coordinated movement when prodded; paralysis of prolegs; and larvae unable to right themselves within 3 s.

Statistical Analysis

Dose-responses were corrected for control mortality using the formula of Abbott (1925). Probit regressions including slope LD_{50} and associated 95% fiducial limits were calculated using stand-alone software developed by Barchia (2001). Significant heterogeneity was identified using a χ^2 test, and, if significant at the 5% level, the variance of the estimated parameter is scaled by the corresponding heterogeneity factor equal to the residual mean deviance (Finney 1971). Toxicity ratio was calculated by dividing the LC_{50} of each field population by the LC_{50} of the laboratory strain New GR (pooled result from bioassays of four nonsynchronous cohorts).

Results

Topical Bioassay

Results from these bioassays are shown in Table 1. The LD_{50} values of field strains ranged from 7 to 65 mg/liter, with a pooled result of 28 mg/liter representing a 9.3-fold range of variation among populations across geographic locations. The most susceptible field strain(s) were more sensitive to cyantraniliprole than laboratory strain New GR, while the most tolerant strain came from Loxton in 2013 (Table 1). Consistency of bioassay was measured in two populations tested in consecutive generations (Boggabri chickpea collected in 2011 and St George chickpea collected in 2012). In both cases, the LD_{50} was similar based on overlap of 95% fiducial limits and demonstrates repeatability of this method of bioassay. The dose-response in topical bioassays was generally accompanied by low slopes ranging from 1.1 to 2.3, with a pooled slope value of 1.5. General goodness-of-fit to the probit binomial model was indicated by significant deviation from the model in five of the 23 field strains tested (St George F_1 chickpea, $P = 0.041$; Cecil Plains cotton, $P = 0.010$; Boggabri chickpea F_1, $P = 0.043$; Walgett chickpea, $P = 0.034$; and Wee Waa pigeon pea, $P = 0.047$; Table 1).

Diet Incorporation Bioassay

Results from these bioassays are shown in Table 2. There was a narrow range of variation amongst field strains (2.6-fold), with the LC_{50} ranging from 0.043 to 0.113 mg/liter (pooled result of 0.068 mg/liter). The most susceptible strain was the laboratory strain New GR, while the most tolerant strains were established from cotton and pigeon pea in the Darling Downs region in early 2013. These two strains (Pampas and Cecil Plains) along with three other strains collected from the Namoi valley of northern NSW were further tested for consistency of bioassay, and in all cases, the LC_{50} of consecutive generations were similar based on overlap of 95% fiducial limits, demonstrating repeatability of this method. The dose-response slopes ranged from 2.6 to 5.1 with a pooled slope value of 3.1, which was twofold higher than the topical bioassays. Goodness-of-fit to the probit model was indicated by significant deviation from the model in five of the 31 field strains tested (Walgett chickpea F_2, $P = 0.017$; ACRI sorghum, $P = 0.047$; Griffith cotton or pigeon pea, $P = 0.014$; Mullaley maize F_2, $P = 0.024$; and Pampas pigeon pea F_1, $P = 0.16$; Table 2).

Comparison of Toxicity in Topical and Ingestion Bioassays

In the laboratory strain, the LC_{50} of cyantraniliprole when administered topically was 20 mg/liter, whereas the LC_{50} when administered by ingestion was 0.03 mg/liter. This demonstrated that cyantraniliprole was 667-fold more toxic by ingestion than by contact in the laboratory strain. Field strains demonstrated a similar response to the susceptible strain. The LD_{50} of cyantraniliprole when administered topically was 28 mg/liter (pooled from 23 strains tested), whereas the LC_{50} when administered in diet was 0.068 mg/liter (pooled from 31 strains tested), demonstrating that cyantraniliprole was 412-fold more toxic by ingestion than by contact.

Establishment of Discriminating Doses

To validate the suitability of candidate discriminating doses, where possible strains were assessed at 1,000 and 2,000 mg/liter in topical bioassays (Table 1) and 0.5 and 1.0 mg/liter in diet incorporation bioassays (Table 2). At the highest dose tested, 100% mortality was observed in both types of bioassay. Empirical data from 1.0 mg/liter of diet-incorporated cyantraniliprole are consistent with the predicted theoretical $LC_{99.9}$ value and indicate that the dose will not exceed 1.3 mg/liter (Table 2). In contrast, the estimate of a discriminating dose from empirical evidence at 2,000 mg/liter of topically applied cyantraniliprole is markedly lower than that predicted by $LD_{99.9}$ values (up to 8,000 mg/liter; Table 1).

Discussion

Topical and ingestion bioassays are both serviceable methods for assessing the sensitivity of lepidopteran pest species to anthranilic diamide insecticides (Temple et al. 2009). However, this is the first study to compare the toxicity of an anthranilic diamide with *H. armigera* measured from both topical and oral administration. Results suggest that there are significant limitations associated with the use of a topical bioassay method for assessing activity of cyantraniliprole against *H. armigera*. This likely relates to mode of action where anthranilic diamides act primarily by ingestion and secondarily by contact. Therefore, topical bioassays may underestimate the total toxicity of insecticide (Jiang et al. 2012). Similarly, results from a study of chlorantraniliprole activity on *H. armigera* found this closely related anthranilic diamide to be 221-fold more toxic when administered orally than by topical application (Bird 2015). Although there was potential for interaction with additive ingredients in the cyantraniliprole formulation used in ingestion bioassays, it is likely that any interaction was minor in comparison with the magnitude of toxicity demonstrated by this method.

Another key indicator of bioassay suitability is probit regression slope value. In some cases, low slope values may be indicative of genetic variance, and in other cases may be due to environmental factors which may be reduced by improved application techniques to increase the predictive value of the regression (Roush and Daly 1990). Slopes were higher in ingestion bioassays compared with topical bioassays, providing further support for the use of a feeding method of delivery in favor of topical application for the purposes of assessing toxicity of this insecticide. Further, ingestion bioassays have previously been found appropriate against chlorantraniliprole and *Heliothis virescens* (F.), *Helicoverpa zea* (Boddie), and *Spodoptera frugiperda* (J.E. Smith) (Temple et al. 2009).

The favorable attributes of anthranilic diamide insecticides can lead to rapid adoption with a high risk for excluding alterative chemistry insecticides (Teixeira and Andaloro 2013). In Australia, a strategy for managing insecticide resistance in *H. armigera* has been in place for over three decades, as resistance was first detected to synthetic pyrethroids in field populations of *H. armigera* (Gunning et al. 1984). The resistance monitoring program for *H. armigera* is an important component of a broader preemptive insecticide resistance management strategy used primarily by the Australian Cotton Industry for formulating responses to emerging resistance issues (Bird et al. 2015).

After several years of commercial use, increased resistance frequencies to anthranilic diamides has yet to be reported from field screening of *H. armigera* (Bird et al. 2015). Low intraspecific variation in baseline sensitivity to cyantraniliprole reported here also suggests that general anthranilic diamide susceptibility remains high. However, high levels of homogeneity and susceptibility in field populations may not preclude the potential to respond to selection pressure. For example, despite a low (<5-fold) range of variation observed in baseline studies of chlorantraniliprole toxicity to *Plutella xylostella* (L.) (Wang et al. 2010), subsequent reports

Table 1. Bioassay on 23 strains of *H. armigera* collected from a range of hosts in 2011–2013, tested as 30–40 mg third or fourth instars with a topical application of cyantraniliprole and assessed for mortality at 4 d posttreatment

Collection data			LD_{50} (mg/liter) (95% FL)	$LC_{99.9}$ (mg/liter)	Toxicity ratio[b]	Fit of probit line			% Mortality (n)[c]	
Origin of field strains (G_a)	Host	Collection date				Slope ± SE	χ^2 (df)	P	1,000 mg/liter	2,000 mg/liter
Walgett NSW (F_2)	Chickpea	Sept. 2012	7.154 (5.536, 8.803)	278.521	0.4	1.9 ± 0.21	2.22 (4)	0.696	100 (60)	–
Felton QLD (F_2)	Canola	Oct. 2012	9.132 (6.777, 11.674)	1087.438	0.5	1.5 ± 0.14	7.51 (6)	0.276	100 (60)	100 (52)
Wee Waa NSW (F_2)	Sorghum	Dec. 2012	12.513 (9.479, 15.895)	2118.966	0.7	1.4 ± 0.12	10.90 (7)	0.143	98.3 (60)	100 (60)
Goondiwindi 1 QLD (F_2)	Pigeon pea	Mar. 2012	14.321 (11.867, 17.198)	1087.200	0.7	1.6 ± 0.12	8.35 (7)	0.303	100 (60)	–
Mullaley NSW (F_1)	Maize	Dec. 2012	15.708 (11.309, 20.773)	7974.336	0.8	1.1 ± 0.10	5.03 (7)	0.656	100 (57)	100 (60)
St George QLD (F_1)*	Chickpea	Sept. 2012	17.206 (10.971, 25.042)	2315.585	0.9	1.5 ± 0.17	14.64 (7)	0.041	100 (60)	–
Cecil Plains QLD (F_2)	Cotton	Feb. 2012	21.822 (13.280, 37.563)	1749.778	1.1	1.6 ± 0.23	16.68 (6)	0.010	100 (39)	100 (60)
ACRI NSW (F_1)	Sorghum	Feb. 2013	25.175 (19.754, 31.568)	3429.341	1.3	1.4 ± 0.12	12.72 (7)	0.079	100 (52)	–
Emerald QLD (F_1)	Chickpea	Sept. 2012	24.846 (19.534, 31.023)	4578.563	1.3	1.4 ± 0.11	6.11 (7)	0.527	98.3 (60)	100 (58)
Boggabri NSW (F_1)*	Chickpea	Oct. 2011	30.629 (22.294, 42.037)	1453.308	1.6	1.8 ± 0.19	14.51 (7)	0.043	100 (60)	100 (60)
St George QLD (F_2)*	Chickpea	Sept. 2012	31.198 (24.674, 38.935)	6563.941	1.6	1.3 ± 0.10	9.75 (7)	0.203	100 (60)	100 (54)
Walgett NSW (F_1)	Chickpea	Sept. 2011	35.113 (25.086, 50.731)	2882.751	1.8	1.6 ± 0.18	13.61 (6)	0.034	100 (60)	100 (60)
Rocky Creek NSW (F_3)	Weeds	Nov. 2011	36.144 (28.796, 44.524)	3673.237	1.8	1.6 ± 0.13	2.80 (6)	0.834	98.4 (64)	100 (60)
Mullaley NSW (F_1)	Chickpea	Oct. 2011	37.164 (30.908, 44.682)	1584.386	1.9	1.9 ± 0.14	8.07 (7)	0.327	100 (60)	100 (60)
Boggabri NSW (F_2)*	Chickpea	Oct. 2011	37.391 (30.908, 44.765)	1553.612	1.9	1.9 ± 0.16	7.23 (5)	0.204	–	–
Wee Waa NSW (F_1)	Pigeon pea	Jan. 2012	38.186 (26.616, 53.224)	842.410	1.9	2.3 ± 0.29	11.25 (5)	0.047	98.3 (60)	100 (57)
Griffith NSW (F_1)	Cotton/Pigeon pea	Mar. 2013	39.156 (32.997, 46.392)	1318.680	2.0	2.0 ± 0.15	9.55 (7)	0.216	100 (60)	–
Bellata NSW (F_1)	Sorghum	Nov. 2011	41.191 (32.996, 51.943)	6498.648	2.1	1.4 ± 0.13	10.37 (5)	0.066	–	–
ACRI NSW (F_1)	Maize	Dec. 2011	41.729 (34.747, 49.802)	1597.674	2.1	2.0 ± 0.16	2.84 (5)	0.725	100 (60)	100 (60)
Goondiwindi 2 QLD (F_2)	Pigeon pea	Mar. 2012	42.704 (35.001, 52.081)	4411.118	2.2	1.6 ± 0.10	5.07 (8)	0.750	100 (60)	–
Narromine NSW (F_1)	Maize	Dec. 2012	56.923 (47.397, 68.324)	3087.184	2.9	1.8 ± 0.12	4.69 (7)	0.698	100 (60)	100 (56)
Moree NSW (F_2)	Chickpea	Oct. 2011	59.537 (49.352, 71.504)	3147.703	3.0	1.8 ± 0.14	3.76 (6)	0.709	100 (60)	100 (60)
Loxton QLD (F_2)	Cotton	Jan. 2013	65.494 (52.642, 81.182)	2565.326	3.3	1.9 ± 0.18	3.60 (6)	0.731	100 (40)	100 (58)
Pooled			27.871 (25.893, 29.967)	3262.360		1.5 ± 0.03				
Laboratory strain New GR			19.656 (17.441, 22.135)	721.638		2.0 ± 0.10	6.82 (7)	0.448	100 (159)	–

G_aGeneration tested.

[b]Toxicity ratio = LC_{50} of population/LC_{50} of New GR strain (average of four nonsynchronous New GR cohorts).

[c]Mortality at highest concentration tested.

* Strains from the same location tested in consecutive generations to test consistency of bioassay.

Table 2. Bioassay on 31 strains of *H. armigera* collected from a range of hosts in 2011–2013, tested as late-second or early-third instars on diet-incorporated cyantraniliprole and assessed for mortality at 7 d posttreatment

Collection data			LC_{50} [mg/liter diet] (95% FL)	$LC_{99.9}$ [mg/liter diet]	Toxicity ratio[b]	Fit of probit line			% Mortality[c]	
Origin of field strains (G_a)	Host	Collection date				Slope ± SE	χ^2 (df)	*P*	0.5 mg/liter diet	1.0 mg/liter diet
Boggabri NSW (F_2)	Chickpea	Oct. 2011	0.043 (0.037, 0.050)	0.667	1.4	2.6 ± 0.21	9.33 (5)	0.097	–	–
Goondiwindi 2 QLD (F_2)	Pigeon pea	Mar. 2012	0.043 (0.038, 0.048)	0.182	1.4	4.9 ± 0.53	4.16 (2)	0.125	–	–
ACRI NSW (F_1)	Maize	Dec. 2011	0.044 (0.039, 0.049)	0.271	1.4	3.9 ± 0.37	3.81 (4)	0.432	–	–
Walgett NSW (F_2)*	Chickpea	Sept. 2011	0.044 (0.032, 0.061)	0.387	1.4	3.3 ± 0.48	13.86 (5)	0.017	–	–
Bellata NSW (F_2)	Sorghum	Nov. 2011	0.045 (0.040, 0.051)	0.268	1.5	4.0 ± 0.38	8.80 (4)	0.066	–	–
North Star NSW (F_1)	Maize or Sorghum	Mar. 2013	0.045 (0.039, 0.052)	0.520	1.5	2.9 ± 0.28	1.82 (3)	0.610	100 (60)	100 (60)
Rocky Creek NSW (F_1)	Weeds	Nov. 2011	0.047 (0.041, 0.053)	0.320	1.6	3.7 ± 0.35	0.92 (4)	0.922	–	–
Walgett NSW (F_1)*	Chickpea	Sept. 2011	0.048 (0.041, 0.056)	0.785	1.6	2.6 ± 0.23	1.85 (4)	0.763	100 (59)	–
St George QLD (F_2)	Chickpea	Sept. 2012	0.050 (0.044, 0.057)	0.360	1.6	3.6 ± 0.34	3.15 (3)	0.369	99.0 (98)	100 (60)
Nandi QLD (F_2)	Cotton or Pigeon pea	Jan. 2013	0.052 (0.046, 0.057)	0.207	1.7	5.1 ± 0.56	0.56 (3)	0.906	100 (60)	100 (60)
ACRI NSW (F_1)	Sorghum	Feb. 2013	0.054 (0.041, 0.072)	0.262	1.7	4.6 ± 0.72	9.63 (4)	0.047	100 (60)	100 (60)
Wee Waa NSW (F_1)	Maize	Nov. 2011	0.056 (0.050, 0.063)	0.281	1.8	4.4 ± 0.45	3.67 (3)	0.299	–	–
Griffith NSW (F_1)	Cotton or Pigeon pea	Mar. 2013	0.058 (0.041, 0.082)	0.350	1.9	4.0 ± 0.68	12.55 (4)	0.014	100 (60)	–
Goondiwindi 1 QLD (F_2)	Pigeon pea	Mar. 2012	0.060 (0.053, 0.067)	0.279	1.9	4.6 ± 0.48	7.31 (3)	0.063	–	–
Wee Waa NSW (F_1)*	Sorghum	Dec. 2012	0.064 (0.056, 0.072)	0.367	2.1	4.1 ± 0.40	3.66 (4)	0.454	–	–
Wee Waa NSW (F_1)	Mung bean	Feb. 2013	0.069 (0.061, 0.078)	0.430	2.2	3.9 ± 0.37	2.72 (4)	0.606	100 (60)	100 (59)
Wee Waa NSW (F_2)*	Sorghum	Dec. 2012	0.071 (0.062, 0.082)	0.726	2.3	3.1 ± 0.27	5.20 (4)	0.267	100 (60)	100 (60)
Warren NSW (F_1)	Sunflower	Mar. 2013	0.073 (0.064, 0.083)	0.564	2.4	3.5 ± 0.32	1.03 (4)	0.905	100 (59)	100 (60)
Emerald QLD (F_1)	Chickpea	Sept. 2012	0.078 (0.069, 0.088)	0.489	2.5	3.9 ± 0.37	2.81 (4)	0.590	98.2 (55)	100 (60)
Narromine NSW (F_2)	Maize	Nov. 2012	0.080 (0.068, 0.094)	1.281	2.6	2.6 ± 0.26	5.19 (4)	0.268	94.9 (59)	100 (60)
Mullaley NSW (F_1)*	Maize	Dec. 2012	0.081 (0.071, 0.092)	0.603	2.6	3.6 ± 0.34	2.62 (4)	0.623	–	–
Breeza NSW (F_1)	Pigeon pea	Feb. 2013	0.081 (0.072, 0.091)	0.433	2.6	4.2 ± 0.43	2.63 (3)	0.452	100 (60)	100 (60)
Mullaley NSW (F_2)*	Maize	Dec. 2012	0.089 (0.064, 0.123)	0.530	2.9	4.0 ± 0.67	11.27 (4)	0.024	98.3 (60)	100 (60)
Walgett NSW (F_2)	Chickpea	Sept. 2012	0.090 (0.078, 0.103)	0.972	2.9	3.0 ± 0.26	9.17 (4)	0.057	100 (59)	–
Pampas QLD (F_2)*	Pigeon pea	Jan. 2013	0.092 (0.080, 0.105)	0.752	3.0	3.4 ± 0.32	4.03 (4)	0.258	100 (60)	100 (60)
Loxton QLD (F_1)	Cotton	Jan. 2013	0.102 (0.088, 0.116)	0.921	3.3	3.2 ± 0.30	2.93 (4)	0.570	96.6 (59)	100 (60)
Pampas QLD (F_1)*	Pigeon pea	Jan. 2013	0.104 (0.071, 0.150)	1.001	3.4	3.1 ± 0.50	12.17 (4)	0.016	94.9 (59)	100 (60)
Cecil Plains QLD (F_1)*	Cotton	Jan. 2013	0.106 (0.094, 0.119)	0.508	3.4	4.6 ± 0.50	2.54 (2)	0.281	–	–
Cecil Plains QLD (F_2)*	Cotton	Jan. 2013	0.107 (0.094, 0.123)	0.900	3.5	3.3 ± 0.31	0.51 (4)	0.972	98.3 (59)	100 (59)
Wee Waa NSW (F_1)	Soya bean	Feb. 2013	0.108 (0.093, 0.124)	1.213	3.5	2.9 ± 0.24	7.04 (4)	0.218	96.7 (60)	100(60)
Felton QLD (F_2)	Canola	Oct. 2012	0.113 (0.101, 0.126)	0.513	3.6	4.7 ± 0.49	2.69 (4)	0.611		
Pooled			0.068 (0.060, 0.077)	0.672		3.1 ± 0.21				
Laboratory strain New GR			0.030 (0.028, 0.032)	0.201		3.7 ± 0.18	8.24 (4)	0.083	100 (120)	–

G_aGeneration tested.

[b]Toxicity ratio = LC_{50} of population/LC_{50} of New GR strain (average of four nonsynchronous New GR cohorts).

[c]Mortality at highest concentration tested.

* Strains from the same location tested in consecutive generations to test consistency of bioassay.

suggest rapid resistance development in this species (Wang and Wu 2012).

Resistance monitoring is a key component of the resistance management strategy for *H. armigera*. The use of discriminating dose techniques in resistance monitoring is a highly efficient method for detecting resistance alleles in insect populations (Roush and Miller 1986, Halliday and Burnham 1990) and has been used effectively in resistance management of *H. armigera* in Australia for over 30 yr. The narrow range of variation in susceptibility to cyantraniliprole observed in field populations of *H. armigera* from diet incorporation bioassays suggests that this method is likely to provide an effective discriminating dose for detecting changes in resistance frequency (Jutsum et al. 1998). This combined with high slope values, goodness-of-fit to the probit binomial model, and enhanced toxicity compared with a topical method of bioassay indicate that an ingestion bioassay using diet-incorporated insecticide is the preferable laboratory method for measuring the dose-response of cyantraniliprole in *H. armigera*.

The estimation of a discriminating dose of cyantraniliprole was based on a theoretical estimate of the highest $LC_{99.9}$ value, while taking into account the empirical mortality observed at the upper limits of the dose-response curve (Robertson et al. 2007). In topical bioassays, empirical mortality at the upper limits of the dose-response suggests that a discriminating dose could be set at 2,000 mg/liter. However, a theoretical estimate based on $LD_{99.9}$ values indicates that the discriminating dose could be as high as 8,000 mg/liter. As noted by Dennehy et al. (1983), this kind of arbitrary approach may greatly reduce the ability to detect resistance rather than increase it. Alternatively, low slope values might suggest that an LD_{95} with a somewhat narrower confidence interval could be a better test of sensitivity for topical bioassays. However, as noted by Roush and Miller (1986), sample size considerations would outweigh the benefits of improving statistical accuracy because this method would require empirical testing of several thousand individuals at the estimated discriminating dose.

In contrast, the dose-response from diet incorporation bioassays of cyantraniliprole produced greater consistency between $LC_{99.9}$ values and empirical estimates of mortality at the upper end of the dose-response curve. Results from testing candidate discriminating dose demonstrated that 1 mg/liter of diet-incorporated cyantraniliprole resulted in 100% mortality. This is consistent with probit model predictions that the $LC_{99.9}$ of the most tolerant strains will not exceed 1.3 mg/liter. Therefore, a discriminating dose of 1.5 mg/liter of diet is recommended for monitoring resistance to cyantraniliprole in Australian populations of *H. armigera*.

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Research Article

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Genetics, cross-resistance and synergism of indoxacarb resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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Abstract

BACKGROUND: The cotton bollworm *Helicoverpa armigera* is a global pest of field and horticultural crops and has developed resistance to insecticides from many chemical classes. Indoxacarb is an important option for selective control of *H. armigera* in a range of crops that play host to this species. A strain of *H. armigera* resistant to indoxacarb (designated GY7-39) was detected from the field by F_2 screening and characterised by comparison with a near-isogenic indoxacarb-susceptible laboratory strain to determine inheritance, cross-resistance profile and synergism of indoxacarb resistance.

RESULTS: The level of indoxacarb resistance in the GY7-39 strain was 139–198-fold compared with the susceptible strain. Genetic analysis showed that resistance was autosomal, incompletely dominant and conferred by one or a few closely linked loci. Indoxacarb resistance in the GY7-39 strain did not confer cross-resistance to chlorantraniliprole. The GY7-39 strain was more susceptible to emamectin benzoate, fenvalerate, Cry1Ac and Cry2Ab compared with the susceptible strain. Indoxacarb resistance was synergised by the metabolic inhibitor PBO.

CONCLUSIONS: Rapid selection of indoxacarb resistance in the GY7-39 strain indicates the potential risk of resistance development to indoxacarb in field populations of *H. armigera*. Lack of cross-resistance indicates that resistance could be managed effectively by the use of rotational strategies that incorporate transgenic technologies. Synergism studies indicate the potential involvement of metabolic detoxification enzymes as the mechanism of resistance to indoxacarb.

Keywords: indoxacarb; cotton bollworm; cross-resistance; synergism; resistance management

1 INTRODUCTION

The cotton bollworm, *Helicoverpa armigera* (Hübner), is a major pest of field and horticultural crops in Australia, Asia, Africa and South America and has developed resistance to a wide range of insecticides, including synthetic pyrethroids,[1] organophosphates,[2] carbamates[3] and spinosad.[4] In Australia, resistance to these insecticides was managed retrospectively by the implementation of a windows-based insecticide resistance management strategy (IRMS) during the mid-1980s, following widespread failure of insecticides to provide control of *H. armigera* in the field.[1] However, if the likelihood of resistance development to novel insecticides can be predicted when resistance alleles are still rare in the population, then it should be feasible to establish an effective pre-emptive resistance management programme to mitigate resistance risk before frequencies reach high levels in the population.

Indoxacarb is a pyrazoline-type insecticide with activity against a range of lepidopteran, coleopteran and sucking insect pests.[5] Metabolism studies in Lepidoptera have shown that indoxacarb is bioactivated by an esterase or amidase type of enzyme(s) to the active *N*-decarbomethoxyllated metabolite, which blocks voltage-dependent sodium channels, preventing influx of sodium into neurons and resulting in insect paralysis and death.[5,6]

Indoxacarb is among the most recent compounds to be used for control of *H. armigera* in Australia, and resistance to indoxacarb has been monitored since it was introduced in 2002. From 2002 to 2012, field resistance was determined from traditional screening methods involving topical application of a discriminating concentration of insecticide to larvae in the F_0 generation.[1] Although this technique has been useful for measuring resistance to broad-spectrum contact insecticides where resistance is mediated by dominant alleles, it may be less effective for determining resistance to indoxacarb, firstly because F_0 screening may underestimate frequency of non-dominant resistance alleles,[7] and secondly because indoxacarb is primarily active by ingestion rather than contact.[6]

In 2013, F_2 screening by diet incorporation was introduced for monitoring resistance to indoxacarb in Australian populations of *H. armigera*. This method increases capacity for detection of low frequency resistance,[7] and has resulted in the first case of genetic indoxacarb resistance isolated from field populations of *H. armigera* in Australia. The aim of the present study was to characterise indoxacarb resistance isolated by F_2 screening. This

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involved (1) investigating the inheritance of indoxacarb resistance by quantitative genetic analysis, where specific crosses and bioassays were used to determine sex-linkage, dominance and the number of loci involved in resistance, (2) determining the degree of cross-resistance to a range of other insecticides and (3) investigating the synergistic effects of PBO on indoxacarb resistance in *H. armigera*.

2 MATERIALS AND METHODS

2.1 Rearing methods

The rearing methods used were the procedures described previously,[8] except that formalin was omitted and soybean flour was baked at 200 °C for 10 min to remove enzyme inhibitors. Rearing trays (Tacca Plastics, Sydney, Australia) were covered and heat sealed with perforated lids (Oliver Products, Grand Rapids, MI). Moths were provided with a 4% honey/sugar solution fed through a cotton wick and housed in containers open at the top and covered with cloth liners secured around the lip of the containers. Eggs were harvested by washing cloth liners in 1% bleach and collecting them on a Whatman No. 54 filter paper by vacuum filtration. Filter papers were allowed to air dry and were then placed in sealed plastic bags until neonates hatched.

In the larval stage, insect strains were maintained in a laboratory environment of 25 ± 2 °C with a 14:10 h (L:D) photoperiod and 45–55% RH. Adults were maintained in a separate facility under the same conditions of light and temperature with RH maintained at 70–80% for the duration of the dark cycle using a steam humidifier.

2.2 Insect strains

The laboratory strain New GR was established from a cohort of a general laboratory strain GR. Progenitor strains of GR were sourced during the mid-1980s from a series of collections from cotton fields in the Namoi Valley, northern New South Wales. The New GR strain was susceptible to indoxacarb and monitored regularly by bioassay to evaluate the response to a diagnostic concentration of indoxacarb (12 µg indoxacarb mL^{-1} in a diet incorporation bioassay performed on late second-instar or early third-instar larvae) that kills 99.9% of susceptible larvae.[9] Bioassays were performed on a minimum of four non-synchronous cohorts of New GR, and the results were pooled in the final analysis because there were no significant differences between cohorts for any of the insecticides tested. The New GR strain was used as the susceptible control in all bioassays and in crosses to the resistant strain.

The resistant strain GY7-39 was established from a single *H. armigera* moth collected as an egg from maize near Moree, New South Wales, in November 2013. Progeny from the mating of this moth with a moth from the susceptible New GR strain were subjected to an F_2 screen. The GY7-39 strain was created from F_2 offspring that survived the diagnostic concentration of indoxacarb.

The GY7-39 strain initially had a restricted gene pool because it had originated from an isofemale line. To reduce founder effects and maintain the indoxacarb-resistant and indoxacarb-susceptible strains in a common genetic background, the GY7-39 strain was introgressed into the susceptible strain (New GR) by repeated backcrossing (at generations 5, 7 and 12) and reselection to produce a near-isogenic resistant strain of *H. armigera*. After the first cross, the strain was maintained without selection for one generation and reselected in the subsequent generation with the discriminating dose of indoxacarb. As there was significant survival of F_1 progeny at this concentration, the F_1 progeny from the second and third backcrosses were directly subjected to selection. The bioassays reported herein were performed on the near-isogenic third backcross to reduce the potentially misleading effects of hybrid vigour.

2.3 Insecticides

Commercial insecticide formulations were used in diet incorporation bioassays: indoxacarb [Steward EC (15% active ingredient)] and chlorantraniliprole [Altacor (35% active ingredient)] (DuPont Australia Ltd, Macquarie Park, Australia), and emamectin benzoate [Affirm (1.9% active ingredient)] (Syngenta Crop Protection, Macquarie Park, Australia). Fenvalerate (95.3%) was provided by Sumitomo Chemical (Sydney, Australia). Synergism bioassays were performed using piperonyl butoxide (PBO) (90%) (Sigma Chemical, Poole, UK).

The Cry1Ac toxin was produced from the HD73 strain by GeneSearch (Arundel, Queensland, Australia). A recombinant clone of the *cry2Ab* gene in *Bacillus thuringiensis* was used for the production of Cry2Ab toxin. The Cry2Ab *B. thuringiensis* strain was grown in nutrient broth (Oxoid Ltd, Basingstoke, UK), with 10 µg mL^{-1} of tetracycline, for 3–4 days in a 28 °C orbital shaker. When sporulation and crystal formation were completed, the suspension was centrifuged and the pellet washed 3 times. The pellet was resuspended in distilled water and sonicated. A sample of the final suspension was transferred into SDS sample buffer and electrophoresed on a 10% polyacrylamide gel with four known concentrations of bovine serum albumin (BSA). The concentration of Cry2Ab was estimated by scanning the gel and analysing the density of the Cry2Ab band relative to the BSA standard using Scion Image 1.62 software (Scion Corporation, Frederick, MD).

2.4 Bioassays

The responses of susceptible and resistant strains to indoxacarb, chlorantraniliprole and emamectin benzoate were measured by performing bioassays on artificial diet into which formulated insecticide was incorporated. A stock suspension of insecticide was diluted with distilled water to produce six or seven twofold dilutions; the ratio of diet to toxin determined the concentration, calculated as micrograms of insecticide per millilitre of diet. Serial dilutions were added to 200 mL of diet and incorporated by vigorous shaking by hand for 30 s to produce a homogeneous mixture which was then dispensed into 45-well bioassay trays (Tacca Plastics, Sydney, Australia). Late second or early third instars were introduced to trays (one larva per well); untreated diet was used as the control.

Cross-resistance to Cry1Ac and Cry2Ab was evaluated using a surface treatment bioassay. Artificial diet was dispensed into 24-well plates (Falcon, Cowley, UK). A stock suspension of the Cry toxins was diluted with distilled water to produce eight twofold dilutions; concentrations were calculated as micrograms of toxin per square metre of diet surface. A pipette was used to transfer 50 µL aliquots of serially diluted Cry toxin to the surface of the diet. The toxin solution was distributed over the diet surface, and the residual surface liquid was allowed to evaporate in a cool air flow. One unfed neonate larva was introduced to each well; distilled water was used as the control.

Table 1. Resistance levels to five insecticides of the resistant strain (GY7-39) and laboratory strain (New GR)

Insecticide	Population	LC_{50} (μg mL^{-1} diet) (95% FL)	Slope ± SE	RR (95% CI)
Indoxacarb	New GR	0.147 (0.137, 0.158)	3.4 ± 0.19	
	GY7-39 (F_5)[a]	25.371 (21.333, 30.056)	3.0 ± 0.26	173
	GY7-39 ($BC3_3$)[b]	24.734 (20.443, 30.316)	2.4 ± 0.21	168
Chlorantraniliprole	New GR	0.020 (0.0173, 0.0234)	4.4 ± 0.48	
	GY7-39	0.024 (0.0200, 0.0277)	2.6 ± 0.26	1.2 (0.958, 1.428)
Emamectin benzoate	New GR	0.009 (0.0777, 0.1061)	5.2 ± 0.56	
	GY7-39	0.006 (0.0472, 0.0646)	4.8 ± 0.54	0.6 (0.518, 0.706)
Fenvalerate	New GR	55.828 (40.006, 79.495)	3.3 ± 0.32	
	GY7-39	44.587 (31.110, 63.774)	3.1 ± 0.29	0.8 (0.656, 0.972)
Cry1Ac	New GR	45.624 (36.715, 56.729)	1.8 ± 0.13	
	GY7-39	17.636 (15.804, 19.675)	2.8 ± 0.17	0.4 (0.318, 0.470)
Cry2Ab	New GR	52.312 (44.442, 61.418)	2.0 ± 0.15	
	GY7-39	41.751 (33.322, 51.621)	1.6 ± 0.09	0.8 (0.643, 0.991)

[a] The GY7-39 strain was isolated by F_2 screening and the survivors were selected for three generations.
[b] The GY7-39 ($BC3_3$) strain was backcrossed to the New GR strain 3 times and then selected for three generations.

Cross-resistance to fenvalerate was evaluated by topical bioassay. Insecticide solutions were prepared from technical material dissolved in analytical-grade acetone to produce six twofold serial dilutions. Larvae within a weight range of 30–40 mg were treated with 1 μL of acetone/insecticide solution by topical application to the dorsal thorax using a 50 μL microsyringe in a repeating dispenser (Hamilton Company, Reno, NV); acetone alone was used as the control.

Synergism bioassays were performed by firstly exposing larvae to a range of concentrations of indoxacarb using the diet incorporation bioassay method described above. After 5 days of exposure, larvae within a weight range of 30–40 mg were selected for testing. Piperonyl butoxide was dissolved in analytical-grade acetone at a concentration known to cause no mortality in *H. armigera* (50 μg μL^{-1}).[1] Larvae were then treated with 1 μL of acetone/PBO solution as above; PBO (50 μg μL^{-1}) alone was used as the control.

In all cases, bioassays were performed on three cohorts of insects with individual treatments (insecticide concentrations) of each cohort consisting of a minimum of 20 individuals. Bioassays were maintained under the same conditions described for larval rearing. The range of concentrations used in each bioassay was expected to induce 1–99% mortality.

Diet incorporation bioassays were assessed at 7 days, and topical bioassays were assessed at 3 days. Larvae were considered to be dead if one or more of the following criteria were demonstrated: larvae unable to perform coordinated movement when prodded; paralysis of prolegs; larvae very slow to right themselves (time exceeding 3 s).

2.5 Genetics of resistance to indoxacarb

Each of the various crosses established to determine the genetic basis of resistance comprised a minimum of 30 female and 30 male moths in each of three cohorts. The response of F_1 progeny from reciprocal mass crosses between the susceptible laboratory strain New GR and the indoxacarb-resistant strain GY7-39 were used to examine the level of dominance of resistance. The data from these tests of dominance were also used to examine whether resistance was autosomal or sex linked.

The degree of dominance for the 50% lethal concentration (LC_{50}) (D_{LC}) was calculated as follows:

$$D_{LC} = \frac{\log LC_{RS} - \log LC_{SS}}{\log LC_{RR} - \log LC_{SS}}$$

where LC_{RR}, LC_{RS} and LC_{SS} are lethal concentrations for the resistant, F_1 hybrid and susceptible strains respectively. The resulting parameters range from 0 (completely recessive resistance) to 1 (completely dominant resistance).[10]

Effective dominance (D_{ML}) was calculated from mortality values at a range of single concentrations as follows:

$$D_{ML} = \frac{ML_{RS} - ML_{SS}}{ML_{RR} - ML_{SS}}$$

where ML_{RR}, ML_{RS} and ML_{SS} are the mortality levels at a particular concentration for the resistant, F_1 hybrid and susceptible strains respectively. The D_{ML} values range from 0 (completely recessive) to 1 (completely dominant).[10]

The number of loci influencing resistance was estimated by a direct test for a monogenic inheritance based on the observed and expected mortalities from a reciprocal backcross to each single dose.[11] This test was based on the goodness-of-fit χ^2 between the F_1 backcross and the New GR susceptible strain, with expected values calculated as described by Sokal and Rohlf:[12]

$$\chi^2 = \frac{(F_1 - pn)^2}{pqn}$$

where F_1 is the observed number of dead larvae in the reciprocal backcross at dose x, p is the expected proportion of larvae dead, n is the number of backcross progeny exposed to dose x and $q = 1 - p$. The null hypothesis was rejected if the test resulted in a significant deviation ($P < 0.05$), comparable with a χ^2 distribution with one degree of freedom.

If resistance is monogenic and strains are homozygous, then New GR are SS, GY7-39 are RR, F_1 progeny are RS, the backcross of F_1 × New GR produces progeny that are 50% SS and 50% RS and the backcross of F_1 × GY7-39 produces progeny that are 50% RR and 50% RS. The backcross of F_1 × New GR was preferred

Table 2. Responses to indoxacarb of the resistant strain (GY7-39), laboratory strain (New GR) and progeny of crosses to indoxacarb following the third backcross

Strain/cross	LC_{50} (μg AI mL^{-1} diet) (95% FL)	Slope ± SE	RR (95% CI)	D_{LC}
New GR (susceptible)	0.1476 (0.137, 0.158)	3.4 ± 0.2		
GY7-39 (resistant)	29.177 (24.695, 34.792)	2.6 ± 0.2	198 (167.582, 233.104)	
F_1: GY7-39♀ × New GR♂	10.691 (8.372, 13.977)	2.1 ± 0.2	72 (60.379, 86.870)	0.81
F_1: GY7-39♂ × New GR♀	8.768 (7.590, 10.128)	2.8 ± 0.2	59 (50.890, 69.321)	0.77
F_1: pooled	9.554 (8.198, 11.174)	2.4 ± 0.1	65 (57.133, 73.316)	0.80
Backcross F_1 × New GR	2.205 (1.855, 2.612)	1.2 ± 0.1		

to the backcross of F_1 × GY7-39 because the F_1 differed more from the susceptible strain than from the resistant strain.[13] Thus, this test increased the power of the backcross for distinguishing among modes of inheritance.[11] The monogenic model was tested by calculating the average of mortality probabilities for RS and SS individuals (Y_x) to each single dose as follows:

$$Y_x = 0.5 \times \left(W_{F_1} + W_{SS}\right)$$

where W_{F_1} is the observed mortality for F_1 pooled progeny of reciprocal mass crosses of the resistant GY7-39 strain and the susceptible New GR strain, and W_{SS} is the mortality of the susceptible strain New GR, at each concentration tested.[11]

2.6 Data analysis

Where necessary, bioassay data were corrected for control mortality.[14] Estimates of LC_{50} values and their 95% fiducial limits (FL) were obtained by probit analysis using POLO-PC software (LeOra Software, Berkeley, CA). Resistance ratios (RRs) were calculated by dividing the LC_{50} value of the resistant strain by the LC_{50} value (average of four non-synchronous cohorts) of the laboratory strain. Significant differences ($P = 0.05$) between LC_{50} values were determined by the lethal concentration ratio test according to Wheeler *et al.*,[15] where, if the 95% confidence interval (CI) includes 1, the LC_{50} values are not significantly different. The synergistic ratio (SR) was calculated by dividing the LC_{50} of the strain tested with indoxacarb alone by the LC_{50} of the strain tested with indoxacarb + PBO.

3 RESULTS

3.1 Response to selection and cross-resistance

After three generations of selection, the field-derived GY7-39 strain (F_5) had 173-fold resistance to indoxacarb compared with the laboratory strain New GR (Table 1). The GY7-39 strain was then crossed with New GR and reselected on three occasions, with reselection of the third backcross resulting in a resistance ratio of 168-fold after three generations (Table 1). It was estimated (by the principle of Mendelian segregation) that the third backcross had produced a strain of GY7-39 that shared >93% of its genome with that of the parental strain New GR.

The third backcross of GY7-39 was bioassayed to determine patterns of cross-resistance to other insecticides. The response of the GY7-39 strain to chlorantraniliprole was similar to that of the New GR strain based on the ratio test ($P = 0.05$) (Table 1), suggesting a lack of cross-resistance. Emamectin benzoate, fenvalerate, Cry1Ac and Cry2Ab had greater toxicity to the GY7-39 strain than to the New GR strain based on the ratio test ($P = 0.05$) (Table 1). These results suggest that indoxacarb-resistant *H. armigera* may be more sensitive to these insecticides.

Table 3. Dominance of indoxacarb resistance in *H. armigera* as a function of indoxacarb concentration

Concentration (μg AI mL^{-1} diet)	Survival (%)			Effective dominance (D_{ML})
	New GR	F_1	GY7-39	
0.75	2	94	0	1.00
1.5	0	87	0	0.96
3	0	74	0	0.87
6	0	52	95	0.78
12	0	12	78	0.67
24	0	2	73	0.16
48	0	1	25	0.07

3.2 Genetics of resistance to indoxacarb

3.2.1 *Sex linkage and dominance*

Bioassays of indoxacarb against the F_1 progeny from reciprocal mass crosses between GY7-39 and New GR were conducted on the third backcrossing event during the creation of near-isogenic strains. At the time of the crossing experiment, the resistance ratio for the GY7-39 strain was 198-fold (Table 2). The LC_{50} values of the F_1 progeny of the reciprocal crosses between the resistant and susceptible strains were similar based on a ratio test ($P = 0.05$) suggesting that inheritance of indoxacarb resistance in the GY7-39 strain was autosomal, with no evidence of sex linkage or maternal effects.

The resistance ratio decreased to 65-fold (pooled from the reciprocal crosses) in the F_1 progeny from the third backcross event (Table 2). The degree of dominance (D_{LC}) for the pooled reciprocal crosses was 0.8, indicating that indoxacarb resistance in the GY7-39 strain was inherited as an incomplete dominant trait.

Estimation of effective dominance (D_{ML}) for six concentrations tested showed that dominance was dose dependent and increased with concentration (Table 3). Resistance was functionally recessive at the highest concentration of 48 μg mL^{-1} ($D_{ML} = 0.07$), partially dominant at intermediate concentrations and functionally dominant at the lowest concentration of 0.75 μg mL^{-1} ($D_{ML} = 1$).

3.2.2 *Number of genes involved*

Results from a direct test of monogenic inheritance suggest that one (or a few closely linked) loci conferred resistance to indoxacarb in the GY7-39 strain.

Table 4. Direct test of monogenic inheritance for resistance to indoxacarb by comparing expected and observed mortality of the backcross (F_1 × New GR) of *H. armigera*

Concentration (μg AI mL^{-1} diet)	Number of larvae tested	Observed mortality (proportion)	Expected mortality (Y_x)	χ^2 (df = 1)	*P*
0.1875	240	3 (0.01)	0.35	120.68	<0.0001
0.375	240	41 (0.17)	0.46	79.67	<0.0001
0.75	240	106 (0.44)	0.49	2.39	0.12
1.5	238	118 (0.50)	0.53	1.06	0.30
3	237	126 (0.53)	0.57	1.18	0.28
6	240	154 (0.64)	0.63	0.16	0.69
12	240	175 (0.73)	0.74	0.09	0.77
24	238	213 (0.89)	0.94	8.56	0.03
48	240	240 (1.00)	0.99	2.02	0.16

The test showed no significant deviation between observed and expected mortality at six of the nine concentrations tested (Table 4). A significant deviation ($P = 0.03$) occurred at 24 μg mL^{-1} of indoxacarb, where observed mortality (89%) was lower compared with expected mortality (94%). Highly significant deviations ($P < 0.001$) between observed and expected mortality also occurred at the two lowest concentrations tested. This may be either an artefact of the bioassay or because the concentrations were too low to discriminate between the susceptible strain and the F_1 backcross progeny. Alternatively, it may indicate the presence of low-level polygenic resistance conferred by generalist mechanisms.

The potential for a type II error leading to acceptance of the null hypothesis (lack of differences between observed and expected outcomes) can be high.[11] Therefore, it may be useful to consider slope values when determining the number of genes involved in inheritance of resistance. The slope of the concentration–mortality response for the backcross progeny (F_1 backcross × New GR) was 1.2, which is threefold lower than the slope of the susceptible strain (3.5) and twofold lower than the slope for the resistant and F_1 strains (2.6 and 2.4 respectively) (Table 2). This pattern suggests increased genetic variation in the backcross compared with the parental and F_1 strains and is consistent with the findings from the direct test for monogenic inheritance, which suggests that resistance to indoxacarb in the GY7-39 strain was conferred primarily by a single locus or several closely linked loci.

3.3 Synergism of indoxacarb resistance

At the time of testing for synergism, the resistance ratio for indoxacarb was 140-fold in the GY7-39 strain compared with the New GR strain (Table 5). Resistance to indoxacarb was reduced from 20.8-fold to 0.92-fold by the addition of PBO to actively feeding *H. armigera* larvae, resulting in a synergistic ratio of 23-fold. A small but significant synergistic effect was observed in the New GR strain (Table 5). Results suggest that PBO-suppressible metabolic processes are important mechanisms for conferring resistance to indoxacarb in *H. armigera*.

4 DISCUSSION

An important feature of the F_2 screening method for estimating resistance frequencies is that alleles that confer resistance may be isolated for further study.[7] This method was used to isolate an indoxacarb-resistant genotype of *H. armigera* in which resistance increased rapidly to 173-fold after three generations of selection at the discriminating dose of indoxacarb. The advantage of selecting resistant strains recently derived from the field is that they are less likely to be confounded by the presence of additive effects. To reduce the potentially misleading effects of hybrid vigour, the indoxacarb-selected strain was serially backcrossed 3 times to a susceptible strain and was expected to produce >93% genetic similarity between the resistant and susceptible strains.

Knowledge of insecticide cross-resistance is important for developing strategies to delay the evolution of field resistance. In the present study, indoxacarb resistance in the GY7-39 strain did not extend cross-resistance to other insecticides specific for *H. armigera*. There was enhanced sensitivity of the GY7-39 strain to emamectin benzoate and the two Cry toxins from *Bacillus thuringiensis* currently deployed in commercial transgenic cotton varieties. Each of these insecticides has a unique mode of action quite different to that of indoxacarb.[16–18] Hence, a rotational strategy that incorporates the use of transgenic technology should be effective for resistance management of *H. armigera*. The GY7-39 strain was also more sensitive to fenvalerate. The target sites for both indoxacarb and pyrethroid are voltage-dependent sodium channels. Although increased target-site insensitivity (knockdown resistance or *kdr*) has contributed to pyrethroid resistance at various times and geographical locations in Australia,[1,19,20] pyrazolines block sodium channels in a manner different from pyrethroids[21] and would therefore not compromise resistance risk for indoxacarb.

The mode of inheritance of resistance is also an important factor for determining the rate of resistance evolution. The reciprocal crosses between the GY7-39 strain and the New GR strain indicated that resistance to indoxacarb in the GY7-39 strain was inherited autosomally and as an incompletely dominant trait. Similarly, deltamethrin resistance in *H. armigera* was also incompletely dominant[22] and resulted in rapid development of pyrethroid resistance in Australia.[1]

In contrast to pyrethroids, indoxacarb resistance is currently at low levels (<2%) in the Australian *H. armigera* population.[23] This is likely attributable to a 90% reduction in the number of sprays to control *Helicoverpa* species following the introduction of dual toxin transgenic cotton in 2003, which significantly reduced selection for resistance to foliar insecticides.[24] Alternatively, the presence of fitness costs associated with resistance could delay the spread of resistance alleles under certain conditions. Especially if resistance alleles are rare, such as for the case with indoxacarb resistance in *H. armigera* in Australia, development of resistance

Table 5. Toxicity of indoxacarb with and without PBO (50 μg μL^{-1}) on laboratory (New GR) and resistant (GY7-39) strains of *H. armigera*

Insecticide	Strain	LC_{50} (μg mL^{-1} diet) (95% FL)	Slope ± SE	RR	SR (95% CI)
Indoxacarb	New GR	0.148 (0.137, 0.158)	3.4 ± 0.2		
Indoxacarb + PBO	New GR	0.112 (0.086, 0.154)	8.3 ± 1.0		1.3 (1.201, 1.455)
Indoxacarb	GY7-39	20.791 (16.959, 25.382)	2.5 ± 0.2	140	
Indoxacarb + PBO	GY7-39	0.918 (0.748, 1.132)	1.8 ± 0.1	6	22.6 (18.020, 28.466)

depends not only on the level of exposure to insecticide but also on the relative fitness of the heterozygotes compared with the susceptible genotype.[25] At low frequencies, resistance alleles are present primarily in heterozygotes, and dominant fitness costs would favour a delay in resistance development.[26] For example, fitness costs are thought to be responsible for a lack of field resistance to indoxacarb in *Heliothis virescens*.[27] Investigations are under way to determine whether fitness costs are involved in delaying the development of indoxacarb resistance in *H. armigera*.

It is generally assumed that monogenic resistance is more likely to spread than polygenic resistance.[28] Results from a monogenic test of inheritance suggests that resistance to indoxacarb was controlled by a single major or a few tightly linked loci. Significant deviation from expected values occurred at the lowest and second lowest concentrations used. One possible explanation for this aberration is the presence of resistant genotypes in the susceptible population. In that case, some of the F_1 progeny would likely be resistant and the dose response confounded by the mixed genotype. However, this is unlikely as the laboratory population used in this study demonstrated full susceptibility to indoxacarb for over 30 generations of screening.

Alternatively, other generalist mechanisms conferring low levels of tolerance to indoxacarb that are only conspicuous at low concentrations may be present in the GY7-39 strain. As this strain originated from a single wild individual crossed with an individual from the laboratory strain, it is likely that levels of insensitivity to a range of insecticidal compounds are also present in this strain by virtue of parentage. It has been reported that field populations of *H. armigera* can respond to selection pressure by multigenic tolerance mechanisms, and acquisition of tolerance may involve a broad range of gene activities including elevated levels of immune gene products[29] and/or metabolic gene products.[30] These mechanisms confer tolerance at pesticide concentrations of low to medium toxicity.[29,31] The presence of generalist mechanisms inherited from either the field-derived parent or the laboratory parent may be responsible for the enhanced survival at low concentrations. Further evidence in support of the single major gene hypothesis is a decrease in the slope values of the concentration–mortality response for the backcross progeny compared with the F_1, susceptible and resistant strains. This increased variance suggests that resistance was due to a major gene rather than to the effects from a quantitative trait.[32]

Dominance of resistance alleles can contribute to the spread of resistance under selection pressure, with the expression of dominance dependent upon the dose of insecticide applied.[10,33] Dominant resistance is considered to favour an increase in resistance heritability because, if the dose applied in the field is not high enough to kill heterozygous individuals, incompletely dominant resistance becomes a functionally dominant trait. On the other hand, when resistance is dominant, theory predicts that susceptible genes can persist for much longer in the population as heterozygotes and therefore slow the time to fixation.[33] In the present study, the degree of dominance of indoxacarb resistance was concentration dependent; resistance was recessive at the highest concentration and dominant at the lowest concentration tested. However, predictions of effective dominance based on laboratory data must be carefully considered because the range of concentrations that establish dominance may differ between laboratory and field populations, and also because of the effect of attenuated insecticidal concentration due to chemical degradation.[13] Nevertheless, results from the present study suggest that the risk for indoxacarb resistance is high owing to enhanced survival of heterozygotes compared with susceptible individuals. It may be possible to delay development of resistance by using management strategies that favour dilution of resistance alleles, and by appropriate rotational strategies. Population models suggest that the presence of refuges, fitness costs (described above) and incomplete resistance (the disadvantage suffered by resistant insects in the presence of insecticide) can delay the development of insecticide resistance[34] and may explain the low frequency of indoxacarb resistance in the *H. armigera* population in Australia. The lack of cross-resistance between indoxacarb and other selective insecticides suggests that rotational strategies could be used effectively for managing resistance to indoxacarb in *H. armigera*.

The most common mechanisms associated with resistance to insecticides in *H. armigera* are metabolism due to oxidation by cytochrome P450 monooxygenases and hydrolysis and/or sequestration by carboxylesterases.[35] The involvement of metabolic mechanisms in indoxacarb resistance has been reported in several insect species, based on synergistic effects of metabolic inhibitors on indoxacarb toxicity. Indoxacarb resistance was associated with P450 monooxygenases in *Spodoptera* litura,[36] *Choristoneura rosaceana*[37] and *Musca* domestica,[38] with little or no evidence of esterase or glutathione *S*-transferase (GST) involvement. On the other hand, there are reports of esterase and GST involvement in indoxacarb resistance in *P. xylostella*. In a field-derived strain from Malaysia, high-level (813-fold) resistance to indoxacarb was largely inhibited by PBO or a PBO analogue specific for esterases, suggesting that indoxacarb resistance in this strain was due to enhanced metabolic detoxification by esterases.[39] In a laboratory-selected strain of *P. xylostella* from India with moderate (31-fold) resistance to indoxacarb, both synergistic suppression by metabolic inhibitors and increased metabolic enzyme activities suggested that resistance was mediated by both esterase and GST activity.[40] Similarly, indoxacarb resistance was found to be associated with GST and esterase activity in *Spodoptera exigua* from China.[41] In the present study, the observed suppression of resistance in the indoxacarb-selected strain by the metabolic inhibitor PBO indicates the likely involvement of metabolic detoxification enzymes in indoxacarb resistance in *H. armigera*. Investigations of specific enzyme systems and their contribution to indoxacarb resistance are the focus of further studies.

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Research article

The Use of F_2 Screening for Detection of Resistance to Emamectin Benzoate, Chlorantraniliprole, and Indoxacarb in Australian Populations of *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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Abstract

The ability to effectively detect changes in susceptibility to insecticides is an integral component of resistance management strategies and is highly dependent upon precision of methods deployed. Between 2013 and 2016, F_2 screens were performed for detection of resistance alleles in *Helicoverpa armigera* (Hübner) to emamectin benzoate, chlorantraniliprole, and indoxacarb in major cropping regions of eastern Australia. Resistance to emamectin benzoate was not detected. There were low but detectable levels of survival at discriminating concentrations of chlorantraniliprole and indoxacarb. Alleles conferring an advantage to chlorantraniliprole were present at a frequency of 0.0027 (95% CI 0.0012–0.0064; $n = 1{,}817$). Alleles conferring an advantage to indoxacarb were present at a frequency of 0.027 (95% CI 0.020–0.035; $n = 1{,}863$). Complementation tests for allelism in six of seven positive indoxacarb tests indicated that resistance was due to alleles present at the same locus. The majority (88%) of lines that tested positive for indoxacarb resistance deviated from a model of recessive inheritance. Pheromone-caught male moths contributed significantly greater numbers of F_2 lines compared with moths derived from field-collected eggs or larvae. There was no difference in the detectability of indoxacarb resistance in F_2 lines from pheromone-caught moths compared with moths derived from immature stages collected from the field and reared to adult under laboratory conditions. Therefore, we recommend the use of pheromone traps for sourcing insects for F_2 screening as a more cost- and time-efficient alternative to traditional methods of sampling.

Key words: *Helicoverpa armigera*, insecticide resistance monitoring, resistance management, pheromone trapping

The *Helicoverpa armigera* (Hübner) is one of the most serious insect pest species to develop insecticide resistance, which has reduced the effectiveness of many commonly used chemistries deployed for its management (McCaffrey 1998, Wang et al. 2009). The pest status of *H. armigera* is further enhanced by its highly polyphagous nature (Zalucki et al. 1986) and ability to migrate long distances (Feng et al. 2005). This species is now becoming a truly global pest having recently extended its considerable geographical range from Europe, Africa, Asia, and Australia to the New World. Initially reported as an incursion in South America (Czepak et al. 2013, Tay et al. 2013, Mastrangelo et al. 2014, Murua et al. 2014), more recent work has confirmed presence of *H. armigera* in Florida (Anonymous 2015).

In Australia, there is considerable experience in managing *H. armigera* in agricultural systems. Following the widespread failure of insecticides to control field populations of *H. armigera* in the 1980s (Gunning et al. 1984), a windows-based insecticide resistance management strategy (IRMS) was implemented along with the establishment of a monitoring program for detecting changes in insecticide resistance frequencies (Forrester et al. 1993). Results from resistance monitoring play a critical role in evaluating the success of resistance management strategies and provide a basis for the timely implementation of tactical responses to control resistant populations. Although the IRMS did not overcome pyrethroid resistance in Australian *H. armigera*, it proved to be a successful tactic for delaying resistance and extending the useful life of these insecticides (Forrester et al 1993). But despite the limited success of retrospective resistance management, if the likelihood of resistance development to insecticides can be predicted when resistance alleles are still rare

in the population, then it should be feasible to establish proactive measures for managing resistance before field control failures occur.

More recently, management of *Helicoverpa* spp. particularly, *H. armigera*, has become increasingly reliant on newer selective insecticides in a range of commodities that play host to this species. These include emamectin benzoate, chlorantraniliprole, and indoxacarb, which are advocated because of their low toxicity to beneficial insects and high compatibility with integrated pest management (IPM) programs (Williams 2016).

Emamectin benzoate is a semisynthetic insecticide derived from the avermectin family of compounds, which are naturally occurring macrocyclic lactones isolated from fermentation products of the soil microorganism *Streptomyces avermitilis*, which acts as an agonist for GABA-gated chloride channels resulting in irreversible activation of chloride channels causing disruption of nerve impulses and rapid paralysis (Kass et al. 1980, Ishaaya et al. 2002). Chlorantraniliprole is an anthranilic diamide which acts as a selective agonist for ryanodine receptors in the muscle cells of insects by stimulating release and depletion of calcium from internal stores in the sarcoplasmic reticulum, causing impaired regulation of muscle function leading to feeding cessation (Cordova et al. 2006). Indoxacarb is a pyrazoline-type sodium channel blocker bioactivated to the active *N*-decarbomethoxyllated metabolite which blocks voltage-dependent sodium channels, preventing influx of sodium into neurons (Wing et al. 2004).

From 2002 to 2012 field resistance to these insecticides in Australian populations of *H. armigera* was determined by the use of topical application of a discriminating dose of insecticide to larvae in the F_0 generation to measure phenotypic frequency of resistance (Rossiter et al. 2008). Although phenotypic characterization is an appropriate method for measuring resistance which has already become established or is conferred by a dominant allele, such as the case with some broad-spectrum contact insecticides (Plapp et al. 1990), it is likely to be less effective for identifying resistance alleles that are rare in populations or where resistance is conferred by nondominant resistance alleles (Roush and Miller 1986, Andow and Alstad 1998). This is because when resistance is rare, resistance alleles are most frequently found as heterozygotes. Individuals heterozygous for nondominant resistance may have a susceptible phenotype and may not survive a discriminating concentration of insecticide. Under these circumstances, the sensitivity for detection of resistance by examining insects directly from the field in the F_0 generation may be compromised by the inability to detect heterozygotes.

To increase the sensitivity for detecting resistance alleles in field populations and therefore the capacity to detect the presence of early stage resistance, an F_2 screening method was introduced as a new standard for monitoring resistance to key selective chemical classes used to control *H. armigera* in Australia. This method is effective for preserving genetic variation in isofemale lines and concentrates the resistance alleles in homozygous genotypes, which can be detected with a discriminating concentration of insecticide (Andow and Alstad 1998).

Although highly effective for recovery of recessive alleles such as those that confer Bt resistance in *Helicoverpa* spp. (Mahon et al. 2007, Downes et al. 2009), F_2 screening is equally efficient for detecting nonrecessive forms of resistance (Andow and Alstad 1998). The benefit of using a genic approach is not only to increase capacity for early detection of rare resistance alleles, but also for capture and isolation of these alleles from field populations, which can then be used for further genetic and molecular characterization. Moreover, Stodola and Andow (2004) indicated that many potential modifications could be made to adapt the F_2 to improve its efficiency.

The primary aim of this study was first to utilize the F_2 screening method to test for the presence of resistance alleles to emamectin benzoate, chlorantraniliprole, and indoxacarb in Australian populations of *H. armigera* and, second, to establish baseline levels of resistance from which to identify major changes over time. A third aim was to compare the relative effectiveness of pheromone trapping with hand sampling of immature life stages of *H. armigera* as a means for enhancing production of isofemale families for F_2 screening.

Materials and Methods

General Rearing Methods

Methods used to rear *H. armigera* were similar to those described by Teakle and Jensen (1985), except that formalin was omitted and soybean flour was baked at 200°C for 10 min to remove enzyme inhibitors which can interfere with bioassays. Neonates were individually transferred to 45-well plastic trays (Tacca Plastics, Sydney, Australia) containing ca. 1.5 ml of diet and heat-sealed with perforated lids (Oliver Products, Grand Rapids, MI). When larvae reached the late fourth–early fifth instar, they were transferred to fresh plastic trays containing diet. Larvae were allowed to pupate in these trays and when hardened they were removed, washed in 1% bleach solution and transferred to 5-liter round, plastic containers (21 cm diameter). A 14-cm-diameter hole was cut into the lid of the container and used to secure a cloth liner which acted as an oviposition substrate for the emerging moths. Moths were provided with a 4% honey/sugar solution held in 50-ml plastic containers with a hole in the lid to accommodate a cotton wick. Eggs were harvested daily by replacing the cloth liners which were washed in a 1% bleach solution and collected onto a Whatman No. 54 filter paper by vacuum filtration. Filter papers were allowed to air dry and were then placed in sealed plastic bags until neonates hatched.

In the larval stage, insect strains were maintained in a laboratory environment of 25 ± 2 °C with a photoperiod of 14:10 (L:D) h and ambient RH. Adults were maintained in a separate facility under the same conditions of light and temperature with RH maintained at 70–80% for the duration of the dark cycle using a steam humidifier.

Laboratory Susceptible Strain

A laboratory susceptible strain was used to establish isofemale lines and to check for consistency of bioassay at weekly intervals throughout each growing season from 2013 to 2016. The laboratory strain, New GR, was established from a cohort of a general laboratory strain GR. Progenitor strains of GR were sourced during the mid-1980s from a series of collections from cotton fields in the Namoi Valley, northern New South Wales.

Resistance Screening Methods

Resistance was tested by using an F_2 screening procedure which generates isofemale lines that produce a proportion of individuals homozygous for haplotypes present in their field-derived parents (Andow and Alstad 1998). This method comprises a stepwise process for generating isofemale lines: 1) collecting the parental (F_0) generation from the field; 2) rearing F_1 offspring for each line; 3) sib-mating F_1 adults; 4) screening F_2 larvae by diet incorporation of insecticide. The method described herein was modified from Stodola and Andow (2004) where field-derived male or female moths were individually mated to moths from a laboratory susceptible strain to generate isofemale lines which were tested for resistance in the F_2 generation.

Establishment of F_0 Generation From Field-Collected Material

Two methods were used to source *H. armigera* from the field for resistance monitoring. First, visual searches for *H. armigera* eggs and larvae were conducted in a range of cultivated hosts (primarily cotton, sorghum, maize, and pulses) growing in the major crop production areas of northern New South Wales and central/southern Queensland, between September and March, over three field seasons (2013–2014, 2014–2015, and 2015–2016). Second, commencing in the 2014–2015 field season, pheromone traps were placed in the above crops growing in the Namoi Valley (NSW), as a method for sourcing live adult male *H. armigera* for mating directly with laboratory-reared female moths.

Eggs and larvae collected from the field by hand were reared individually to adults in the laboratory on artificial diet as described above. Following pupation insects were washed in a 1% bleach solution, dried, sexed and transferred to 850-ml round, plastic containers (WF Plastics Australia Pty. Ltd., Warwick Farm, NSW, Australia) open at the top and covered with cloth liners. Following emergence moths were single-pair mated with an individual of opposite sex from the laboratory susceptible strain (New GR).

Pheromone trapping was conducted using Heliothis net traps (Scentry Biologicals Inc., Billings, MT), baited with *H. armigera* female pheromone lures (InSense Pty. Ltd. Cobram, VIC, Australia). Trapped moths were removed daily and transported to the laboratory where they were individually placed into single-pair mating containers along with a recently emerged female moth from the New GR strain.

F_1 Generation

Single-pair mating containers were checked daily for the presence of eggs. Cloth liners with fertile eggs were collected and stored at 25°C. A cohort comprising 135 neonates from each single pair was used to establish individual isofemale lines.

F_2 Generation

Larvae were reared to adults and bulk-mated with siblings. A minimum of 40 moths (20 males and 20 females) constituted each isofemale line. Eggs from each line were harvested daily for up to 4 d to ensure that at least 300 eggs had accumulated for testing.

Bioassay Method

All bioassays were performed on artificial diet into which formulated insecticide was incorporated. The ratio of diet to toxin determined the concentration calculated as micrograms of insecticide per milliliter of diet.

For F_2 bioassays, formulated insecticides were diluted in distilled water to produce a concentration expected to induce 99.9% mortality of susceptible insects. The discriminating concentration for emamectin benzoate, chlorantraniliprole, and indoxacarb was 0.2, 1, and 12 µg of insecticide/ml of diet, respectively (Bird 2015). Diluted insecticide was added to 1 liter of diet and incorporated by using a stick blender to produce a homogenous mixture. Insecticide-incorporated diet was then dispensed into 45-well bioassay trays as described above. We aimed to expose 90 larvae (two 45-well trays) from each isofemale line to each of the three insecticides. A minimum of 500 lines were screened for each insecticide in each season.

For complementation tests for allelism, stock suspension of insecticide was diluted with distilled water to produce six or seven twofold dilutions which were added to 200 ml of diet and incorporated by vigorous shaking by hand for 30 s to produce a homogenous mixture then dispensed into 45-well bioassay trays as above. Bioassays were performed on three cohorts of insects with individual treatments (insecticide concentrations) of each cohort consisting of a minimum of 20 individuals; untreated diet was used as the control.

Insects used in all bioassays were reared to the late second or early third larval instar on untreated diet. To ensure the fitness of test insects, F_2 progeny of isofemale families that did not reach the required stage of development within four to five days were discarded. Test larvae were introduced to trays containing bioassay diet (one larva per well) and covered with heat-sealed, perforated lids.

Bioassays were maintained for 7 d under the same conditions described above for larval rearing. Larvae were considered dead if one or more of the following criteria were demonstrated: larvae unable to perform coordinated movement when prodded; paralysis of prolegs; larvae very slow to right themselves (time exceeding 3 s).

Isolation of Resistance

If resistance is recessive, 6.25% of F_2 progeny will be homozygous (*rr*) for resistance. However, if resistance is inherited as a nonrecessive trait the heterozygote is more to likely survive a discriminating concentration of insecticide in the F_1 and the proportion of survival in the F_2 will also be increased depending on the degree of dominance of resistance. All surplus larvae were retained as a "back-up" cohort of each isofemale family and stored at 15°C pending the result of bioassays. They were discarded only if the screens of emamectin benzoate, chlorantraniliprole, and indoxacarb produced no survivors. If, on the other hand, one or more survivors were detected, a process of validation was undertaken which comprised two steps. First, a minimum of 90 additional larvae of the back-up cohort from isofemale lines that tested positive were retested on the discriminating concentration of insecticide. Second, if there was more than one survivor in the retested cohort, all surplus material along with survivors of the F_2 screens were reared to adult. Progeny of surviving larvae were mated among themselves or crossed to the untreated cohort. The subsequent F_3 generation was retested and the line was retained for further characterization if it demonstrated survival at the discriminating concentration. In some cases, where there were insufficient numbers of F_2 larvae for retesting, validation of resistance was determined from the results of the F_3 bioassays.

Seven lines that tested positive for indoxacarb resistance (three from 2013–2014 and four from 2014–2015) were retained in laboratory culture for further analysis. In the F_4, each strain was outcrossed (except EM14-1) to the laboratory susceptible New GR strain to reduce the effects of inbreeding depression which can influence the outcome of bioassays (Gahan et al. 2005). Each strain was then reselected with the discriminating dose of indoxacarb for four to six generations. Complementation tests for allelism were performed by backcrossing to the reference strain GY7-39, which was the first indoxacarb strain isolated from the field in 2013 (Bird 2016). Male and female reciprocal crosses within each test were pooled because there was no difference in the median lethal concentration when bioassayed by diet incorporation of insecticide.

Insecticides

Commercial insecticide formulations were used in all bioassays: indoxacarb (Steward EC [15% active ingredient], Du Pont Australia Ltd., Macquarie Park, Australia), emamectin benzoate (Affirm [1.9% active ingredient], Syngenta Crop Protection), and chlorantraniliprole (Altacor [35% active ingredient], Du Pont Australia Ltd.).

Table 1. Establishment of isofemale lines of *H. armigera* and progression through an F_2 screen

Year	F_0 lines started	F_1 generation		F_2 lines screened		
		No. of isolines that produced larvae	No. of isolines that produced moths	Emamectin benzoate	Chlorantraniliprole	Indoxacarb
2013–2014	1,595	875	570	500	525	548
2014–2015	1,733	874	727	652	656	665
2015–2016	2,612	1,001	782	628	636	650
Total	5,939	2,750	2,079	1,780	1,817	1,863

Data Analysis

Where necessary, bioassay data were corrected for control mortality (Abbott 1925). Estimates of LC_{50} values and their 95% fiducial limits (FL) were obtained by probit analysis using POLO-PC software (LeOra Software, Berkeley, CA). Toxicity ratios (RR) were calculated by dividing the LC_{50} value of the resistant strain GY7-39 by the LC_{50} value of the test strains. Significant differences ($P = 0.05$) between LC_{50} values were determined by the lethal concentration ratio test according to Wheeler et al. (2006) where, if the 95% confidence interval (CI) includes 1 then the LC_{50}s were not significantly different. Resistance ratios were calculated by dividing the LC50 value of the selected strains by the LC50 value of the laboratory susceptible strain.

A χ^2 goodness-of-fit test (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) was used to compare the proportion of survivors within isofemale lines that scored positive for resistance relative to the proportion of survivors that would be expected under a hypothesis that resistance was recessive. If resistance is completely recessive, 6.25% of larvae would be expected to survive a discriminating concentration of insecticide.

For comparisons of mating success of moths sourced from pheromone traps and moths sourced from the field as eggs or larvae, and comparisons of proportion of positive tests from moths sourced from pheromone traps and moths sourced from the field as eggs or larvae, we used logistic regression (generalized linear model with logistic transformation) with the two-level factor (pheromone male, nonpheromone male) as the explanatory term (R Development Core Team 2008). The change in deviance by adding the explanatory term was compared to a χ^2_1 distribution to determine if proportions were significantly different.

Results

Progression to F_2 Generation

From 2013 to 2016, a total of 1,780, 1,808, and 1,863 isofemale lines were examined for resistance to emamectin benzoate, chlorantraniliprole, and indoxacarb, respectively, using an F_2 screening method (Table 1). Details of the number of F_0 lines that were established as single-pairs, and the number of isofemale lines that resulted from these pairs and passed through a defined stage over the course of this study are also shown in Table 1. Approximately 54% of lines were lost during the F_0 generation through failure to mate or where mating had resulted in either nonviable eggs or too few viable eggs to establish an F_1 strain. Of the 2,750 lines that were initiated from field-derived eggs, larvae, or moths, ~30% were bioassayed with one or more insecticides. Of the F_1 larvae produced, 76% successfully emerged as F_1 moths and were established with a minimum of 20 males and 20 females.

Detection of Resistance

The combined testing data from all years and collecting sites is shown in Table 2. None of the 1,780 lines bioassayed against emamectin benzoate scored positive for resistance to this insecticide. From the three-year combined total of 1,817 lines tested with chlorantraniliprole, five scored positive, resulting in an average resistance frequency of 0.0027 with a 95% CI between 0.0012 and 0.0064. Of 1,863 lines tested with indoxacarb 50 lines scored positive, resulting in an average resistance frequency of 0.027 with a 95% CI between 0.020 and 0.035 over the period of this study.

F_2 Screens From 2013–2014

A total of 1,595 single-pair crosses were set up from eggs and larvae collected in 2013–2014. Progression of these isofemale lines through to the F_2 screen is summarized in Table 1. Among these, 875 pairs (55%) produced sufficient numbers of eggs for establishment of an F_1 generation. A total of 570 lines then went on to produce F_1 adults, which represents 36% recovery from the original F_0 lines started. Further losses were incurred due to the failure of F_1 moths to mate and produce sufficient numbers of larvae for bioassay. However, the majority (96%) of F_1 larvae that successfully emerged as F_1 moths then went on to be tested by one or more insecticide. While no isofemale lines scored positive for emamectin benzoate or chlorantraniliprole resistance, 9 of the 548 lines examined for resistance to indoxacarb scored positive in 2013–2014, resulting in a frequency of 0.016 (95% CI 0.009–0.031; Table 2). This result was confirmed by validation bioassays in the F_3 generation.

F_2 Screens From 2014–2015

Progression of isofemale lines through to the F_2 screen in 2014–2015 is summarized in Table 1. Insect sampling by the use of pheromone traps was introduced to supplement egg and larval collection in the 2014–2015 season. From 1,733 single-pair crosses, 50% (874 pairs) produced sufficient numbers of viable eggs for establishment of an F_1 generation. The majority (91%) of F_1 larvae went on to provide F_2 larvae for testing of one or more insecticides. Again, no isofemale lines scored positive for emamectin benzoate resistance, while a single line scored positive for chlorantraniliprole resistance from 656 tests resulting in a resistance frequency of 0.0015 (95% CI 0.0003–0.0086). A total of 665 lines were tested with indoxacarb. Of these, 25 lines scored positive for indoxacarb resistance after being rescreened in the F_3 generation resulting in a resistance frequency of 0.038 (95% CI 0.026–0.055).

In 2014–2015, 62% of F_0 lines established from pheromone trap-caught male moths and crossed with laboratory susceptible females went on to produce viable F_2 isofemale lines tested for resistance to one or more insecticides. In contrast, only 32% of F_0 lines established from male and female moths collected as eggs or larvae progressed to the F_2 for testing (Table 3). Furthermore, nonpheromone trap males mated with laboratory susceptible females

Table 2. Number of isofemale lines from F_2 screens that scored positive for resistance to emamectin benzoate, chlorantraniliprole, and indoxacarb

Year	Location	Emamectin benzoate F_2 screen		Chlorantraniliprole F_2 screen		Indoxacarb F_2 screen	
		Total tested	Total positive	Total tested	Total positive	Total tested	Total positive
2013–2014	Namoi	295	0	310	0	321	5
	Gwydir	22	0	23	0	23	2
	Macquarie	25	0	25	0	28	0
	Mungindi	15	0	15	0	16	0
	Darling Downs	27	0	31	0	31	0
	St George	3	0	3	0	3	0
	Emerald	20	0	24	0	27	0
	MacIntye	16	0	16	0	17	0
	MIA	26	0	27	0	28	1
	Burdekin	43	0	44	0	46	1
	Lachlan	8	0	7	0	8	0
	Season total	500	0	525	0	548	9
2014–2015	Namoi	149	0	153	0	155	2
	Namoi pheromone traps	225	0	228	1	229	10
	Gwydir	81	0	80	0	82	2
	Macquarie	16	0	16	0	16	2
	Mungindi	35	0	35	0	38	1
	Darling Downs	14	0	12	0	12	0
	St George	38	0	38	0	37	4
	Emerald	47	0	49	0	50	4
	Macintyre	24	0	23	0	23	0
	MIA	5	0	4	0	5	0
	Kununurra	18	0	18	0	18	0
	Season total	652	0	656	1	665	25
2015–2016	Namoi	67	0	70	1	73	4
	Namoi pheromone traps	494	0	501	1	510	11
	Gwydir	9	0	9	1	9	0
	Macquarie	1	0	1	0	1	0
	Mungindi	3	0	3	0	3	0
	Darling Downs	32	0	32	0	32	0
	St George	16	0	16	1	16	1
	Emerald	4	0	2	0	4	0
	Macintyre	2	0	2	0	2	0
	Season total	628	0	636	4	650	16

Table 3. Comparison of testing efficiency from pheromone trap caught male moths, and moths sourced from egg and larval samples in 2014–2015 and 2015–2016

Moth source	2014–2015			2015–2016		
	No. F_0 lines started	No. F_2 tests conducted	% Tested	No. F_0 lines started	No. F_2 tests conducted	% Tested
Pheromone trap males	371	229	62[a]	1,600	510	32[a]
Nonpheromone trap males	674	305	45[b]	531	81	15[b]
Nonpheromone trap females	688	131	19[c]	480	59	12[b]

Proportions followed by the same letters are not significantly different ($P < 0.05$).

produced more viable F_2 lines (45%) compared with field-derived females mated with laboratory susceptible males (19%). The difference between pheromone trap caught males and moths from other sources was highly significant in all cases ($P < 0.0001$; Table 3), indicating that male moths sourced from pheromone traps had a greater degree of efficiency for producing F_2 strains, particularly in the F_0 generation, than their male and female counterparts sourced from the field as eggs and larvae.

The number of positive indoxacarb tests from pheromone trap-caught males (4.4%) was not significantly different ($P = 1.0$) from the male cohort sourced from eggs and larvae (4.3%; Table 4). There was also no significant difference ($P = 0.2546$) between the number of positive indoxacarb tests from pheromone trap males and female moths sourced as eggs and larvae (1.5%; Table 4), suggesting that the source of moths may not influence the presence of resistance alleles.

F_2 Screens From 2015–2016

Only 38% (1,001/2,612) of single-pairs initiated from the F_0 insects produced viable F_1 larvae (Table 1). Of the 782 lines that progressed to the moth stage, 83% went on to produce sufficient larvae for

Table 4. Comparison of indoxacarb positive tests from pheromone trap-caught male moths, and moths sourced from egg and larval samples in 2014–2015 and 2015–2016

Moth source	2014–2015				2015–2016			
	Total tests	Positive tests	% R	*P*	Total tests	Positive tests	% R	*P*
Pheromone trap male	229	10	4.4	–	510	11	2.2	–
Nonpheromone trap male	305	13	4.3	1.0	81	2	2.5	1.0
Nonpheromone trap females	131	2	1.5	0.2546	59	3	5.1	0.3521

testing of one or more insecticides. No lines scored positive for emamectin benzoate resistance. Four lines scored positive for chlorantraniliprole resistance from a total of 636 tested, resulting in a resistance frequency of 0.0063 (95% CI 0.0024–0.0161; Table 2). A total of 650 lines were tested with indoxacarb and, of these, 16 lines scored positive after being rescreened in the F_3 generation, resulting in a resistance frequency of 0.025 (95% CI 0.015–0.040; Table 2).

Although the number of tests conducted (as a proportion of the F_0 lines started) was lower than in the previous year, the results demonstrated a similar increase in capacity for pheromone trap male moths to produce viable F_2 isofemale lines. Significantly more of the pheromone trap-collected moths progressed to the testing stage (32%), compared with male moths and female moths that had been collected as immature life stages and reared to adults in the laboratory (15 and 12%, respectively) and produced successful F_2 isofemale lines ($P < 0.001$; Table 3). As in the previous year the number of positive indoxacarb tests from pheromone trap males (2.2%) was not significantly different from the male cohort sourced from eggs and larvae (2.5%) ($P = 1.0$; Table 4). There was also no significant difference between the number of positive indoxacarb tests from pheromone trap males and female moths derived from field-collected eggs and larvae (5.1%) ($P = 0.3521$; Table 4), suggesting that the source of moths may not influence the presence of resistance alleles.

Survival in Isofemale Lines That Carried Resistance Alleles

A total of 50 isofemale lines tested positive in indoxacarb screens over the three years of this study (Table 2). In the first strain that tested positive, GY7-39, the proportion of larvae that survived the discriminating concentration of indoxacarb was statistically greater than the 6.25% expected if resistance is recessive (proportion observed = 25.7, $\chi^2 = 231$, $P < 0.0001$). This was also the case for another 44 lines (proportion observed = 10–33, $\chi^2 > 4.1$, $P < 0.0433$). These results suggest that resistance is not completely recessive in the majority of cases where isofemale lines scored positive for indoxacarb resistance. On the other hand, in three lines (from Burdekin in 2013, from Emerald in 2014, and from Namoi in 2016) the proportions of larvae that survived were not statistically different from the 6.25% expected if resistance is completely recessive (proportion observed = 7.4–8.9, $\chi^2 < 1.07$, $P > 0.301$). In a further three lines isolated in 2016 (two from the Namoi and one from St George) survival was also consistent with recessive inheritance (proportion observed = 11.1, $\chi^2 = 3.63$, $P = 0.0568$).

Five isofemale lines scored positive for chlorantraniliprole resistance (Table 2). In four lines, the proportion of larvae that survived a discriminating concentration of chlorantraniliprole was not statistically different from the 6.25% expected if resistance is recessive (proportion observed = 5.6–8.7, $\chi^2 < 0.074$, $P > 0.1906$). In one case, the proportion of larvae that survived was statistically greater than the proportion expected for recessive resistance (proportion observed = 17.8, $\chi^2 = 40.824$, $P < 0.0001$), suggesting that more than one mechanism of resistance may be involved. Complementation tests were not performed on any lines that demonstrated reduced susceptibility to chlorantraniliprole, as it was difficult to maintain these strains in laboratory culture beyond one or two generations.

Complementation Tests for Allelism in Strains Positive for Indoxacarb Resistance

Strains that were selected for resistance and then backcrossed to an indoxacarb resistant strain, GY7-39, were bioassayed with indoxacarb and results from the interstrain complementation tests presented in Table 5. All selected strains exhibited resistance ratios of > 100-fold compared with the laboratory strain, New GR. The F_1 progeny from crosses between five of the six strains (UN29-4, LN12-1, MQ6-4, and EM14-1) and the GY7-39 strain were not significantly more susceptible to indoxacarb compared with the parental strain of GY7-39, suggesting that a major resistance locus may be common to these strains. In one strain, LN16-40, the F_1 progeny from crosses of this and the GY7-39 strain were significantly more susceptible to indoxacarb than the parental GY7-39 strain (Table 5), which may have been the result of inbreeding depression following six generations of selection in the LN16-40 strain.

Discussion

The time frame to respond to insecticide resistance before field failures occur largely depends on the precision with which resistance alleles can be detected in the population and the dominance of resistance alleles (Andow and Ives 2002). Prior to the present study there were no documented cases of resistance to emamectin benzoate, chlorantraniliprole, or indoxacarb isolated from Australian populations of *H. armigera*, even after commercial use of these products over many years (Rossiter et al. 2008). It was therefore presumed that alleles that confer resistance to these insecticides are currently rare in Australian *H. armigera* and is supported by baseline studies which show high susceptibility in geographically diverse populations (Bird 2015). We made no presumption about the dominance of resistance to these insecticides and adopted the F_2 approach as a means of increasing the limits of detectability if recessive resistance alleles were present in the population.

The F_2 screen was originally designed to use mated field-caught females (Andow and Alstad 1998). However, as noted by Stodola and Andow (2004), the F_2 screen is sufficiently flexible as to allow modification of the breeding steps without compromising the effectiveness of the technique. A notable methodological variation on the theoretical basis of the F_2 screen used herein involved a combination of wild-caught males and moths derived from natural populations as field collected eggs and larvae, which were then individually mated to a laboratory susceptible strain. Although this modification reduces by half the number of genes screened, it nevertheless enhances the likelihood of obtaining offspring from heterozygous moths, as we had experienced very poor mating efficiency where both parental

Table 5. Indoxacarb bioassays performed on strains isolated for indoxacarb resistance by F_2 screening between 2013 and 2016 and then backcrossed an indoxacarb-resistant strain GY7-39

Strain	Date collected	Number of generations selected	LC_{50} [μgAI/ml diet] (95% FL)	Fit of probit line			Toxicity Ratio (95% CI)	Resistance Ratio
				Slope	X^2 (df)	*P*		
GY7-39	Nov. 2013		21.0 (18.0, 24.6)	2.6 ± 0.22	3.622 (4)	0.4596	–	117
UN29-4	Feb. 2014	5	20.8 (15.7, 28.0)	2.9 ± 0.26	7.658 (4)	0.1049	1.01 (0.82, 1.25)	116
UN29-4 x GY7-39			20.5 (18.6, 22.7)	2.8 ± 0.17	1.573 (4)	0.8136	1.03 (0.86, 1.24)	114
GY7-39			18.8 (10.1, 40.1)	2.2 ± 0.22	15.36 (4)	0.0040	–	104
LN49-9	Mar. 2014	4	27.5 (20.2, 37.6)	2.8 ± 0.27	4.482 (3)	0.2139	0.76 (0.62, 0.93)	153
LN49-9 x GY7-39			29.2 (23.1, 37.4)	2.7 ± 0.27	9.834 (4)	0.0433	0.72 (0.60, 0.86)	162
GY7-39			37.1 (31.3, 44.9)	2.2 ± 0.21	2.647 (4)	0.6185	–	206
LN12-1	Sept. 2014	4	34.1 (30.1, 38.5)	3.9 ± 0.40	0.520 (2)	0.7711	1.09 (0.87, 1.35)	189
LN12-1 x GY7-39			29.0 (25.8, 32.9)	2.3 ± 0.14	4.486 (5)	0.4818	1.28 (1.00, 1.59)	161
GY7-39			37.1 (31.3, 44.9)	2.2 ± 0.21	2.647 (4)	0.6185	–	206
LN16-40	Nov. 2014	6	18.2 (9.7, 40.8)	1.6 ± 0.14	26.22 (5)	0.0001	2.04 (1.54, 2.69)	101
LN16-40 x GY7-39			22.3 (19.7, 25.4)	2.0 ± 0.13	2.442 (4)	0.6551	1.66 (1.33, 2.08)	124
GY7-39			29.8 (26.4, 33.5)	4.3 ± 0.44	2.221 (3)	0.5278	–	166
MQ6-4	Dec. 2014	4	33.3 (25.3, 45.0)	3.2 ± 0.30	7.668 (4)	0.1045	0.89 (0.74, 1.07)	185
MQ6-4 x GY7-39			28.8 (26.4, 31.4)	3.8 ± 0.26	2.320 (3)	0.5087	1.03 (0.89, 1.20)	160
GY7-39			24.8 (19.0, 33.2)	2.4 ± 0.20	8.085 (5)	0.1516	–	138
EM14-1	April 2015	5	27.8 (18.6, 45.1)	2.1 ± 0.24	6.923 (5)	0.2265	0.89 (0.68, 1.17)	154
EM14-1 x GY7-39			34.4 (28.1, 42.8)	2.7 ± 0.18	7.197 (4)	0.1258	0.72 (0.59, 0.88)	191
New GR (laboratory susceptible strain)			0.18 (0.11, 0.28)	4.8 ± 0.54	4.176 (2)	0.1239	–	–

moths were derived from field collected eggs and larvae (L.J.B unpublished data). The capability for detecting resistance using this method was demonstrated by the isolation of genetic indoxacarb resistance from F_2 screens (Bird 2016).

Regardless of the methods chosen, a statistical problem associated with any resistance screening procedure relates to the possibility of false inference, either the chance of detecting a false positive or a false negative result in error. In the case of the former scenario, we minimized the chance of identifying an isofemale line as carrying a resistance allele when it is actually susceptible (detection of a false positive) by using a screening method sensitive enough to discriminate between lines with and without resistance. In the present study, we used a discriminating concentration based on the 99.9% lethal concentration in geographically diverse field strains (Bird 2015), and retested all potential positives in the F_2 and/or the F_3 generation to confirm the presence of resistance.

The other possibility is identification of an isofemale line as susceptible when it actually carried a resistance allele (detection of a false negative) which can occur in F_2 screens when an isofemale line starts with a resistance allele which is subsequently lost or reduced to a low frequency in the F_1 or F_2 generation. First, to reduce the probability of false negatives in F_2 screens, all isofemale lines were established from a minimum of 20 males and 20 female moths in order to produce sufficiently large F_1 populations and maximize the number of RS × RS matings. However, this may not preclude differences in mating behaviors among moths with or without resistance alleles which may influence proportions of resistant larvae detected in F_2 screens (Higginson et al. 2005, Blanco et al. 2008). Second, we included in the analysis only F_1 parents that contributed to the F_2 screens, and we generated large F_2 cohorts of >800 larvae from which a random selection was tested. Third, a minimum of 500 lines for each insecticide were screened in each year.

Another factor that may influence the ability to detect rare resistance alleles in the F_2 screen is whether resistance is monogenic or polygenic. The F_2 screen is designed to estimate monogenic inheritance (Andow and Alstad 1998). Genetic characterization of indoxacarb resistance indicates monogenic inheritance of this resistance allele in *H. armigera* (Bird 2016). In contrast, analysis of chlorantraniliprole resistance in *Plutella xylostella* suggests that resistance may be conferred by a combination of multiple metabolic factors and/or target site resistance due to reduced binding affinity at the ryanodine receptor (Guo et al. 2014, Liu et al. 2015). Likewise, resistance to emamectin benzoate resistance in *Spodoptera* spp. was found to be polygenic (Shad et al. 2010, Che et al. 2015), suggesting that the F_2 screen may not be as effective for detection of resistance to these insecticides.

The lack of recessive inheritance of indoxacarb resistance implied from this study is consistent with previous findings from characterization of the first isolated indoxacarb-resistant strain of *H. armigera* (Bird 2016). However, deviations from a nonrecessive model of indoxacarb resistance in *H. armigera* were evident in six out of 50 lines. It is unclear if this represents a genetic variant of the first indoxacarb-resistant strain isolated. However, complementation tests for allelism were performed on six other strains isolated between 2013 and 2016 and, in five of these cases, results support the conclusion that resistance was due to alleles present at the same locus. Strains that tested positive for chlorantraniliprole resistance were difficult to maintain in laboratory culture, which may indicate the presence of a fitness cost associated with enhanced survival to this insecticide in *H. armigera*. Reduced fitness has also been observed in strains of *P. xylostella* resistant to chlorantraniliprole (Ribeiro et al. 2014) and cyantraniliprole (Liu et al. 2015).

Mating capacity in the F_1 generation was significantly enhanced where pheromone-caught males were involved. Field-caught male moths were used in favor of field-caught female moths because multiple mating occurs in this species (Coombs et al. 1993), which could lead to misinterpretation of resistance data. Significantly more isofemale lines were generated from pheromone-caught moths compared with moths reared from field-collected eggs and larvae. In addition, females derived from immature life stages produced significantly

fewer F_2 lines than males derived from immature life stages, suggesting progeny of field-derived females had lower viability than laboratory adapted females. Adaptation to laboratory environments has also been attributed to improved performance of F_1 progeny of *Ostinia nubilalis* (Stodola et al. 2006).

The improvements in efficiency for producing F_2 lines associated with wild-caught male moths were likely due to the elimination of losses from egg and larval parasitism, pathogenic infection, infertility, mixed species composition, spray drift, and temperature-induced stress associated with transport of egg and larval samples from remote locations. An additional advantage of a method that uses field-caught moths instead of immature life stages is that it circumvents the need for extended laboratory rearing of insects and hence further enhances the economic efficiency with which samples can be processed (Bolin et al. 1998).

Levels of detection for indoxacarb resistance were not significantly different between pheromone and nonpheromone trapped moths, suggesting that the source of moths may not influence the presence of resistance alleles. The increased efficiency of a pheromone-based sampling procedure combined with increased sensitivity of the F_2 screening technique should enhance our ability to identify resistance risk to key selective insecticides for control of *H. armigera*. Results from the present study demonstrate low but detectable levels of resistance to indoxacarb and chlorantraniliprole in Australian populations of *H. armigera* and highlights the need for continued use of methods with a high level of sensitivity for resistance detection. As noted by Gould (1998), this will be particularly important for monitoring the development of resistance conferred by nonrecessive alleles, such as the case with indoxacarb resistance, which favor resistance evolution and therefore pose more of a challenge for implementing management and mitigation tactics.

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PLOS ONE

RESEARCH ARTICLE

Multiple recombination events between two cytochrome P450 loci contribute to global pyrethroid resistance in *Helicoverpa armigera*

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Abstract

The cotton bollworm, *Helicoverpa armigera* (Hübner) is one of the most serious insect pest species to evolve resistance against many insecticides from different chemical classes. This species has evolved resistance to the pyrethroid insecticides across its native range and is becoming a truly global pest after establishing in South America and having been recently recorded in North America. A chimeric cytochrome P450 gene, *CYP337B3*, has been identified as a resistance mechanism for resistance to fenvalerate and cypermethrin. Here we show that this resistance mechanism is common around the world with at least eight different alleles. It is present in South America and has probably introgressed into its closely related native sibling species, *Helicoverpa zea*. The different alleles of *CYP337B3* are likely to have arisen independently in different geographic locations from selection on existing diversity. The alleles found in Brazil are those most commonly found in Asia, suggesting a potential origin for the incursion of *H. armigera* into the Americas.

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Introduction

In agriculture, insecticides are commonly used to control insect pest species. However, pest management is hindered by the increasing level of insecticide resistance in various pest species worldwide. One of the most serious insect pest species to evolve resistance against many insecticides from different chemical classes is the Old World cotton bollworm, *Helicoverpa armigera* (Hübner). Its pest status is further strengthened by its highly polyphagous nature and extremely wide geographical distribution, with the latter due to its ability to migrate long distances [1–4]. Recent work confirmed a major incursion of *H. armigera* into Brazil, as well as Argentina, Uruguay and Paraguay and it has been detected in Puerto Rico and Florida [5–8].

Pyrethroid resistance of *H. armigera* was first reported in Australian populations in 1983, six years after the introduction of these insecticides [9]. Since then, pyrethroid resistance has been reported from various populations globally but, in most cases, the molecular resistance mechanism in *H. armigera* is yet to be determined. The two major pyrethroid resistance mechanisms common to other species are target site insensitivity, and metabolic resistance by cytochrome P450 monooxygenases (P450s) and carboxylesterases [10, 11]. Target site resistance against pyrethroids, also known as knockdown resistance (*kdr*), is based on one or more point mutations in the insect sodium channel protein, which is the target of pyrethroid insecticides. In *H. armigera*, two mutations (D1549V and E1553G) in the sodium channel protein have been described [12, 13]. In the case of metabolic resistance, 18 P450s have been identified as capable of metabolizing one or more pyrethroid insecticides after heterologous expression [11, 14–19]. One of them is the chimeric *CYP337B3*, recently identified in Australian *H. armigera* and thought to have arisen through an unequal crossing-over between the parental genes, *CYP337B1* and *CYP337B2* [18].

Joussen et al. [18] demonstrated that, after heterologous expression, *CYP337B3* is capable of metabolizing fenvalerate, a type II pyrethroid ester, to the nontoxic 4'-hydroxyfenvalerate. In contrast, the parental enzymes exhibit no detectable fenvalerate metabolism. *CYP337B3* has been shown to confer 42-fold resistance towards fenvalerate in Australian *H. armigera* lines differing solely in the presence of *CYP337B3* and its parental genes [18]. Similarly, a 49-fold resistance factor was reported by Forrester et al. [20] for an Australian field-collected population, indicating that metabolism of fenvalerate by *CYP337B3* is the detoxification mechanism *in vivo*.

Recently, *CYP337B3* was also identified in field-collected populations from Pakistan [21] and China [22], confirming its presence outside Australia. Sequence analysis revealed a distinct *CYP337B3* allele (*CYP337B3v2*), in the Pakistani population and three distinct alleles in the Chinese populations (*CYP337B3v2*, *CYP337B3v3*, *CYP337B3v4*) that differ from the Australian allele (*CYP337B3v1*), by a number of synonymous and non-synonymous SNPs, in addition to variability of the intron sequence and size. This variation may result from different crossing-over positions during recombination of the *CYP337B1* and *CYP337B2* parental genes, with different alleles of *CYP337B1* and *CYP337B2* involved in the crossing-over. Such a pattern is indicative of independent origins of the various *CYP337B3* alleles, however further work would be required to determine whether this is accurate across a wide geographic distribution of samples. Given the high frequency of this allele around the world, it is possible that the population of *H. armigera* now established in South America [5, 23, 24] may be carrying this allele and that the possession of *CYP337B3* may confer a selective advantage over local species.

Previous studies with allozymes and mitochondrial DNA indicated that very little population structure among *H. armigera* populations, though more recent work using many thousand genomic markers has shown that the population of *H. armigera* in Australia can be

differentiated from the rest of the world [25, 26]. This lack of structure makes the identification of the *CYP337B3* chimeric origin difficult, as well as more generally hampering the identification of source populations in the case of incursions into the New World. However, examining patterns of distribution and diversity among recently selected genes can shed light on such processes. *CYP337B3* is a strong candidate for having undergone rapid selection due to its role in pyrethroid resistance, which has a short (approximately 50 year) history across much of the globe [27].

Here, we use field-collected populations from 17 countries distributed through Africa, Asia, Oceania, Europe, and South America to first examine the evolutionary history of the chimeric *CYP337B3* gene and the global spread in *H. armigera*. We use global allelic frequency data to show that *CYP337B3* is commonly found around the world, with at least six likely independent unequal crossing-over events giving rise to the same resistance phenotype in different regions. We also confirm recent data showing evidence of hybridisation between invasive *H. armigera* from Brazil and the local *Helicoverpa zea* population, including the exchange of the *CYP337B3* gene [26, 28]. Furthermore, we then use the different *CYP337B3* alleles to shed light on the nature of selection of *CYP337B3*, finding evidence to support recent selection operating on standing variation as opposed to novel mutations arising as a result of pyrethroid application.

Materials and methods

Population samples

A total of 1063 insect samples were used in the current study, including several specimens collected and used in previous work [5, 25, 26, 28, 29]. *H. armigera* was collected from 17 countries, *H. zea* from two (Fig 1; S1 Table). All insect samples were collected from agricultural areas with the permission of the landholder or by trapping using species-specific pheromones. No collecting permits were required because both species are common agricultural pests. No endangered or protected species were used in this work. Samples were usually collected as larvae from wild and crop host plants, as adult moths via light/pheromone traps or as larvae after bioassay. For one set of samples from China (n = 240), larvae were collected from colonies derived from eight recently collected field samples (established with 20 to 50 individuals). Collection dates range from 2002 to 2013. Samples were preserved in ethanol (>95%), in RNAlater, or at -20˚C prior to DNA extraction. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) or the Fast Tissue-to-PCR Kit (Fermentas, Thermo Scientific).

The species status of several preserved specimens was confirmed by mitochondrial gene (*COI* and *Cytb*) sequencing, either from previous work [5, 29] or, where new samples were available, by amplifying and sequencing the same regions (S2 Table). PCR amplification followed the protocols of Behere et al. [29] and Tay et al. [5]. PCR products were sequenced at Macrogen (Seoul, Korea) and the Biological Resources Facility (Australian National University, Canberra, Australia). Assembly of DNA trace sequences was performed using CLC Genomics Workbench version 8.0.

Screening for *CYP337B3*

Following species identification, samples were screened for the presence of *CYP337B3* using the CYP337B2F and CYP337B1R primers described in Joussen et al. [18]. Heterozygote/homozygote status was determined through relevant band detection on 1.5–2% agarose gels containing 1% (w/v) of GelRed (Biotium) and visualised under UV light. Initial sequence was generated from these short fragments for a subset of samples following the PCR amplification protocol of Joussen et al. [18]. However, in order to generate more information, primers were designed to amplify the intron of the gene (S2 Table). In the chimeric *CYP337B3* gene,

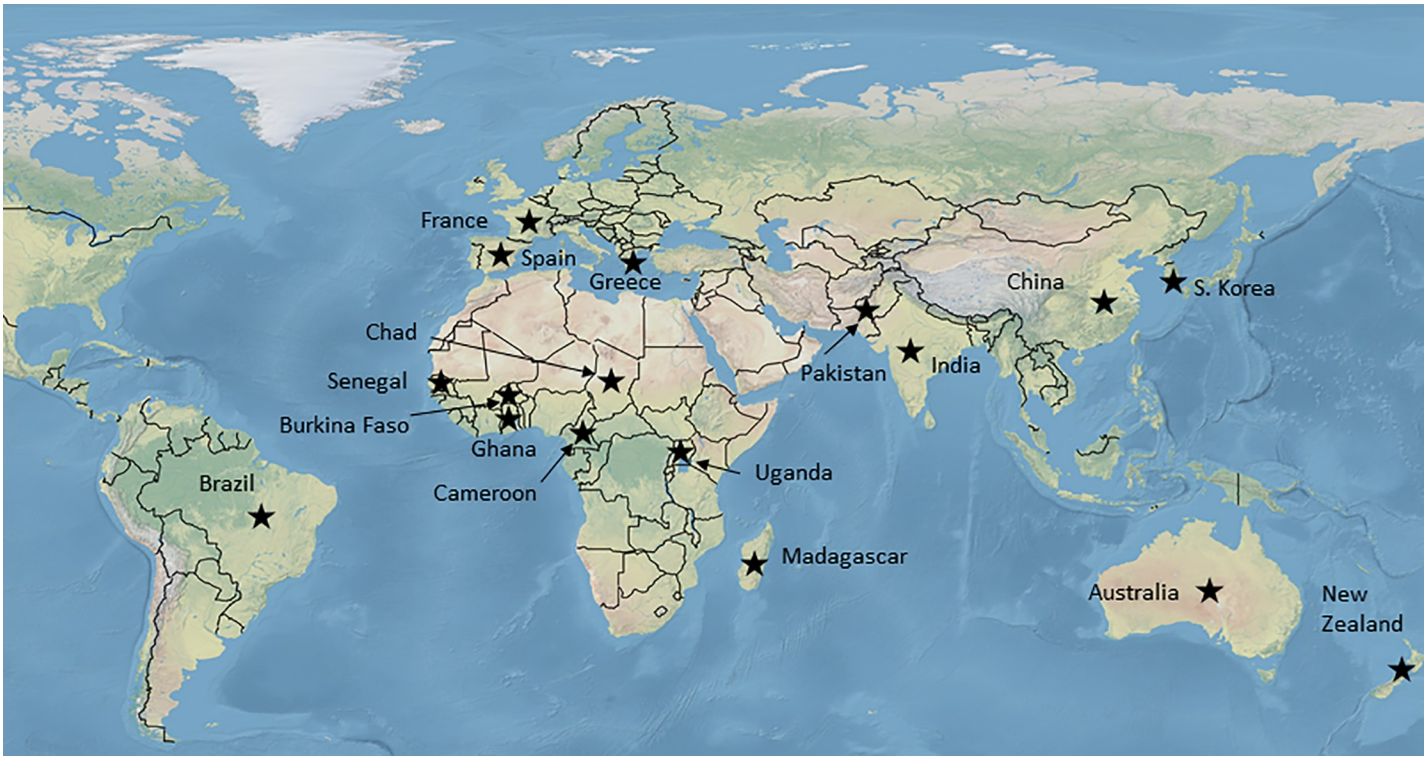

Fig 1. Collection localities. *H. armigera* (n = 986) were sampled from 17 countries (black stars): Australia, New Zealand, China, Korea, India, Pakistan, Greece, Spain, France, Burkina Faso, Cameroon, Chad, Ghana, Madagascar, Senegal, Uganda, and Brazil. *H. zea* were sampled from Brazil (n = 43) and the United States of America (n = 16). Modified from public domain map from Natural Earth (http://www.naturalearthdata.com).

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CYP337B1 contributes the intron and several hundred base pairs of coding sequence depending on the allele, while *CYP337B2* contributes much of the 5' end of the coding sequence. Thus, the *CYP337B3* intron corresponds to the *CYP337B1* intronic sequence. We also screened samples from Brazil (n = 181) and Australia (n = 97) for the reciprocal recombination event using CYP337B1F and CYP337B2R primers (S2 Table) but no products were detected with this primer combination.

Further amplification of full-length genes and transcripts was performed for selected individuals with representative alleles. PCR products and cloned fragments were sequenced at several institutes, including: Macrogen (Seoul, Korea), Biological Resources Facility (Australian National University, Canberra, Australia), UC Davis Genome Center (University of California, Davis, US), StarSEQ (Mainz, Germany), Bioneer Corporation (Daejeon, Korea) and the Department of Entomology at the Max Planck Institute for Chemical Ecology (Jena, Germany). Assembly and sequence analysis of the PCR products was done using CLC genomics workbench v8.0 and T-Coffee (http://www.ebi.ac.uk/Tools/msa/tcoffee/).

Resistance bioassays

To establish the role of *CYP337B3* in pythrethroid resistance, fenvalerate field resistant and susceptible individuals were identified by bioassay of field-collected material. Fenvalerate (95.3%) was provided by Sumitomo Chemical (Sydney, Australia) and dissolved in analytical grade acetone to produce a diagnostic concentration (0.125 μg/μL). A 1 μL volume of the discriminating concentration was applied to the dorsal thorax of late 3rd/early 4th instar larvae within a weight range of 30-40mg using a 50 μL micro-syringe in a repeating dispenser

(Hamilton Company, Reno, NV, USA). Bioassays were maintained for 7 d at 25˚C with a 14:10 (L:D) h cycle. Relative humidity (RH) was not controlled. Dead and moribund larvae were collected and described as sensitive by using one or more of the following criteria: larvae unable to demonstrate coordinated movement when prodded; paralysis of prolegs; larvae very slow to right themselves (time exceeding 3 s). Larvae that were actively feeding and developing normally were described as resistant.

Population genetic analysis

The elimination of variation in regions linked to an adaptive allele on one or even a few haplotypes is referred to as a "selective sweep" [30]. This process will lead to low nucleotide diversity (π) across the region of the genome under selection as well as an excess of rare variants as highlighted by Tajima's D [31]. This statistic identifies deviation from a neutral model of evolution, whereby a negative score signifies purifying selection or population expansion that one would expect following a selection event. To provide evidence that the *CYP337B3* gene is under selection in *H. armigera*, we searched for genomic signals of a recent selection event using high-throughput sequencing data.

DNA samples from 12 Australian *H. armigera* homozygous for *CYP337B3v1* were sequenced on an Illumina HiSeq. Briefly, Nextera libraries were produced following the manufacturer's instructions and sequence was generated as 100 bp PE reads (Illumina HiSeq 2000, Biological Resources Facility, Australian National University, Canberra, Australia). Raw reads were aligned to the bacterial artificial chromosome (BAC) 33J17 (JQ995292.1) sequence using BBMAP v33.43 (http://sourceforge.net/projects/bbmap/), trimming reads when quality in at least 2 bases fell below Q10. We did not align reads to the reference *H. armigera* genome sequence, which contains only *CYP337B1* and *CYP337B2* but not *CYP337B3* [32]. Only uniquely aligning reads were included in the analysis, to prevent spuriously inferring evolutionary processes occurring independently on each BAC. Outputted BAM files were sorted before duplicate reads were removed and files were annotated with read groups using Picard v1.138 (http://picard.sourceforge.net). The reference sequences were indexed using Samtools [33]. UnifiedGenotyper in GATK v3.3–0 [34] was used to estimate genotypes across all individuals simultaneously, implementing a heterozygosity value of 0.01. Genotypes annotated as "LowQual" were removed prior to subsequent analysis with VCFtools v0.1.12b [35], whereby π and Tajima's D were calculated in sliding windows of 2500 bp that progressed by 1250 bp across biallelic sites of 33J17. Results were plotted in R v3.1.2 using ggplot2 v1.0.1 [36], while gene annotations were derived via tblastx [37] and visualised with CLC Genomics workbench v8.0.

To examine potential mechanisms underlying the origin of the *CYP337B3* alleles, an intron dataset containing sequences for representative *CYP337B1* and *CYP337B3* individuals (since the chimeric *CYP337B3* intron comes from the *CYP337B1* parental gene; see above) was created. MAFFT ver. 7.182 [38] was used to align these sequences using the -linsi option. Subsequently, IQ-TREE ver. 1.3.0 [39] was used, first in the -m TESTNEWONLY mode to determine the appropriate nucleotide substitution model, and then in full mode to generate a maximum likelihood tree. Subsequently 10,000 bootstrap replicates were carried out, and the bootstrap values transferred from the consensus tree to corresponding nodes of the maximum likelihood tree.

BEAST ver. 2.3.0 [40] was used for the intron-only dataset to test whether the *CYP337B3* allele is monophyletic in origin (i.e., has arisen once from a *CYP337B1*/*CYP337B2* cross-over and subsequently diverged independently). Two analyses were performed–one, in which monophyletic taxon sets for each of the *CYP337B1* and *CYP337B3* introns was enforced, and

the other in which no monophyly was assumed. In BEAST, all runs used the evolutionary model identified in IQ-TREE (HKY + G4), a constant population coalescent tree prior, and a chain length of 10 x 10^6. Other coalescent tree priors were also tested, but these did not quantitatively impact the results. BEAST results were examined in Tracer to confirm convergence and check ESS values (all exceeded 1,500). The likelihood of each analysis was then compared using Bayes Factors and the ACIM model, with 1,000 replicates, in Tracer ver. 1.6 [41].

Results

A total of 1063 insect samples was collected from 18 countries around the world (Fig 1). The identification of *H. armigera* was initially based on morphological assessments by collectors and subsequently confirmed with the mitochondrial markers, *cytochrome oxidase I* and *cytochrome b* [29]. Any individuals not identified as *H. armigera* by mitochondrial DNA were excluded from subsequent analysis (*H. armigera* n = 999, *H. zea* n = 59, *Chloridea* (*Heliothis) virescens* n = 5).

A PCR assay using one primer from each of the two parental genes *CYP337B1* and *CYP337B2* was used to identify the presence of the chimeric *CYP337B3* gene. *CYP337B3* was extremely common in field collected *H. armigera* throughout its native range (Fig 2 and S1–S5 Figs). From a total of 999 individuals tested, *CYP337B3* was present in 969 (97%) and only 30 (3%) individuals were homozygous for *CYP337B1* or *CYP337B2*. 878 individuals were *CYP337B3* homozygotes (88%) and 91 were heterozygotes, meaning they carried one chromosome carrying *CYP337B3* and the other chromosome with the two parental genes *CYP337B1* and *CYP337B2* (9%). In some countries, almost all individuals were at least heterozygous for the *CYP337B3* gene, but homozygous *CYP337B3* individuals were the most common

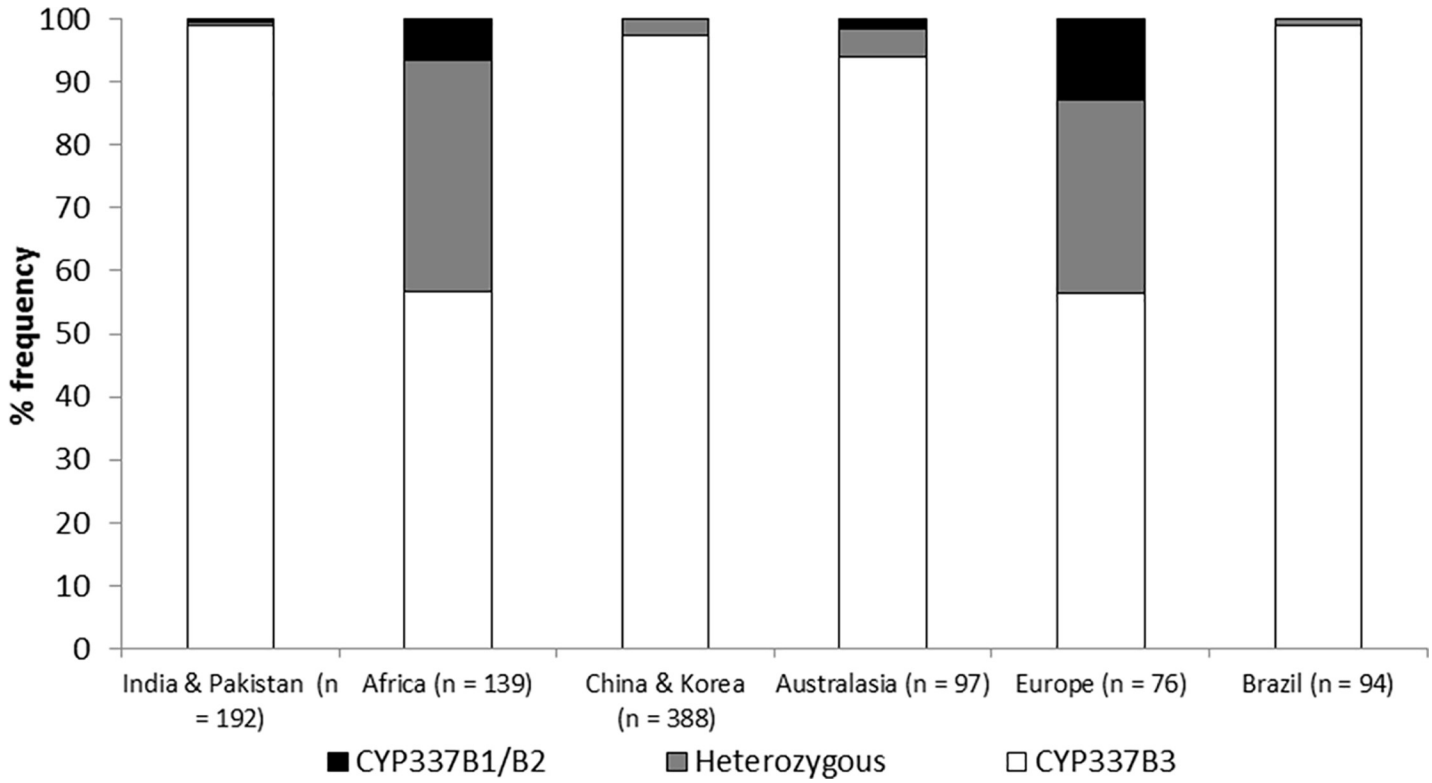

Fig 2. The frequency of *CYP337B* genotypes in selected geographic regions. For a more detailed breakdown see S1 Fig through S5 Fig.

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everywhere. In India, Pakistan, Korea, China, and Australia, almost all individuals were homozygous for *CYP337B3*, while heterozygotes (*CYP337B1*, *CYP337B2* and *CYP337B3*) at the *CYP337B3* locus were more common in European and African samples (Fig 2 and S1–S5 Figs).

The PCR product of *CYP337B3* used in the screening PCR reaction [18] was sequenced from positive individuals (alleles n = 460) and a pattern of single nucleotide polymorphisms was identified. By comparing this short (355 bp) sequence between individuals, 6 alleles (*CYP337B3v1-2*, *5–8*) were identified worldwide (Fig 3). An allele originally identified in an Australian (Toowoomba) strain [18] was found only in Australia in the current study, where it was the most common allele (*v1*: n = 91), and *v2* the next most common allele (*v2*: n = 23). In Asian samples, *v2* was the most common allele (n = 203) and two other rare alleles were identified (*v7* and *v8*; n = 1 each) in addition to *v3* and *v4* identified by Han et al. [22]. Three alleles were found in Africa including two that were new (*v2*: n = 11, *v5*: n = 79, *v6*: n = 8). In the European samples, we identified an approximately 50:50 ratio of *CYP337B3v2* and *v5* (v2: n = 20; v5 n = 17). In addition, a proportion of samples from Australia (n = 11), Africa (n = 2), and Europe (n = 7) appeared from the sequencing traces to be heterozygous for different *CYP337B3* alleles. Overall, the various *CYP337B3* alleles appeared to show distinct patterns for each region, suggesting different alleles are present for pyrethroid resistance around the world.

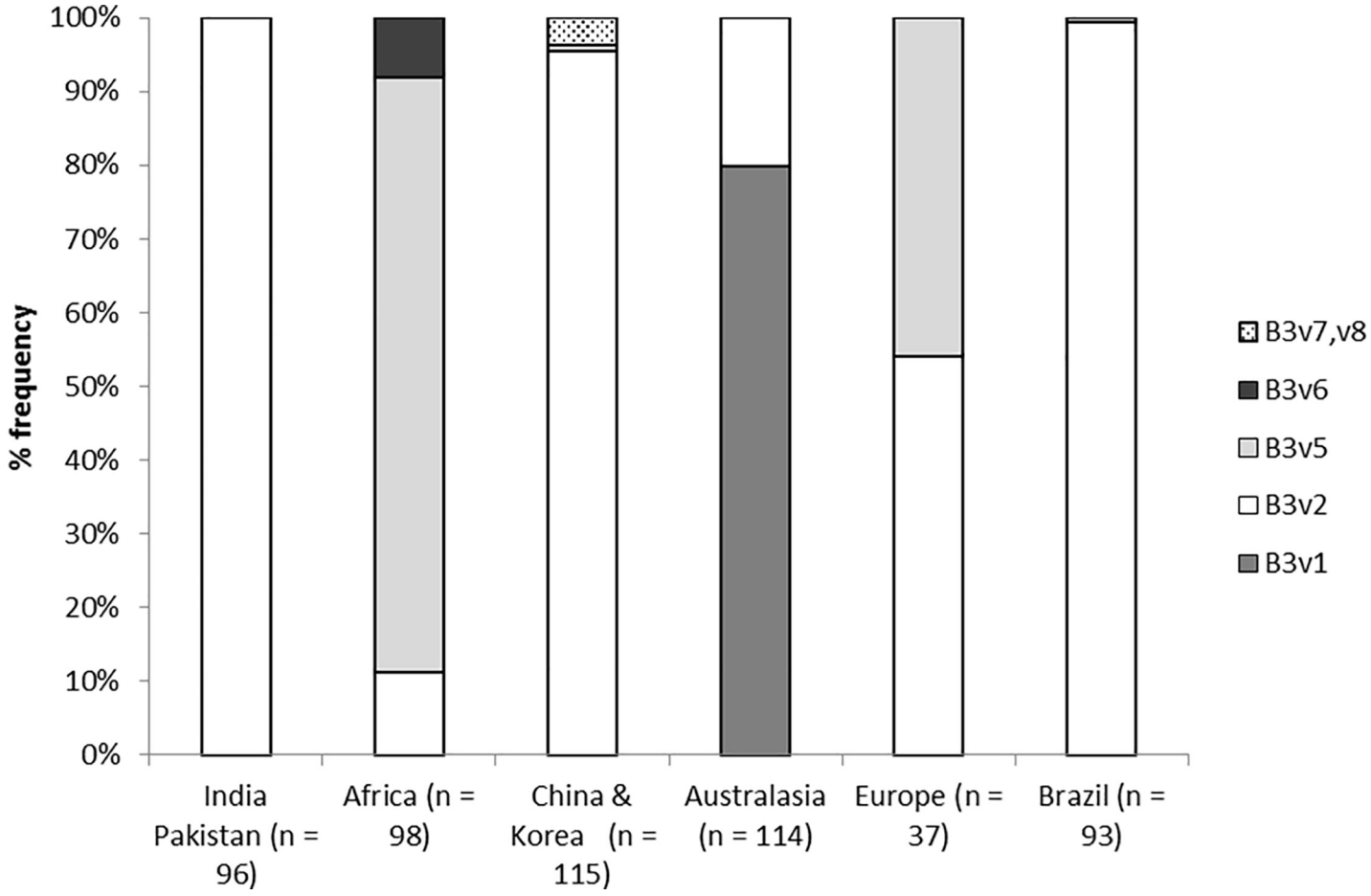

Fig 3. Frequencies of the different alleles of *CYP337B3* (B3vX) in different geographic regions. Africa = Burkina Faso, Cameroon, Chad, Ghana, Madagascar, Senegal, Uganda; Australasia = Australia and New Zealand; Europe = Greece, France and Spain. n = the number of alleles sequenced, homozygotes and heterozygote alleles summed.

https://doi.org/10.1371/journal.pone.0197760.g003

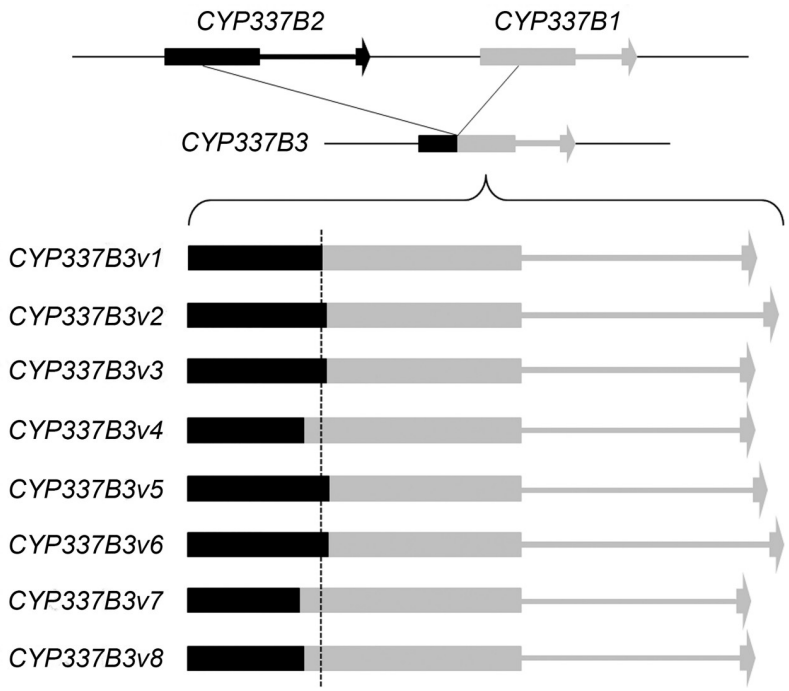

Fig 4. A schematic of the different crossing-over points and intron sizes observed in the chimeric *CYP337B3* gene.

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Sequencing of the entire coding sequences of all variants revealed a number of non-synonymous SNPs resulting in different predicted proteins when compared to *CYP337B1v1*: 3 in *v2* [21], 3 in *v5*, 8 in *v6*, 9 in *v7*, and 11 in *v8*. All variants of the chimeric gene appear to show a similar pattern with slightly different cross over points between *CYP337B1* and *CYP337B2* during the formation of *CYP337B3* and different intron sequences and sizes that derived from *CYP337B1* determined by sequencing genomic DNA (Fig 4; S6 Fig; S3 Table). This suggests that multiple origins of the same functional phenotype from different genetic backgrounds define the development of this chimeric gene.

All of the 93 *H. armigera* individuals collected in Brazil were positive for *CYP337B3*, and all except one were homozygous. The majority of *CYP337B3* alleles detected in Brazilian *H. armigera* were identical to *CYP337B3v2*, which is predominantly found in Asia. However, the single heterozygous individual was carrying an African allele (*CYP337B3v5*). This suggests that Asia may be the most likely source for the Brazilian incursion of *H. armigera*, however, other source populations are also possible, as is the occurrence of multiple incursions. As well as examining *H. armigera*, we also examined *H. zea* from Brazil (n = 59) and we were able to amplify *CYP337B3* from one individual from Goias while from the others (n = 23) only *CYP337B1* provided consistent results. DNA from the suspect individual (S1 Table) was re-extracted twice in a different laboratory, using new reagents (extraction kit, primers, PCR reagents) and PCR was performed for the *CYP337B3* gene, intron and the mitochondrial marker COI. Extraction blanks were performed and were negative for both the COI sequence and the *CYP337B3*. Sequencing of both fragments from the three different extractions confirmed the presence of the *CYP337B3v2* allele (as found in the Brazilian *H. armigera*) in an individual with a mitochondrial sequence from *H. zea*.

Bioassays for pyrethroid resistance on *H. armigera* from cotton growing areas in eastern Australia using the discriminating dose clearly showed a higher frequency of homozygotes for

CYP337B3 in survivors (100% *CYP337B3* homozygous; n = 26) than in dead or dying insects (9 *CYP337B3* homozygotes, 2 heterozygotes and 1 homozygous *CYP337B1*/*B2* individual; n = 12), although a subset of the dead or dying caterpillars was actually positive for *CYP337B3*. In addition to bioassayed individuals, samples were collected from crops in Kununurra, Ord River Irrigation Scheme, Western Australia seven days after a field application of fenvalerate on cotton. Survivors were found to possess *CYP337B3*, with a 100% homozygous frequency (n = 7). Insects collected from the same region on unsprayed crops (cotton, chickpea and chia) showed a lower frequency of *CYP337B3* (2 homozygote *CYP337B3*, 1 heterozygote and 3 homozygote *CYP337B1*/*B2* individuals; n = 6). Although these very small samples from field populations are insufficient for claims of statistical significance, they are consistent with the significant genotypic differences previously found in the Toowoomba laboratory population derived from Australia [18].

Using individuals from Australia that were homozygous for the *CYP337B3v1* allele, high throughput sequencing data was aligned to the BAC clone *33J17* which was the original source of the *CYP337B3v1* identification and originates from the Australian Toowoomba strain [18]. Of the 100,377 genotypes called across *33J17*, 32,381 remained after "LowQual" SNPs were removed. Both π and Tajima's D were variable, however values were decisively differentiated where *CYP337B3* lies; nucleotide diversity reaches its lowest point at the *CYP337B3* locus (0.00057) relative to the BAC-wide mean at 0.0179 (Fig 5; S4 Table). This is the expectation following a "hard" selective sweep, whereby adaptive alleles appear as a single haplotype before increasing in frequency [30]. Tajima's D is also lowest across the region where *CYP337B3* is located (-2.18) and points towards the process of purifying selection. It is worth noting that the BAC-wide mean value for Tajima's D is -0.43; such a low negative value may signify that other evolutionary processes have also acted upon this region [42]. While this variability is likely a result of small sample sizes and relatively small sliding window values, the small size (~ 5 kbp) of the region around *CYP337B3v1* may signify ongoing high recombination rates and gene flow [43, 44].

To establish the potential mechanisms that underlie the creation of the chimeric gene and account for its global distribution, we sequenced the intron of *CYP337B3*. This region should contain more polymorphisms and allow a better diagnosis of the frequency of the different genotypes. As for the coding region data (above), we found a pattern of eight alleles in the intron data. Introns sequenced from *B3v3*, *v4* and *v8* are very similar and differ only by two SNPS whereas introns from *B3v1*, *v2*, *v5*, *v6* and *v7* show more significant differences in both length and sequence (Fig 4). Individuals that were homozygous for the *CYP337B3* gene were predominantly homozygous at the allele level, but as in the coding region, heterozygotes were again observed in the sequence traces of a small number of samples from Africa, Australia and Europe. Cloning and sequencing of the heterozygous PCR products showed that, in Australia, *v1/v2* (Australia/Asia) heterozygotes were present, while in Africa, there were heterozygotes of *v2/v5* (Asia/Africa) and *v5/v6* (both African). European heterozygotes possessed *v2/v5* (Asia/Africa).

Phylogenetic relationships were inferred for the intronic region of a subset of representative *CYP337B1* and *CYP337B3* sequences. If the chimeric *CYP337B3* were due to a single cross-over event followed by subsequent divergence of allelic variations, then the phylogenetic estimates should show a monophyletic clade with the *CYP337B3* clade nested within the *CYP337B1 clade*. Our tests with BEAST indicated that this was not the case; specifically Bayes Factor (BF) analysis found overwhelming support for a paraphyletic origin of *CYP337B3* (BF score for paraphyly *vs*. monophyly = 494.412, with BF $>$ 10 indicating very strong evidence against the null model of monophyly). This does not rule out the possibility of some subsequent recombination between derived *CYP337B3* alleles and either parent gene.

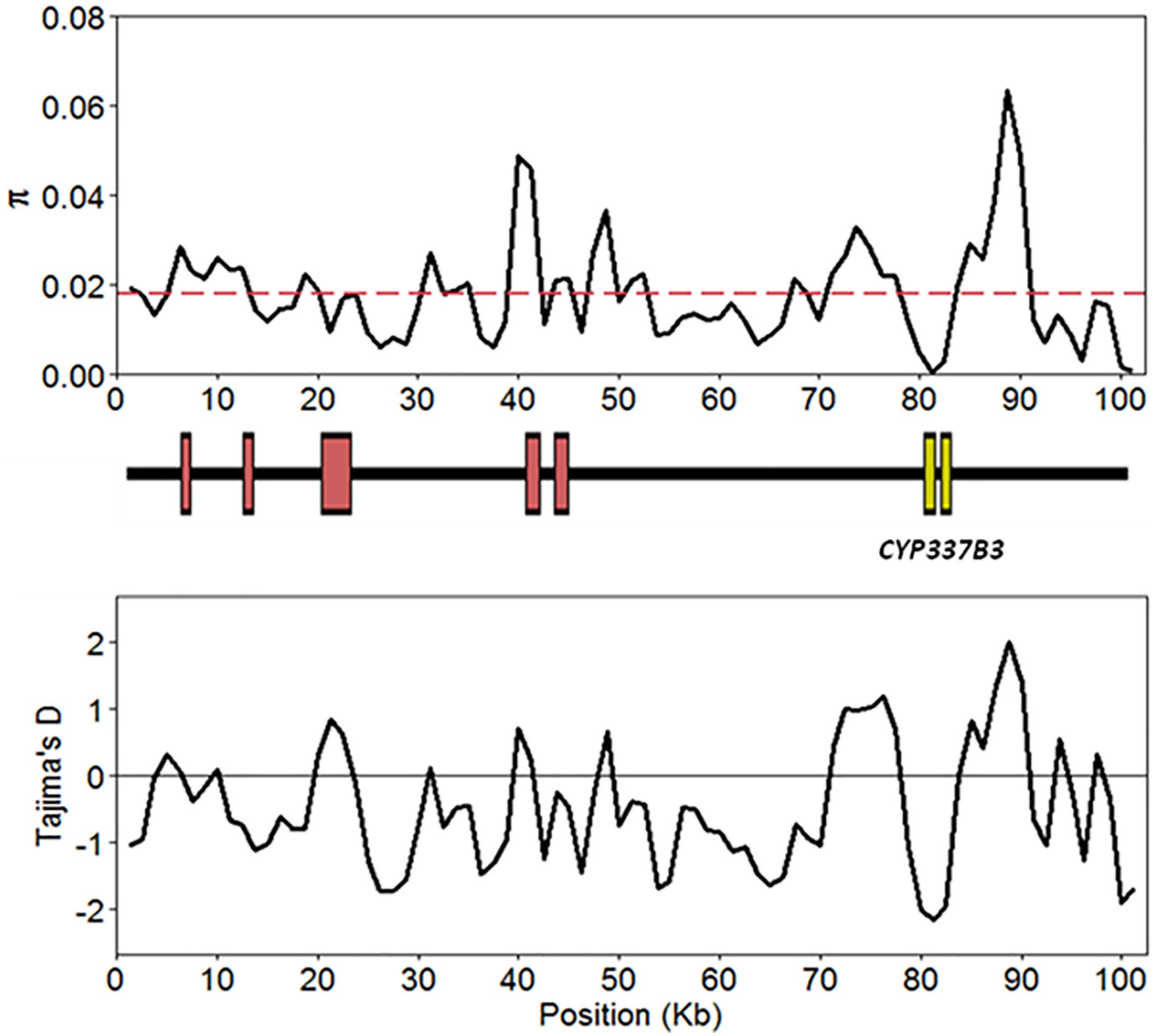

Fig 5. Selective sweep among Australian *H. armigera* homozygous for *CYP337B3v1*. (a) Nucleotide diversity (π) and (b) Tajima's D calculated across sliding windows of the 33J17 BAC (JQ995292.1), with the red dashed line showing the average value of π (0.0179). Locations of gene bodies are indicated between the plots, with those in red identified as potential reverse transcriptases and those in yellow as exons of *CYP337B3v1*.

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Fig 6 presents the results of the maximum likelihood phylogenetic analysis, which also suggests multiple origins of the various *CYP337B3* alleles. Although bootstrap support values for some nodes of the tree are quite low, several well-supported relationships are present, and these are consistent with multiple geographic origins for the *CYP337B3* alleles. For example, *CYP337B3v1* (found only in Australia) is most closely related at the sequence level to a *CYP337B1v1* allele from Australia. Furthermore there is a cluster of closely related but rare *CYP337B3* alleles found in China (*CYP337B3v3*, *v4*, and *v7*) which could indicate local origins for those alleles. Thus, while some *CYP337B3* alleles may be recently derived from local

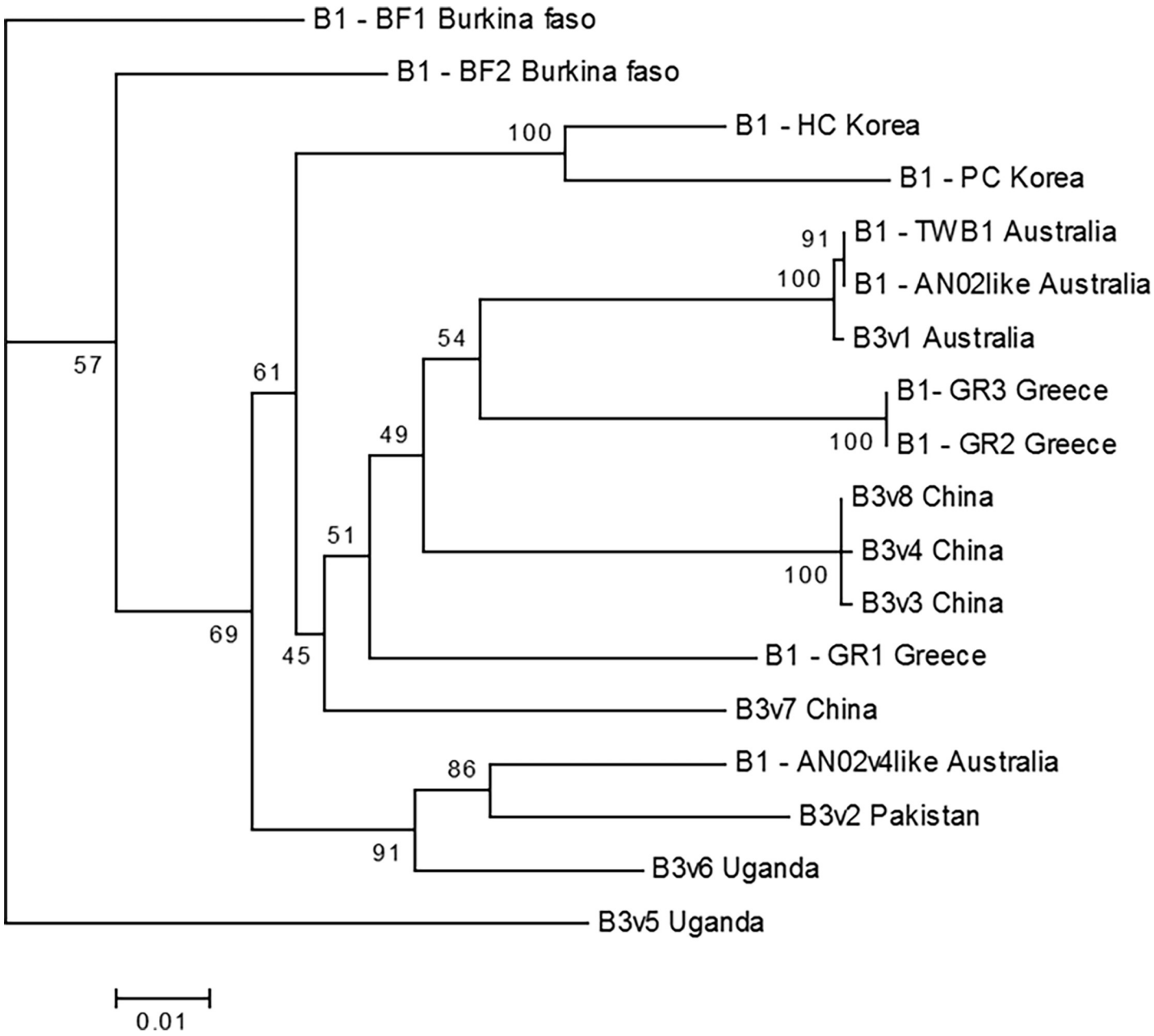

Fig 6. Maximum likelihood phylogeny of CYP337B1 and CYP337B3 alleles. The maximum likelihood tree was generated in IQ-TREE with the HKY+G4 model of sequence evolution, using representative *CYP337B1* and *CYP337B3* intron sequences. Bootstrap values from 10,000 replicates are given on tree nodes. The eight *CYP337B3* alleles are indicated with labels (v1:v8), different CYP337B1 alleles are indicated by B1- followed by an allele ID and the country of origin. Sequences and related NCBI identifiers can be found in S3 Table and S1 File.

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CYP337B1 alleles, the overall signal indicates that multiple unequal crossing-over events are responsible for the geographic distribution of chimeric *CYP337B3* while gene flow as well as some intergenic recombination may also have played a role producing the current geographic distribution patterns of *CYP337B3* alleles.

We performed an additional calculation to determine the molecular rate that would be required to produce the measured *CYP337B3* allelic diversity if it had all arisen in the last ~50

years. Even using the rapidly evolving mitochondrial divergence rate of 1.5–2.5% divergence per million years as a baseline [45], we found that a 1000-fold increase in this rate would be required to place all *CYP337B3* divergence events inside a 50-year time window. This rate is so high, it suggests that rather than recently arising through *de novo* mutation in response to pyrethroid applications and then spreading around the world, cytochrome P450-mediated pyrethroid resistance is most likely the result of selection acting over the last 50 years on existing genetic variation present in the populations at low frequency.

Discussion

Helicoverpa armigera is one of the world's most destructive pests and has developed resistance to numerous pesticides over the years [27]. Globally, pyrethroids are one of the most commonly used insecticides and resistance occurred rapidly at a global scale after the first use of pyrethroid insecticides in the late 1970s [27]. A number of mechanisms have been proposed for this resistance, including target site insensitivity [27, 46, 47], and metabolism by carboxylesterases [48, 49] and P450s [50–56]. Recent work suggests that a chimeric gene, *CYP337B3*, may play an important role in cytochrome P450-mediated pyrethroid resistance and we set out to examine the frequency of this gene worldwide [18, 21].

We detected the chimeric *CYP337B3* gene in every population examined and found a total of eight allelic variants of *CYP337B3* globally. Of the eight global alleles identified, one was found only in Australia (*CYP337B3v1*), one was dominant across Asia (India, Pakistan, China, Korea), and Brazil (*CYP337B3v2*), four were rare and uniquely found in China (*CYP337B3v3*, *v4*, *v7 and v8*) and two were predominantly African (*CYP337B3v5*, *v6*). European samples showed a mixture of alleles (*CYP337B3v2*, *v5*). *CYP337B3v2* was the most widespread allele, identified on every continent examined but was present at the highest frequencies in Asia and Brazil.

One issue in using *CYP337B3* as a population marker is that diversity in *CYP337B3* is not new diversity, but rather a re-shuffling of diversity already present in the parental *CYP337B1* and *CYP337B2* genes. However, the presence of multiple alleles at the *CYP337B3* locus suggests that this chimeric gene has formed independently several times over the evolutionary history of the locus. In the overall patterns, we found that three alleles, *v1*, *v2* and *v5*, together account for ~98% of all detected *CYP337B3* alleles. The geographic distribution of these common alleles may suggest older recombination events in Australia, Asia, and Africa producing the common alleles and potentially more recent additional events in Asia and Africa producing the rare alleles. The alternative explanation that a single event has occurred, and then been distributed globally by migration and subjected to subsequent local recombination, is much less likely as it is not supported by the phylogeny or the molecular rate analysis. Indeed, our phylogenetic analysis found strong support for a paraphyletic origin of *CYP337B3* rather than the alternative of a single unequal cross-over event followed by subsequent allelic divergence. In addition, our calculations suggest that a 1000-fold increase in a commonly accepted baseline divergence rate (1.5–2.5% divergence per million years) would be required to place all *CYP337B3* divergence events inside a 50-year time window [45]. Taken together, the allele frequencies and phylogenetic analysis suggest that, rather than recently arising through *de novo* mutations in response to pyrethroid application, cytochrome P450-mediated pyrethroid resistance is most likely the result of recent selection acting on existing genetic variation maintained in the population at low frequency. Our selective sweep analysis supports this scenario, with patterns of estimated π and Tajima's D across the *33J17* BAC indicating that the *CYP337B3* locus has undergone recent selection, at least in Australia and with regard to

CYP337B3v1. Recent work confirms that this can also be shown for the other alleles [26]. Further work will reveal how the diversity in this one region compares to the rest of the genome.

There are a number of examples in the literature where the same insecticide resistance mechanisms have developed independently in different locations. In particular pyrethroid resistance (perhaps because of the short period between the introduction and the development of resistance) associated with identical point mutations in the voltage-gated sodium channel has developed in different haplotypes with different geographical origins in mosquitoes and houseflies [57, 58]. In the sheep blowfly, the same mutations in an esterase gene conferring organophosphate resistance were identified in very different haplotypes [59].

Although further work incorporating whole genome scale markers will be necessary to determine the origin of *H. armigera* in the New World and global patterns of movement in this species [26, 28], we gain insight into some of these processes here. The vast majority of the Brazilian insects examined possessed the allele *CYP337B3v2* found predominantly in Asia. However, we also detected the *CYP337B3v2* allele in other regions of the world, and so it is possible that the Brazilian populations of *H. armigera* could have come from localities where the *CYP337B3v2* allele also exists. For example, the closest native population of *H. armigera* to the New World is in West Africa and it is possible that the New World *H. armigera* are the result of a similar incursion from West Africa. However, only Asia has close to the same proportion of *CYP337B3v2* alleles. A recent survey [8] showed that the pattern of mitochondrial markers in *H. armigera* populations collected from southern Brazil, Argentina, Uruguay and Paraguay is very different from *H. armigera* populations from central and northern regions of Brazil, suggesting multiple incursions of this global pest into the New World [60]. It would be interesting to examine the *CYP337B3* locus in these individuals to see if they share the same pattern as found in Brazil.

Recently, evidence for other pyrethroid detoxicative mechanisms in addition to *CYP337B3* has been found in China [22], and our own data showed some mortality in *CYP337B3*- homozygous individuals. This might suggest that possession of the *CYP337B3* is not enough for resistance alone and other mechanisms related to expression or other genes may exist. However, as the resistance based on *CYP337B3* is metabolic, it is not unusual that a high dose of insecticide will kill even homozygous individuals, for example if the enzyme is not abundant enough to decrease the amount of insecticide at its target site beneath the toxic threshold. Furthermore, we clearly see evidence of conservation of the *CYP337B3* alleles at this locus across the globe and we would not expect the observed level of conservation in the absence of selection, particularly in the non-coding regions. Indeed, when we examined individuals from Australia we found evidence of a selective sweep around the gene, indicating maintenance of the allele at this locus. Throughout its native range, phenotypic pyrethroid resistance is very common in *H. armigera* and it is of course possible that other or additive resistance mechanisms are at work in populations around the world.

The arrival of *H. armigera* in the New World represents a significant expansion of its geographc range. It remains to be seen how large the problem *H. armigera* will become but, clearly, arriving with a resistance gene already in place would provide *H. armigera* a selective advantage over local susceptible populations of pests such as *H. zea* and *Chloridea* (*Heliothis*) *virescens*. One further complication is that *H. zea* has been shown to interbreed with *H. armigera* in the laboratory to produce viable offspring [61–63] and there is evidence of hybridisation in Brazil using genome wide markers [28]. In the current study, we applied the same PCR reaction conditions to individuals identified as *H. zea* from Brazil and we able to amplify *CYP337B3v2* from one individual. Clearly this one result is not enough to suggest that hybridisation is widespread between the two species but the detection of a presumably highly selectable gene indicates that this is something that should be considered a risk. While it is unclear

what risk hybrids may pose, it is certain that an exchange of resistance and host association genes could lead to significant problems in control. The fact that the presence of the chimeric resistance gene *CYP337B3* can be ascertained by a simple screening PCR enables the rapid determination of susceptible *vs*. pyrethroid-resistant populations, which could lead to improved pest management strategies and the detection of hybrid individuals in the field.

This work highlights a case of resistance alleles in *H*. *armigera* evolving on a local scale in a global context and provides an example of a species invading new continents, already able to overcome some of the control measures deployed for other species. We also show that resistance genes can arise locally and then mix on a global scale as a result of either natural movement or human-mediated transport. The implications for *H*. *armigera* and potentially *H*. *zea* as species are unclear but what is not in doubt is that the pesticide resistance selection pressure on *H*. *armigera*, in terms of numbers exposed, has dramatically increased with this incursion. This is true not just for conventional pesticide resistance but also for transgenic insecticidal traits. Transgenic cotton and especially corn and soybean which express Bt toxins are grown over a far larger area in the New World and the management of Bt and conventional pesticide resistance should be a priority.

Ultimately, we grow the same crops around the world, control pests using the same chemistries, and transport commodities through global trade routes, so it is not a surprise that we are developing global, well-adapted pests. Our paper not only sets the scene for further work in this area but also highlights the value of considering biosecurity threats in terms of both the actual pest as well as its resistance profile (both phenotype and genotype).

Supporting information

S1 Fig. The frequency of *CYP337B3* on the Asian Sub-continent by region. Northern, Central and Southern represent general geographic regions of India.
(TIF)

S2 Fig. The frequency of *CYP337B3* in selected African countries. Homozygous and heterozygous frequencies are shown.
(TIF)

S3 Fig. The frequency of *CYP337B3* in China and Korea. *Larvae from lab colonies derived from each field collection (20–50 individuals) and maintained at a population size of 300–500 adults per generation without exposure to any insecticide for 3–5 generations.
(TIF)

S4 Fig. The frequency of *CYP337B3* in Australia and New Zealand. Homozygous and heterozygous frequencies are shown.
(TIF)

S5 Fig. The frequency of *CYP337B3* in Europe. Homozygous and heterozygous frequencies are shown.
(TIF)

S6 Fig. Alignment of the crossover points for the various *CYP337B3* alleles. *CYP337B1* is in red, *CYP337B2* is green and the boxed sequence represents crossover point for each *CYP337B3* allele.
(TIF)

S1 Table. Collection sites of *H. armigera* and *H. zea* from 18 different countries.
(DOCX)

S2 Table. PCR primers used in this work.
(DOCX)

S3 Table. Sequence identifiers, gene names, origin and NCBI numbers used for the maximum likelihood estimate of phylogenetic relationships.
(DOCX)

S4 Table. Sequencing and alignment statistics for the 12 individuals used to demonstrate the selective sweep around *CYP337B3v1*.
(DOCX)

S1 File. Intron sequence data used for the maximum likelihood estimate of phylogenetic relationships.
(DOCX)

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Pyrethroid and carbamate resistance in Australian *Helicoverpa armigera* (Lepidoptera: Noctuidae) from 2008 to 2015: what has changed since the introduction of Bt cotton?

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Abstract

Pyrethroid and carbamate resistance was evaluated in *Helicoverpa armigera* from 2008 to 2015. Insects were collected as eggs primarily from cultivated hosts in the major cropping areas of New South Wales and Queensland, Australia. Larvae reared from eggs were tested for resistance to fenvalerate, bifenthrin or methomyl in the F_0 generation using a topical application of a discriminating dose of insecticide. In 2008–2009, resistance to fenvalerate was 71% and no resistance to bifenthrin was recorded. In the following two seasons, resistance to pyrethroids was relatively stable with fenvalerate resistance ranging from 63% to 67% and bifenthrin resistance ranging from 5.6% and 6.4% in 2009–2010 and 2010–2011, respectively. However, in 2011–2012, pyrethroid resistance had increased to 91% and 36% for fenvalerate and bifenthrin, respectively. Resistance remained above 90% for fenvalerate and above 35% for bifenthrin in the following three seasons from 2012 to 2015. In 2008–2009, methomyl resistance was 33% and declined to 22% and 15% in 2009–2010 and 2010–2011, respectively. Methomyl resistance remained at moderate levels from 2011–12 to 2014–15, ranging from 21% to 40%. Factors that influenced selection pressure of pyrethroid and carbamate insecticides and impacted resistance frequency in *H. armigera* may have been associated with changes in the composition of the cropping landscape. The rapid expansion of the pulse industry and the commensurate increased use of insecticide may have played a role in reselection of high-level pyrethroid resistance, and highlights the need for an urgent and strategic response to insecticide resistance management in the Australian grains industry.

Keywords: cotton bollworm, transgenic crops, insecticide resistance monitoring, resistance management

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Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner) is one of the most serious global insect pest species of agriculture having by far the most reported cases of insecticide resistance worldwide, including field-evolved resistance to pyrethroids, carbamates, cyclodienes, and organophosphates (McCaffery, 1998), spinosad (Gunning, 2002), indoxacarb (Bird, 2016) and toxins derived from *Bacillus thuringiensis* (Mahon *et al.*, 2007). This capacity to develop resistance is associated with its highly polyphagous nature, wide geographical distribution (Zalucki *et al.*, 1986; Fitt, 1989) and an ability to migrate long distances (Feng *et al.*, 2005). Historically, *H. armigera* had a range of distribution throughout the Old World, with more recent work confirming a major incursion into South America (Czepak *et al.*, 2013; Mastrangelo *et al.*, 2014; Murúa *et al.*, 2014).

In Australia, there is considerable experience in managing *H. armigera* in agricultural systems. Following the widespread

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failure of insecticides to control field populations of *H. armigera* in the 1980s (Gunning *et al.*, 1984), a windows-based Insecticide Resistance Management Strategy (IRMS) was designed and implemented to manage resistance by restricting the use mode-of-action group by rotation, and the number of applications (Forrester *et al.*, 1993). In addition, a monitoring programme was established to detect changes in insecticide resistance frequencies based on a discriminating dose technique highly effective for detecting incipient resistance. This programme was important for assessing the effectiveness of IRMS and also provided a basis for the timely implementation of pre-emptive responses to control resistant populations (Forrester & Bird, 1996; Rossiter *et al.*, 2008). Although the IRMS did not overcome pyrethroid resistance in Australian *H. armigera*, it proved to be an effective tactic for delaying resistance and extending the useful life of these insecticides prior to the commercialization of genetically modified cotton producing the δ-endotoxin genes of *B. thuringiensis* subsp. *kurstaki* (Bt). The phased introduction of commercial scale Bt cotton to control *H. armigera* and the native *Helicoverpa punctigera* (Wallengren) began with the single gene variety, Ingard® (producing Cry1Ac toxin) in 1996, followed by two gene cotton, Bollgard II® (producing Cry1Ac and Cry2Ab) in 2003.

Concurrent with the introduction of Bt cotton was a reduction in the number of insecticide sprays for *Helicoverpa* spp. on a per hectare basis by 59% in the first 8 years of deployment when the cotton industry was reliant upon the single gene variety Ingard® (Fitt, 2008). During this time, Ingard® cotton was restricted to 30% of the total area of the cotton crop in Australia as a precautionary measure to reduce the risk of resistance to the Cry1Ac toxin. However, strong reliance on insecticides continued on the remaining 70% of non-Bt cotton, particularly during the latter part of the growing season due to the gradual decline of expression of Cry1Ac in Ingard® cotton, resulting in ongoing selection for resistance to insecticides in *H. armigera*. Following the introduction of Bollgard II®, which provided season-long protection of the crop from *Helicoverpa* spp., there was a further reduction (up to 90%) in the total number of spray applications required (Fitt, 2008). This resulted in tangible benefits to the environment (Knox *et al.*, 2006), provided a platform for integrated pest management (IPM) programmes and reduced selection pressure for conventional insecticide resistance in *H. armigera* (Wilson *et al.*, 2013).

Prior to the introduction of Bt cotton, heavy reliance on broad-spectrum insecticides selected for high resistance to pyrethroids (Forrester & Bird, 1996) and moderate resistance to carbamates (Gunning *et al.*, 1992) in *H. armigera*. Preferential use of relatively new classes of insecticides such as spinosad, emamectin benzoate and indoxacarb led to incipient resistance to all three insecticides by 2002. However, following the reduction in insecticide sprays targeting *Helicoverpa* spp. associated with high uptake of transgenic varieties, and particularly after commercialization of Bollgard II®, resistance to all chemical classes of insecticide declined and in some cases reverted to baseline levels. For example, resistance to bifenthrin increased steadily from 30% to over 60% over six seasons from 1996 to 2002 and then declined steadily during the following five seasons to 2007 when resistance to bifenthrin could not be detected (Rossiter *et al.*, 2008).

The present study reports pyrethroid and carbamate resistance collected from a wide range of cropping hosts intensively managed for *H. armigera* in eastern Australia from 2008 to 2015, and compares annual frequencies over this 7-year period to frequencies observed prior to the introduction of Bollgard II®. Methods of resistance monitoring have remained consistent with those used since the monitoring programme was first implemented, therefore ensuring continuity and compatibility of monitoring outcomes from this and previous studies. The work was also interested in exploring factors that may have influenced selection pressure imposed by pyrethroids and carbamates and associated changes in resistance frequency to these insecticides, particularly changes in the proportion of alternative host crops managed for *H. armigera* as a possible explanation for increased insecticide use.

Materials and methods

General rearing methods

Methods used to rear *H. armigera* were similar to those described by Teakle & Jensen (1985), except that formalin was omitted, and soybean flour was baked at 200°C for 10 min to remove enzyme inhibitors which can interfere with bioassays. Neonates were individually transferred to 45-well plastic trays (Tacca Plastics, Sydney, Australia) containing approximately 1.5 ml of diet and heat-sealed with perforated lids (Oliver Products, Grand Rapids, MI, USA). When larvae reached the late fourth or early fifth instar, they were transferred to fresh plastic trays containing diet. Larvae were allowed to pupate in these trays and when hardened they were removed, washed in 1% bleach solution and transferred to 5 litres round, plastic containers (21 cm diameter). A 14 cm diameter hole was cut into the lid of the container and used to secure a cloth liner which acted as an oviposition substrate for the emerging moths. Moths were provided with approximately 30 ml of 4% honey/sugar solution held in 50 ml plastic containers with a hole in the lid to accommodate a cotton wick. Eggs were collected daily by replacing the cloth liners which were washed in a 1% bleach solution and collected onto a Whatman No. 54 filter paper by vacuum filtration. Filter papers were allowed to air dry and were then placed in sealed plastic bags until neonates hatched.

In the larval stage, insect strains were maintained in a laboratory environment of 25 ± 2°C with 14:10 (L: D) hour photoperiod and ambient RH. Adults were maintained in a separate facility under the same conditions of light and temperature and RH.

Laboratory reference strains

Discriminating doses of pyrethroids and carbamates used in this study were based on data accumulated during the 1980s and 1990s (Gunning *et al.*, 1987, 1992; Forrester *et al.*, 1993) which was similar to a laboratory reference strain, known as the SUS strain, established from *H. armigera* collected from sorghum at Gatton, southern Queensland in 1979. The SUS strain was replaced by the New GR strain established from a series of collections made in cotton fields in the Namoi Valley, northern New South Wales during the mid-1980s and was supplemented with field-collected insects intermittently from 1990 to 2000. The New GR stain was bioassayed at intervals throughout this study to compare the dose response with that of the original SUS strain.

Sampling locations

Six geographically distinct areas across New South Wales and Queensland were sampled between September 2008 and March 2015 (fig. 1). The majority of samples were collected in

Fig. 1. Map of eastern Australia depicting the major cropping areas, which correspond to those listed in table 2.

the Namoi and Gwydir valleys in northern New South Wales (30°19′57″S, 149°46′52″E; elevation 216 m), with smaller scale sampling conducted in the central New South Wales region of the Macquarie valley (31°42′9″S, 147°48′58″E; elevation 198 m) and in the Murrumbidgee Irrigation Area (MIA) (34°16′48″S, 146°2′44″; elevation 144 m) in the south of the state. Sampling locations close to the New South Wales and Queensland border are hereafter referred to as the Border Rivers region (28°29′12″S, 149°28′12″E; elevation 189 m). A further two regions were regularly sampled throughout the study; the Darling Downs region in southern Queensland (27°11′40″S, 151°15′57″E; elevation 341 m) and the Emerald region in central Queensland (23°31′38″S, 148°9′52″E; elevation 179 m).

Field sampling procedure

Visual searches for *Helicoverpa* spp. eggs were conducted in a range of cultivated hosts (primarily cotton, sorghum, maize and pulses) and the scrophulariaceous weed host, *Verbascum virgatum*. Collection sites had not been treated with insecticide at the time of sampling. Eggs (comprising a mixture of both *H. armigera* and *H. punctigera*, in unknown proportions) were collected at random across a wide geographical range in each sampling region and from a range of *H. armigera* host crops. The objective of each field collecting trip was to source between 100 and 200 eggs from any one individual farm location.

Field-collected eggs were removed from plant material using a fine hair paintbrush, transferred, one egg per well, to 45-well plastic trays and sealed, as above. Eggs were checked for the presence of parasites. The egg parasite, *Trichogramma* spp., occasionally caused high levels of egg mortality in samples. The solitary egg-larval braconid wasp parasite, *Chelonus* spp. was also occasionally present in samples but easily identified by characteristic developmental arrest in *H. armigera* larvae and the precocious onset of metamorphosis in parasitized larvae which were subsequently removed from the test cohort. Hatched larvae were identified to species at the second or third instar as either *H. armigera* or *H. punctigera*. The *H. armigera* larvae were then reared, as described above, to the appropriate size for resistance testing.

Insecticides

Insecticide solutions used in all bioassays were prepared from technical material dissolved in analytical grade acetone. Fenvalerate (95.3%) was provided by Sumitomo Chemical (Sydney, Australia); bifenthrin (93.3%) was provided by FMC (Brisbane, Australia); methomyl (98%) was provided by Bayer CropScience (Melbourne, Australia). Discriminating dose bioassays were performed using concentrations previously determined for fenvalerate as 0.125 µg/larva (Gunning *et al*., 1984), bifenthrin as 0.1 µg/larva (Forrester *et al*., 1993) and methomyl as 1 µg/larva (Gunning *et al*., 1992). The insecticide solutions used in control bioassays of the susceptible strain were prepared as twofold serial dilutions corresponding to six or seven insecticide concentrations which were expected to induce 1–99% mortality in 30–40 mg *H. armigera* larvae.

The insecticidal classes available for management of *H. armigera* throughout the duration of this study were widely registered for use across all crop types sampled, with the exception of pigeon pea when used a structured refuge for transgenic cotton. Registered insecticidal classes included pyrethroids, carbamates, organophosphates, spinosad and indoxacarb. During the span of this study, there were no additional registrations of pyrethroid or carbamate insecticidal classes. The diamide class of insecticides was introduced in Australia 2008 with chlorantraniliprole initially registered in cotton and then extended to pulses in mid-2014. A second diamide insecticide cyantraniliprole was also first registered in cotton in late-2013.

Bioassays

Larvae collected as eggs from the field were reared to the third or fourth instar. Larvae within a weight range of 30–40 mg were transferred to fresh diet and allocated randomly to insecticide treatment groups. Each larva was treated by topical administration of 1 µl of acetone/insecticide solution applied to the dorsal thorax using a 50 µl micro-syringe in a repeating dispenser (Hamilton Company, Reno, NV, USA). Trays containing tested larvae were covered with heat-sealed perforated lids.

Control bioassays were performed in triplicate with individual treatments (insecticide concentrations) in replicates consisting of a minimum of 20–30 individuals; acetone alone was used as the control. Control bioassays were performed on three non-synchronous cohorts of New GR and the results

were pooled in the final analysis because there were no significant differences between cohorts for any of the insecticides tested. The LD_{50} generated from the pooled result was used as the estimate of baseline susceptibility for the New GR strain. Pyrethroid and methomyl bioassays were maintained for 3 and 4 days, respectively, under the same conditions described above for larval rearing and assessed for mortality based on the inability to demonstrate coordinated movement when prodded with a blunt probe.

Insecticide usage data and cropping statistics

Data for the volume of pyrethroid and carbamate insecticides used by the cotton industry during the study period were provided by the association of Crop Consultants Australia Incorporated (CCA). The CCA coordinated the collection of survey data across key cotton growing regions of Australia through its annual cotton market audit survey. Data for the hectares sown to the major broad-acre crops in New South Wales and Queensland from 2001 to 2014 were obtained from the Australian Bureau of Agriculture and Resource Economics (ABARES, 2016).

Data analysis

The dose responses from bioassays of *H. armigera* to fenvalerate, bifenthrin and methomyl were corrected for control mortality using the formula of Abbott (1925). Slope, LD_{50} and $LD_{99.9}$ estimates, and associated 95% fiducial limits (FLs) were calculated by probit analysis using the POLO-PC software (LeOra Software, Berkeley, CA, USA). Toxicity ratios were calculated by dividing the LD_{50} value of the New GR strain (pooled result of three non-synchronous cohorts) by the LD_{50} value of the SUS laboratory strain. The proportions of annual larval survival at the discriminating dose for each insecticide were adjusted using a generalized linear model with year as the explanatory factor (R Development Core Team, 2012) to account for unequal sample size between regions and within years. Binomial standard errors were calculated using the formula of Forrester *et al.* (1993).

Results

Discriminating dose calibration

The comparison of dose responses of the reference strains, New GR and SUS, is shown in table 1. The median lethal concentration of fenvalerate was similar in both strains with LD_{50} values of 0.043 and 0.034 in the New GR and SUS strains, respectively, and resulted in a toxicity ratio of 1.3. There was significantly higher sensitivity to bifenthrin in the New GR strain compared with the SUS strain, which resulted in a toxicity ratio of 0.5. Tolerance to methomyl was considerably higher in the New GR strain compared with the SUS strain, with a toxicity ratio of 4.8.

Insect sampling

The regions sampled were largely mixed cropping landscapes comprising a continuum of hosts including cotton, pulses and coarse grains which were high value commodities managed intensively for *H. armigera*. The crops sampled annually in each region are shown in table 2 with data combined for all crop types, as in other similar studies (Forrester *et al.*, 1993; Mahon *et al.*, 2007) based on relative spatial homogeneity of resistance frequency due to the absence of reproductive isolation between cohorts of adults and a high level of gene flow between habitats (Gunning & Easton, 1989; Glenn *et al.*, 1994).

The range of crops sampled within regions varied between years with cropping diversity dependant largely on factors such as rainfall and commodity prices. A high level of sampling diversity was achieved in the Namoi/Gwydir region with insects routinely collected from cotton, maize, sorghum, mung beans, soya beans, pigeon pea, sunflowers and *V. virgatum*. There was moderate sampling diversity in the Macquarie Valley, Border Rivers and Darling Downs regions with regular seasonal planting of cotton, maize and pigeon pea and opportunistic plantings of sorghum and summer pulses in some years when timing of rainfall was favourable. The regions with the lowest sampling diversity were Emerald and the MIA. The total number of sites sampled in each region annually is shown in table 2. The numbers of insects sourced from sampling regions was influenced by population abundance but generally ranged between 100 and 200 eggs from each individual farm location; the minimum number sampled from any individual farm location was 20 eggs and the maximum number sampled from any individual farm location was 1482 eggs. The most intensely sampled region was the Namoi/Gwydir valley with the highest number of sites sampled in 2011–12. Extensive sampling was also conducted in the Darling Downs and Border Rivers regions in most years. However, due to low population abundance across many regions during 2013–14 testing was restricted to insects collected in New South Wales only.

Fenvalerate resistance

Annual resistance frequencies for fenvalerate at each region are shown in table 2. The average annual survival of larvae from all regions and crop types at the discriminating dose of fenvalerate are shown in fig. 2. During the seasons from 2008–09 to 2010–11, fenvalerate resistance ranged from 61% to 70% in the Namoi/Gwydir region and was similar to that recorded from populations sampled from the Border Rivers area, the only other region where fenvalerate resistance was monitored during this time period (table 2). In 2011–12, resistance increased to 90% in the Namoi/Gwydir region, which was consistent with elevated resistance in the other locations sampled in both New South Wales and southern Queensland. Average resistance to fenvalerate persisted at levels above 90% for the remainder of the study (fig. 2).

Bifenthrin resistance

While no resistance was detected to bifenthrin in 2008–09, resistance levels in 2009–10 and 2010–11 increased to 5.6% and 6.4%, respectively (fig. 2). In contrast to low-level bifenthrin resistance experienced prior to 2011, resistance to this insecticide increased markedly in 2011–12 to 39% in the Namoi/Gwydir region, 38% in the Macquarie valley and MIA, 45% in the Darling Downs and 50% in the Border River region (table 2). Elevated levels of resistance persisted in all locations during 2012–13 ranging from 30% in the Darling Downs to 48% at Emerald. Average annual bifenthrin resistance increased further in 2014–15 to 51% (fig. 2) with marked between-site sample size variability due to low insect pressure in some regions such as the Darling Downs and MIA (table 2).

Table 1. Bioassays of fenvalerate, bifenthrin and methomyl against a susceptible laboratory strain of *Heliothis armigera*, New GR compared with historical baseline data for the SUS laboratory susceptible strain of *H. armigera*.

Insecticide	Strain	LD_{50} [μg ai/larva] (95% FL)	$LD_{99.9}$ [μg ai/larva]	Slope ± se	χ^2 (df)	*P*	Toxicity ratio[1]	Reference
Fenvalerate	New GR	0.043 (0.038, 0.048)	0.2691	3.9 ± 0.35	8.64 (4)	0.071	–	–
Bifenthrin	New GR	0.012 (0.010, 0.013)	0.0561	4.5 ± 0.49	6.84 (3)	0.077	–	–
Methomyl	New GR	1.067 (0.853, 1.421)	63.925	1.7 ± 0.22	2.82 (3)	0.420	–	–
Fenvalerate	SUS	0.034 (0.028, 0.040)	–	3.0	–	–	1.3	Forrester *et al.* (1993)
Bifenthrin	SUS	0.026 (0.023, 0.030)	–	3.7	–	–	0.5	Forrester *et al.* (1993)
Methomyl	SUS	0.220 (0.170, 0.280)	–	3.1	–	–	4.8	Gunning *et al.* (1992)

[1]Toxicity ratio is LD_{50} value of New GR/LD_{50} value of SUS strain.

Table 2. Pyrethroid and carbamate resistance in *Heliothis armigera* collected from a range of hosts in six sampling areas (Namoi/Gwydir and Macquarie River valleys, and Murrumbidgee Irrigation Area (MIA) in New South Wales, Border Rivers regions of northern New South Wales and southern Queensland, Darling Downs region of southern Queensland, and the Emerald irrigation area of central Queensland). Results are expressed as the percentage of larvae (reared from field-collected eggs) surviving the discriminating dose (0.125, 0.1 and 1.0 μg/larva of fenvalerate, bifenthrin and methomyl, respectively) ± pooled binomial standard error. *n* = number of larvae tested.

Sampling area	Year	Collection data		Fenvalerate		Bifenthrin		Methomyl	
		Number of farms sampled	Crop type sampled[1]	% survival ± se	*n*	% survival ± se	*n*	% survival ± se	*n*
Namoi/Gwydir	2008/09	48	C, M, S, PP, SF	70.4 ± 2.9	240	0	423	34.0 ± 2.0	544
	2009/10	28	C, M, S, PP, SF, V	64.8 ± 4.0	145	6.3 ± 1.7	205	24.8 ± 3.8	133
	2010/11	63	C, M, S, MB, PP, SF, V	61.4 ± 3.7	176	12.0 ± 2.3	200	17.3 ± 1.7	504
	2011/12	82	C, M, S, MB, PP, SF	89.6 ± 1.1	714	39.4 ± 1.6	923	34.3 ± 1.7	792
	2012/13	49	C, M, S, MB, SB, PP, V	89.3 ± 0.8	1547	38.5 ± 1.3	1320	22.1 ± 1.2	1196
	2013/14	26	C, M, S, PP	90.8 ± 1.3	491	44.1 ± 2.1	540	27.2 ± 2.0	504
	2014/15	42	C, M, S, MB	95.4 ± 0.8	755	58.3 ± 1.8	750	51.6 ± 1.9	701
Macquarie	2011/12	12	C, M, CP	95.6 ± 2.2	90	37.8 ± 4.5	119	28.1 ± 4.0	128
	2012/13	30	C, M, S, MB, PP	91.8 ± 1.3	417	43.0 ± 2.5	291	22.5 ± 2.2	351
	2013/14	2	M, PP	84.0 ± 5.2	50	44.6 ± 6.7	56	31.5 ± 6.4	54
	2014/15	8	C, M, PP	82.4 ± 6.6	34	44.4 ± 9.7	27	39.1 ± 10.4	23
MIA	2011/12	6	C, PP	90.0 ± 6.9	20	37.9 ± 9.2	29	–	–
	2013/14	4	C, PP	77.4 ± 7.6	31	22.6 ± 7.6	31	22.6 ± 7.6	31
Border Rivers	2008/09	8	C, M, S, PP	–	–	0	20	28.9 ± 4.3	114
	2009/10	18	C, PP	–	–	12.2 ± 5.2	41	29.3 ± 4.8	92
	2010/11	29	C	66.0 ± 6.6	53	3.1 ± 1.5	129	15.2 ± 4.4	66
	2011/12	12	C, M, PP	85.7 ± 7.8	21	50.0 ± 8.2	38	46.2 ± 9.9	26
	2012/13	52	C, M, PP	92.9 ± 1.4	336	38.7 ± 2.6	354	30.5 ± 2.7	292
	2014/15	19	C, M, S, PP	89.2 ± 2.4	166	51.1 ± 3.9	176	51.4 ± 4.2	144
Darling Downs	2010/11	11	C, M	–	–	0	60	12.0 ± 6.6	25
	2011/12	13	C, M, PP	92.9 ± 4.0	42	44.9 ± 6.0	69	42.6 ± 6.4	61
	2012/13	17	C, M	95.0 ± 2.0	119	30.2 ± 4.1	129	18.0 ± 3.7	111
	2014/15	10	C, M, MB, PP	75.9 ± 8.1	29	32.0 ± 9.5	25	26.1 ± 9.4	23
Emerald	2009/10	9	C, CP	85.3 ± 6.2	34	0	29	–	–
	2011/12	7	C	–	–	–	–	24.0 ± 8.7	25
	2012/13	5	C, CP	92.6 ± 5.1	27	47.6 ± 11.2	21	–	–
	2014/15	7	C, MB	92.8 ± 3.1	69	46.4 ± 6.0	69	30.0 ± 6.0	60

[1]Initials indicate crop types sampled.
C, cotton; M, maize; S, sorghum; CP, chickpeas; MB, mung beans; SB, soya beans; PP, pigeon pea; SF, sunflowers; V, *Verbascum virgatum*.

Methomyl resistance

Resistance declined from 29% in 2008–09 to 15% in 2010–11 (fig. 2). In 2011–12, industry-wide resistance levels increased to 32%, ranging from 24% in Emerald to 46% in the Border Rivers region (table 2). Average annual resistance declined in the seasons between 2012 (21%) and 2014 (25%), increasing again in 2014–15 to 40% (fig. 2).

Pyrethroid and carbamate use in Australian cotton production 2000–2014

Annual usage of pyrethroids and carbamates over a 15-year period between 2000 and 2014 is shown in figs 3 and 4, respectively, and was summarized from a cotton market audit survey conducted annually to provide data on product usage across the Australian cotton industry. Hence spray

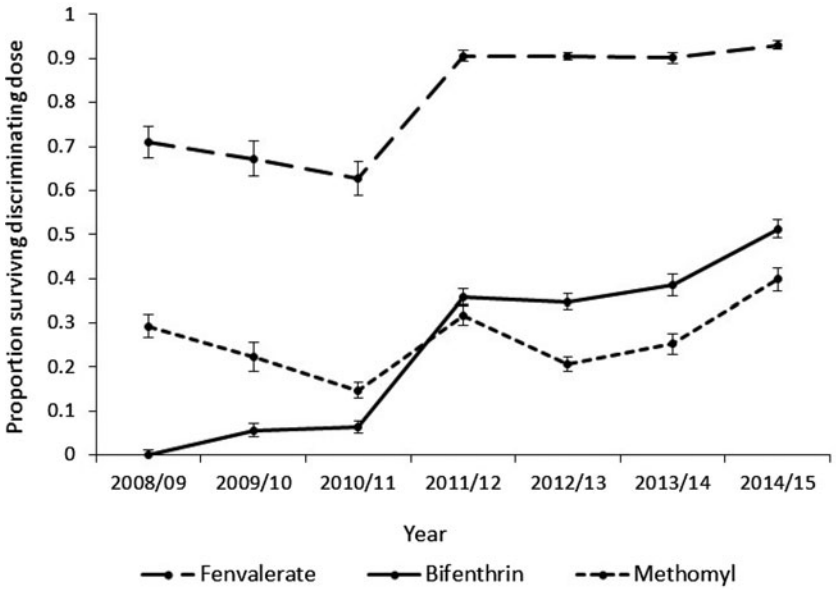

Fig. 2. Proportion of *Heliothis armigera* larvae surviving a discriminating dose of fenvalerate, bifenthrin or methomyl ± pooled binomial standard error. The data for each insecticide have been summed across sampling locations and combined for all crop types within each season from 2008 to 2015.

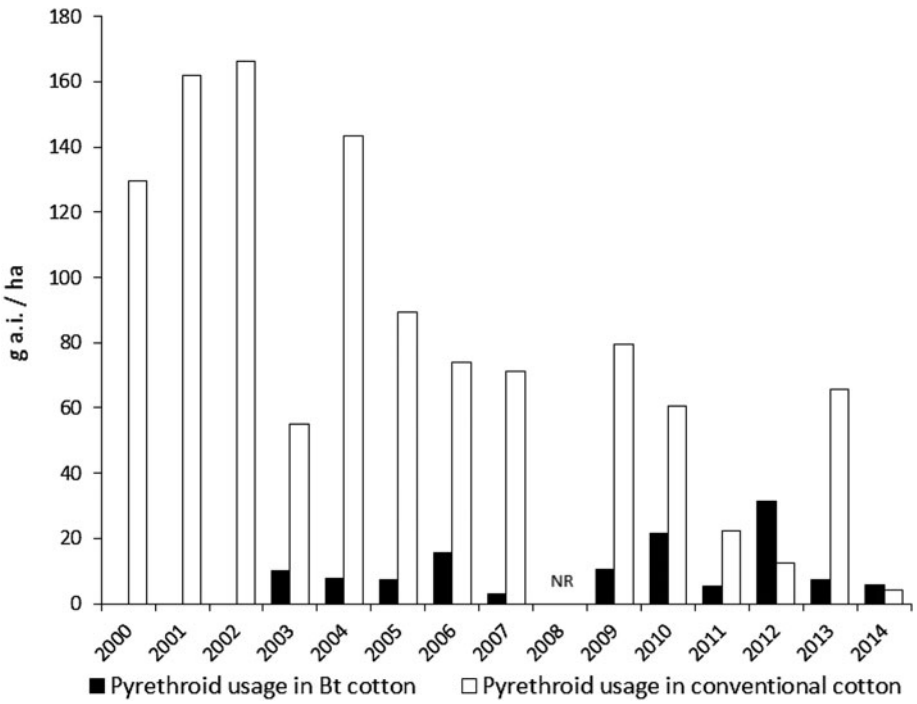

Fig. 3. Pyrethroid use on Bt (Bollgard II®) cotton and conventional cotton. NR = not recorded because cotton area was very small due to drought. Source: Cotton Research and Development Corporation (CRDC) Summary of Crop Consultants Australia (CCA) market audit, 2014.

volume data for pyrethroid and carbamate insecticides is restricted to usage in the cotton industry only as no data were available for insecticide use in the grains industry. Conventional cotton is grown by a small proportion of the industry and accounts for the majority of industry-wide pyrethroid and carbamate use. In the 3 years prior to the introduction of Bollgard II® in 2003, pyrethroid usage ranged from 129 to 166 g of active ingredient per hectare in conventional cotton

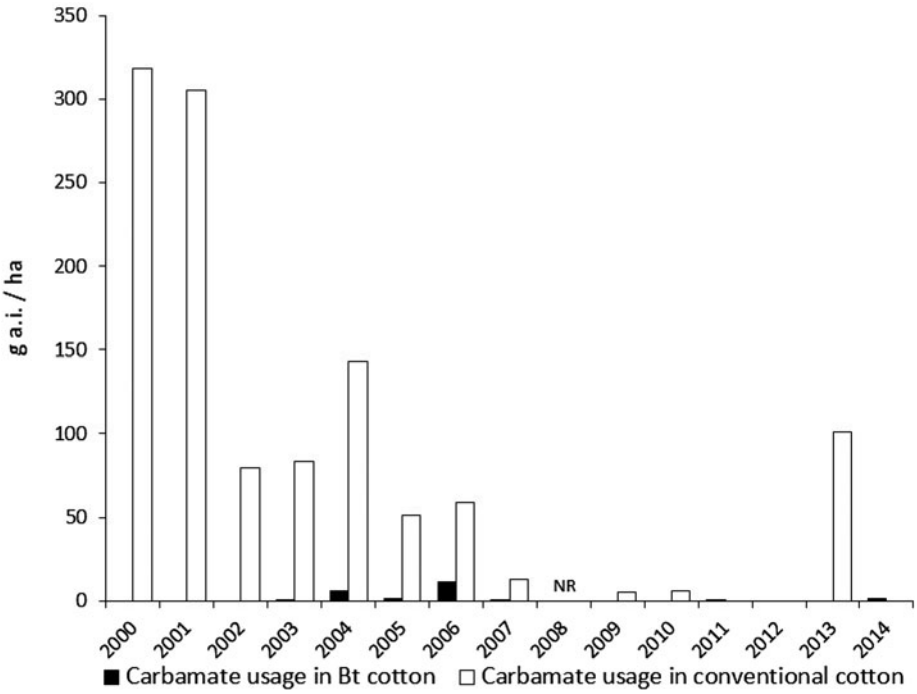

Fig. 4. Carbamate use on Bt (Bollgard II®) cotton and conventional cotton. NR = not recorded because cotton area was very small due to drought.
Source: Cotton Research and Development Corporation (CRDC) Summary of Crop Consultants Australia (CCA) market audit, 2014.

(fig. 3). Variation in annual pyrethroid use was presumably due to fluctuations in seasonal insect pressure, influenced largely by host availability. Nevertheless, there was an overall reduction in pyrethroid use in cotton of approximately 60% in the years following the commercialization of Bollgard II® (fig. 3). The decline in use of carbamate insecticides in conventional cotton was even more pronounced with an overall reduction of 80% in total amount of active carbamate applied to cotton following the introduction of Bollgard II® (fig. 4).

Cropping statistics and pyrethroid resistance in New South Wales and Queensland 2003–2015

The major broad-acre production systems managed for *H. armigera* by the use of insecticides in New South Wales and Queensland are shown in fig. 5. Cotton hectares were influenced by a period of prolonged drought and produced a corresponding trough in production from 2006 to 2010. Sorghum was the dominant summer crop prior to 2008–09, when water for irrigation was less assured for cotton growers. The total area planted to pulse crops (chickpeas, faba beans, field peas, lentils, lupins, mung beans and vetch) increased from the early 2000s and onward, peaking in 2010–11. Chickpea production accounted for 60–70% of pulse total pulse production in New South Wales and Queensland during the period of this study and is represented separately from other pulses in fig. 5.

Bifenthrin resistance in the 14 years from 2001–02 to 2014–15 is presented in fig. 5 to illustrate the long term trends in resistance spanning the period before the introduction Bollgard II® cotton, and comparing this with the results from the present study. In 2001–02, bifenthrin resistance was 60% (Gunning, 2002) and declined steadily over the following five seasons. Resistance could not be detected in the three seasons from 2006–2007 to 2008–2009 (Rossiter *et al.*, 2008) but re-emerged in the second year in the present study.

Discussion

There is little doubt that the introduction of transgenic cotton has reduced the overall need for insecticides that target *Helicoverpa* spp. The commensurate decline in insecticide resistance levels in Australian *H. armigera* were presumed to be associated with reduced selection for resistance in an industry dominated by transgenic cotton (Fitt, 2008; Wilson *et al.*, 2013); there was considerably lower survival at the discriminating dose of bifenthrin following the 2004–05 season, with resistance returning to baseline levels for two seasons from 2006 to 2008. Results from the present study indicate that, although *H. armigera* was still fully susceptible to bifenthrin in 2008–09, incipient resistance was detected in the following season at a level of 6%, increasing to 36% in 2011–12. This was accompanied by a similar increase in resistance to fenvalerate which exceeded 90% and which would have resulted in field failures of ester-bonded phenoxybenzyl alcohol pyrethroids (Forrester *et al.*, 1993). Moderate resistance to methomyl was also consistent with unreliable levels of control observed under field conditions (Gunning *et al.*, 1992).

Despite the dominance of transgenic varieties in Australian cotton production, conventional varieties are still grown by a small proportion (<10% since 2010) of the industry in regions with a preference for non-transgenic varieties (Constable *et al.*, 2011). However, the marked increase in bifenthrin and fenvalerate resistance occurred despite relatively low levels of

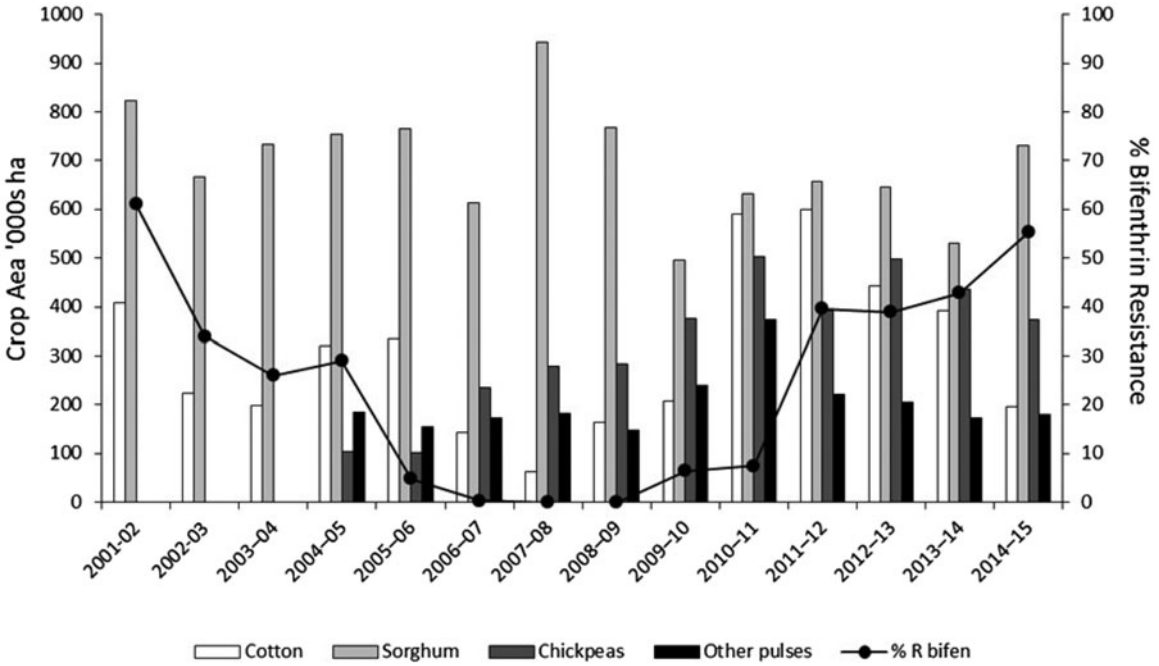

Fig. 5. Hectares of pulses, cotton and sorghum grown annually (2003–2015) in New South Wales and Queensland compared with annual bifenthrin resistance levels (resistance data have been summed across sampling locations and combined for all crop types). Sources: Commodities data from Australian Bureau of Agricultural and Resource Economics (ABARES, 2016); Bifenthrin resistance frequency data 2001–2002 to 2007–2008, from Rossiter *et al.* (2008).

pyrethroid use to target *Helicoverpa* spp. in cotton and was unlikely to have provided sufficient selection pressure to account for the magnitude of increase in resistance frequency. Likewise, but to lesser extent, resistance to methomyl, although variable over time, had increased significantly in 2014–15 despite very low levels of carbamate use in cotton, notwithstanding the exception of increased usage in 2013.

Although use of these insecticides declined in cotton, they remained important management options in the Australian grains industry which was reliant upon broad-spectrum insecticides largely because of the lack of selective options registered in summer and winter pulse crops at that time and also because broad-spectrum insecticides were a cost-effective measure for control of the susceptible species *H. punctigera* (Murray *et al.*, 2005; Brier *et al.*, 2008). The release of high yielding, disease-resistant chickpea varieties and expanding export markets for this and other pulses led to increased production in Australia during the mid-2000s, with pulse hectares peaking in 2010–11. However, pulse crops are hosts for both *H. armigera* and *H. punctigera*, with the crop most at risk from both species during the reproductive phase of plant development (Brier *et al.*, 2008). A mixed *Helicoverpa* spp. composition in flowering and podding winter pulses is a common occurrence (L.J.B. unpublished data) and the use of pyrethroids and methomyl for their control (based either on economic thresholds or used as a prophylactic application) may have provided a scenario for resistance selection in *H. armigera* and contributed to the observed increases in resistance frequency to pyrethroids and carbamates. Resistance management has been supported by the Australian cotton industry over the last 30 years by the development and implementation of a voluntary IRMS. On the other hand, the Australian grains industry has not had access to a formal strategy for mitigating resistance in *H. armigera*. Unregulated insecticide use during the rapid expansion of the pulse industry as a possible source of selection for resistance highlights the urgent need for a strategic approach to insecticide management to preserve the efficacy of other insecticides which are at similar risk of resistance from over-reliance in grains production systems.

The incremental increases in resistance observed in this study contrast markedly with the resistance trends observed in the preceding seven seasons from 2001–2002 to 2007–2008. The use of pyrethroids and carbamates was generally higher in the period before the introduction of Bollgard II® cotton in 2003, which likely reflect the continued reliance on these insecticides in Ingard® cotton, resulting in ongoing selection for resistance in *H. armigera*. It has been suggested that the replacement of Ingard® with Bollgard II® cotton may have contributed to population suppression by removal of a precautionary 30% cap on total area of Ingard® cotton grown (Baker & Tann, 2017). However, *H. armigera* population abundance may have also been impacted by prevailing climatic conditions at that time (Zalucki & Furlong, 2005). The size of *H. armigera* populations depends upon the availability of suitable hosts, which is closely linked with the quantity and timing of rainfall (Fitt, 1989). In temperate eastern Australia, *H. armigera* moths are generated from both immigrant origins and emergence from local overwintering pupae (Fitt & Daly, 1990) with establishment of spring cohorts on winter pulses (Murray *et al.*, 2005) and, to a lesser extent, uncultivated hosts such as roadside weeds (Wilson, 1983). During droughts, spring abundance of *H. armigera* is likely to be reduced due to resource bottlenecks such as the absence of suitable non-crop hosts and reduced plantings of cultivated hosts (Gregg *et al.*,

1995), particularly dryland crops such as chickpeas and sorghum.

Plantings of major *H. armigera* hosts were relatively low from 2006 to 2009, with the exception of sorghum. Notwithstanding the challenging conditions for irrigated crops, plantings of sorghum, although variable, were relatively high through the period of this study. Although sorghum has a high carrying capacity for *H. armigera*, it can only support a single generation per season. Furthermore, without a sequence of suitable hosts for cohorts from sorghum to move into, the suppressive effect from Bollgard II® acting as a sink for the *H. armigera* population may have been compounded by bottlenecking events in years with low rainfall. The abundance of *H. armigera* may have also been impacted by an increased abundance of natural enemies such as pupal parasitoids due to reduced insecticide use in cotton (Baker & Tann, 2014) or selective mortality due to lower fitness of pyrethroid-resistant individuals following pupal diapause (Daly & Fisk, 1995).

The effect of population suppression, either by a landscape dominated by transgenic cotton, or by drought conditions which limit the abundance and quality of host crops, may have contributed to a reduction in resistance through founder effects and genetic drift. Reductions in the size of populations and subsequent genetic bottlenecks can result in changes in common allele frequency, leading to shifts in gene diversity (England *et al*., 2003). Genetic diversity is then predicted to influence responses to selection (Allendorf, 1986). A severe reduction in the size of the *H. armigera* population, such as was reported by in the Namoi valley during the seasons from 2006 to 2008 (Baker & Tann, 2017), may have impacted the frequency of common alleles, such as those that confer resistance to pyrethroids, through loss of genetic variation and allelic decay. Normally, the effect of this could be mitigated by immigration of populations from other areas which may have experienced greater suitability of rainfall and breeding conditions, ensuring sufficient levels of migration to maintain genetic similarity and widespread distribution of alleles (Daly & Gregg, 1985; Gregg *et al*., 1995). However, when host availability and diversity are severely limited by widespread and prolonged periods of drought, we might also expect immigration rates to be substantially affected.

Resistance monitoring is a key component of the IRMS in the Australian cotton industry. Ideally, the establishment of baseline susceptibility data and calibration of discriminating doses for resistance monitoring should be determined from a large number of geographically diverse field susceptible populations before product commercialization (ffrench-Constant & Roush, 1990). In the case of monitoring for resistance in Australian *H. armigera*, there were clear benefits associated with this approach as it provided a high level of confidence that survival at this dose indicated resistance evolution in the field (Forrester & Cahill, 1987). It is equally important to maintain a laboratory reference strain representative of baseline susceptibility to ensure validity of comparisons of long-term monitoring programmes over time. However, susceptible strains of Lepidoptera held for long periods in the laboratory may bear little resemblance to susceptible strains in the field because of the high likelihood of inbreeding depression which can lead to loss of hybrid vigour and impact on the outcome of bioassays (Santos *et al*., 2012). The laboratory reference strain used in this study (New GR) appeared to be distinct from the reference strain (SUS), previously described as having full susceptibility to broad-spectrum insecticides (Gunning *et al*., 1992; Forrester *et al*., 1993). The New GR strain demonstrated an increased sensitivity to bifenthrin and an increased tolerance to methomyl compared with the SUS strain. Presumably, this was due to the introduction of field insects into the New GR strain as a source of genetic variation, resulting in genetic divergence. Continued resistance monitoring will be important for assessing the effectiveness of the cotton IRMS. However, the results from this study provide evidence that the cotton IRMS alone is not sufficient to delay resistance development in insecticides that are utilized to target *H. armigera* in other commodities. Hence, there is an imperative for the Australian grains industry to support the development and implementation of management strategies to delay the development of resistance to key selective *Helicoverpa* spp. insecticides that are currently at high risk from over-reliance in grains production systems.

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Insecticide resistance in *Helicoverpa armigera*: update and implications for management

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Key findings

- Indoxacarb (e.g. Steward®) resistance across northern NSW and southern Qld in 2018 was 5.6%.
- Indoxacarb resistance in central Qld in 2018 was 11%, which was significantly higher than the industry-wide average.
- Chlorantraniliprole (e.g. Altacor®) resistance across all regions in 2018 was low (<1%).
- No resistance was detected to emamectin benzoate (e.g. Affirm®) in 2018.

Introduction

Insecticide resistance is one of the greatest limitations to broadacre agricultural production in Australia. In particular, the cotton industry's long-term over-reliance on insecticides to control *Helicoverpa armigera* has resulted in wide-spread product failures due to insecticide resistance. The introduction of selective insecticides and *Bacillus thuringiensis* (Bt) cotton reduced the industries dependence on insecticides for control of *H. armigera* and resulted in a gradual decline in resistance to synthetic pyrethroids (Figure 1).

During the mid-to-late 2000s, as the pulse industry expanded, broad-spectrum pesticide use increased and resulted in rapid reselection for pyrethroid resistance (Figure 1). This highlighted the need for a strategic approach to insecticide use in grains, particularly for selective products with broad registration across different summer and winter crops. Indoxacarb and chlorantraniliprole are of particular concern because they are pivotal insecticides for Helicoverpa management across a range of farming systems and are now at increased resistance risk from over-use in the pulse industry.

Resistance surveillance is a key component of resistance management. NSW DPI provides a fully independent assessment of annual resistance frequency for selective Helicoverpa products. The results inform the ongoing process for responding to changes in resistance, including cross-industry engagement for mitigating risk of field failures and minimising economic losses across multiple commodities.

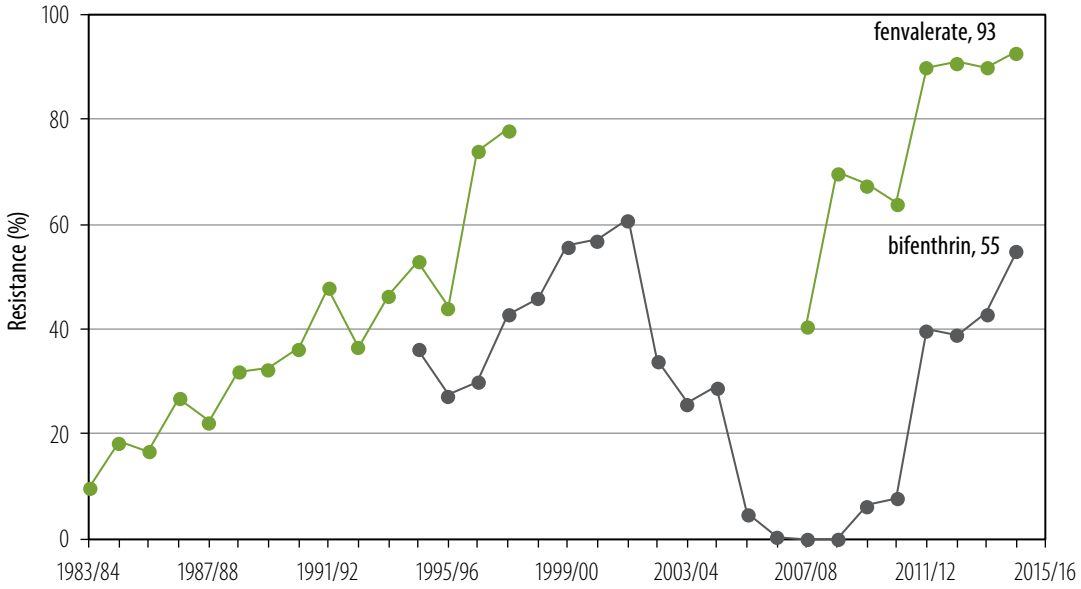

Figure 1. Historical pyrethroid resistance in eastern Australia (average of all regions). Frequency was measured by topically applying a diagnostic dose of insecticide that induces 99.9% mortality in a susceptible strain.

Surveillance methods

Sampling

Pheromone trapping systems (InSense Pty Ltd.) were used to source moths for resistance screening. Traps were located at sites in northern and central NSW: the Macquarie, Namoi and Gwydir valleys; southern Queensland (Qld): the Macintyre valley and Darling Downs region; and Emerald irrigation area (EIA) in central Qld. All traps were located close (<2 m) to known *H. armigera* crop hosts. Sampling was performed every 2–3 weeks throughout the growing season.

Establishing F_2 iso-female lines

Resistance was tested by an F_2 screening procedure based on Andow and Alstad's (1998) methods to generate iso-female lines, a proportion of which are homozygous for haplotypes present in their field-derived parents (Figure 2). This method is highly effective for detecting resistance alleles, regardless of genetic dominance (a scenario for recovering recessive alleles is shown in Figure 2) and involves the following stepwise process:

1. Collecting the parental (F_0) moths from the field;
2. Rearing F_1 offspring for each line;
3. Sib-mating F_1 adults.

We further optimized our methods by using the techniques of Stodola and Andow (2004), where field-derived male moths were individually mated to female moths from a laboratory reared susceptible strain.

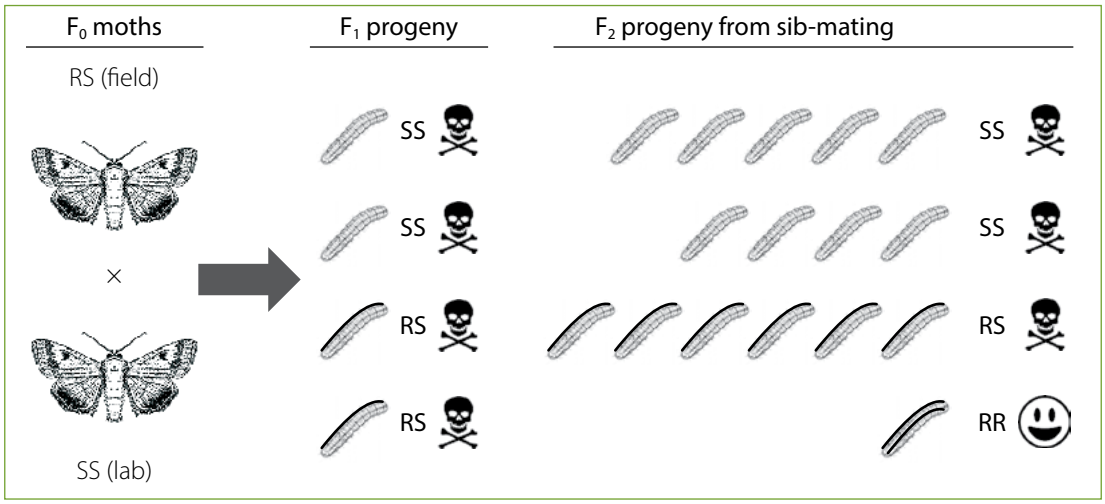

Figure 2. F_2 screen for detection of resistance alleles. Moths are collected from pheromone traps and, in this example one parent has one copy of the resistance gene (RS). Their F_1 progeny are sib-mated to produce the F_2 generation. If resistance is completely recessive then only 1 in 16 of the F_2 progeny will be homozygous (RR) for the resistance gene and will survive a diagnostic dose of insecticide. The remaining susceptible (SS) and heterozygous (RS) progeny will be killed.

Product screening

F_2 larvae were screened by bioassay using an artificial diet into which insecticide had been incorporated. Commercial formulations were used in all screening procedures: indoxacarb (Steward EC [15% active ingredient], and chlorantraniliprole (Altacor [35% active ingredient], both from DuPont Australia Ltd.,); emamectin benzoate (Affirm [1.9% active ingredient], Syngenta Crop Protection).

The ratio of diet to toxin determined the concentration and was calculated as µg insecticide/mL of diet. Insecticides were diluted in distilled water to produce a concentration expected to induce 99.9% mortality of susceptible insects. The discriminating concentration for emamectin benzoate, chlorantraniliprole, and indoxacarb was 0.2, 1 and 12 µg of insecticide/mL of diet, respectively (Bird 2015). Diluted insecticide was added to 1 L of diet and incorporated using a stick blender to produce a homogenous mixture. The insecticide-incorporated diet was then dispensed into 45-well bioassay trays. A total of 90 larvae from each iso-female line was screened against each of the three insecticides. A minimum of 500 lines was screened for each insecticide in each season.

Results

Emamectin benzoate and chlorantraniliprole

As in previous seasons, no resistance was detected to emamectin benzoate in 2017–18. Chlorantraniliprole resistance was low (0.8%, $n = 970$) and not significantly different from the previous season. Although regional frequencies were generally consistent with this industry average, elevated chlorantraniliprole resistance was found in the Darling Downs region in 2017–18 (2.6%, $n = 56$) (Figure 3A).

Indoxacarb

Industry-wide indoxacarb resistance in 2017/18 was 6.5% ($n = 988$) and was not significantly different from the previous season (6.1%, $n = 1022$). Indoxacarb resistance in 2017–18 was significantly higher in the EIA at 11% ($n = 156$), and was similar to the industry average in 2016 –17 (9.2%, $n = 425$) (Figure 3B).

Resistance increased in the lower Namoi to 8.9% ($n = 157$) compared with the previous year, with frequencies for Macintyre and Darling Downs regions similar to the industry average at 6.7% ($n = 134$) and 6.2% ($n = 97$), respectively. Resistance in the upper Namoi, Gwydir and Macquarie valleys was below the industry average in 2017–18 (Figure 3B).

Although levels of indoxacarb resistance in Emerald were over two-fold higher than those in southern Qld and NSW, it is unlikely this was a result of substantially different management practices. It is more likely associated with ecological differences unique to northern regions such as a longer growing

season. A higher number of insect generations per year compared with southern regions, and the lack of an overwintering phase in the lifecycle of *H. armigera*, may be factors that contribute to elevated resistance frequencies in northern populations. The impact of winter diapause (arrested development) in indoxacarb resistance strains of *H. armigera* is currently under investigation.

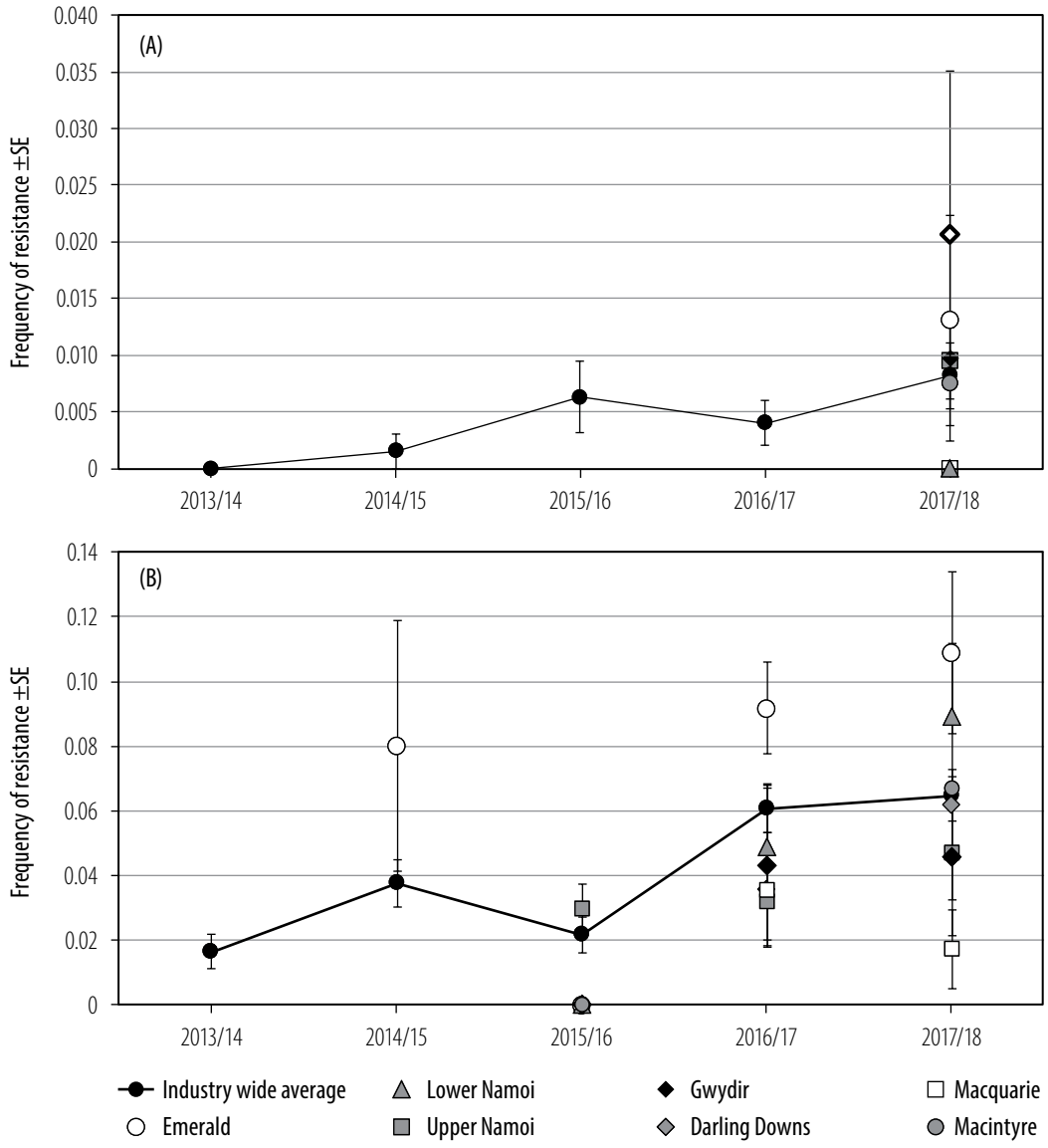

Figure 3. Annual regional frequency of chlorantraniliprole (A) and indoxacarb (B) resistance (± binomial standard error).

Conclusions and implications for management

H. armigera has a very strong track record for developing resistance in response to selection pressure from insecticides. The risk of spray failures is high for pyrethroids and carbamates due to historical resistance to these products, but still low for the selective products indoxacarb, chlorantraniliprole and emamectin benzoate, based on current resistance frequency data. However, it is highly likely that resistance will increase rapidly due to selection pressure from over-reliance on these selective products.

The grains industry is now a major user of Helicoverpa insecticides with product use especially high in pulse crops such as chickpeas. To support the grains industry in mitigating the risk of lost production due to insecticide resistance development, a resistance management strategy (RMS) has recently been produced for *H. armigera* in Australian grain crops. The National Insecticide Resistance Management

(NIRM) working group of the Grains Pest Advisory Committee (GPAC) developed the RMS which is endorsed by CropLife Australia.

The RMS is designed to minimise selection pressure for resistance to the same chemical group across consecutive generations of *H. armigera*. Rotating a broad range of selective options will reduce over-reliance on any one chemical group. Growers should use economic thresholds and avoid prophylactic sprays. Following these recommendations and complying with label instructions will minimise the risk of spray failures occurring as the result of insecticide resistance and maintain effective insecticide control of *H. armigera* into the future.

The RMS is available at: https://grdc.com.au/GRDC-FS-Helicoverpa-resistance-management

For more information on the science behind the strategy go to: https://ipmguidelinesforgrains.com.au/ipm-information/resistance-management-strategies/

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Relative fitness and stability in a near-isogenic strain of indoxacarb resistant Helicoverpa armigera (Lepidoptera: Noctuidae)

--Manuscript Draft--

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Abstract:	A strain of Helicoverpa armigera, demonstrating 171-fold resistance to indoxacarb, was isolated from a field population of survivors from an F2 screen. Introgression of the resistance trait into a susceptible strain by serial backcrossing and reselection with indoxacarb resulted in creation of the near-isogenic GY7-39BC4 strain. Fitness was compared in resistant, susceptible and F1 progeny from a reciprocal backcross of the two strains using life history trait analyses on cotton plants and artificial diet, and selection experiments to determine stability of resistance. There were no significant differences between strains in survival, female fertility or realized fecundity. A comparison of intrinsic rate of population increase (rm) showed similar relative fitness between strains. Lower male fertility and male longevity in the resistant strain and one of the F1 strains compared with the susceptible strain suggests small non-recessive costs may be associated with male reproductive capacity in individuals with indoxacarb resistance alleles. However, there was no significant decline in resistance in the GY7-39BC4 strain when reared in the absence of insecticide for seven generations. These results suggest indoxacarb resistance does not confer a major fitness cost under standard laboratory conditions on either cotton or artificial diet. Survival was reduced by 52% and pupal weights were significantly lower in the resistant strain compared with the susceptible strain following an artificially induced diapause suggesting there may be a recessive overwintering cost associated with indoxacarb resistance.

Dear Editor,

Helicoverpa armigera is a major insect pest in Australian agriculture with a strong track record for developing resistance to insecticides. Indoxacarb is a pivotal Helicoverpa insecticide across a range of farming systems because of its high efficacy and high selectivity for the target pest. Isolation and quantitative genetic analysis of indoxacarb resistance in *H. armigera* was first published in 2016 and this manuscript expands on resistance characterisation by investigating pleiotropic effects (the influence of one gene on two or more seemingly unrelated phenotypic traits) of indoxacarb resistance in near-isogenic strains of *H. armigera*.

Studies fitness costs of resistance are an important part of building risk assessment profiles for key insecticides and for informing insecticide resistance management strategies. The objective of this study was to use two methods to determine the presence of differential fitness between near-isogenic indoxacarb resistant and susceptible strains. Firstly, comparisons of fitness components in life-history trait analyses were conducted on glasshouse-grown cotton and artificial diet in the laboratory. Secondly, population cage experiments were conducted in the laboratory in which strains with known resistance frequencies were maintained with and without selection for six generations and screened each generation to test for stability of resistance.

We found no evidence of a major cost associated with survival, development time or female reproductive potential in indoxacarb resistant *H. armigera*. As a result there were no between-strain differences in net replacement rate or intrinsic rate of natural increase in either cotton or diet experiments. Male fertility and male moth longevity was consistently lower in the resistant strain compared with the susceptible strain reared on cotton or diet. Reduced reproductive capacity in male moths from one of the F_1 hybrid strains reared on cotton and diet suggests that a non-recessive fitness cost may be a force for delaying resistance through reduced reproductive capacity in males with alleles for indoxacarb resistance. However, these costs were not expressed as a reduction in overall fitness because resistance was found to be stable for six generations in the absence of insecticide. A 52% reduction in survival of larvae to post-diapaused adults and significantly reduced male pupal size in the resistant strain compared with the susceptible strain suggests there may be reduced physiological capability of indoxacarb resistant *H. armigera* to survive in overwintering conditions and suggests that there is a greater imperative for adoption of management strategies in northern regions of Australia where a winter diapause does not occur.

Regards,

Lisa Bird

Relative fitness and stability in a near-isogenic strain of indoxacarb resistant *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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Abstract

A strain of *Helicoverpa armigera,* demonstrating 171-fold resistance to indoxacarb, was isolated from a field population of survivors from an F_2 screen. Introgression of the resistance trait into a susceptible strain by serial backcrossing and reselection with indoxacarb resulted in creation of the near-isogenic GY7-39BC4 strain. Fitness was compared in resistant, susceptible and F_1 progeny from a reciprocal backcross of the two strains using life history trait analyses on cotton plants and artificial diet, and selection experiments to determine stability of resistance. There were no significant differences between strains in survival, female fertility or realized fecundity. A comparison of intrinsic rate of population increase (r_m) showed similar relative fitness between strains. Lower male fertility and male longevity in the resistant strain and one of the F_1 strains compared with the susceptible strain suggests small non-recessive costs may be associated with male reproductive capacity in individuals with indoxacarb resistance alleles. However, there was no significant decline in resistance in the GY7-39BC4 strain when reared in the absence of insecticide for seven generations. These results suggest indoxacarb resistance does not confer a major fitness cost under standard laboratory conditions on either cotton or artificial diet. Survival was reduced by 52% and pupal weights were significantly lower in the resistant strain compared with the susceptible strain following an artificially induced diapause suggesting there may be a recessive overwintering cost associated with indoxacarb resistance.

Keywords: cotton bollworm, diapause, fitness costs

Key message

- Fitness costs associated with resistance alleles can delay evolution of insecticide resistance and can be informative for improving resistance management strategies.
- Here we determined relative fitness of near isogenic indoxacarb-susceptible, -resistant and F_1 hybrid strains of *Helicoverpa armigera* on cotton plants and artificial diet.
- There was no major fitness cost associated with indoxacarb resistance under standard rearing conditions. A survival cost associated with simulated diapause highlights the importance of resistance management in regions where diapause does not occur.

Author contribution statement

LB and PW conceived and designed research. LB, PW and LD conducted experiments. PW analysed data. LB wrote the manuscript. All authors read and approved the manuscript.

Introduction

Helicoverpa armigera is a global pest of agriculture with an impressive record for developing resistance to a broad range of insecticidal classes such as synthetic pyrethroids (Forrester et al. 1993), organophosphates (Gunning et al. 1998), carbamates (Gunning et al. 1996), spinosad (Wang et al. 2009) and toxins produced by *Bacillus thuringiensis* (Bt) (Mahon et al. 2007). Indoxacarb is used widely for control of *H. armigera* in Australia across a diverse range of commodities (Brier et al. 2008). Indoxacarb is a pyrazoline-type insecticide bioactivated in the insect gut to the active *N*-decarbomethoxyllated metabolite which blocks voltage-dependent sodium channels and prevents influx of sodium into neuron (Wing et al. 2004).

In Australia the levels of indoxacarb resistance reported in *H. armigera* are generally low (Bird et al. 2017). However, reduced levels of susceptibility have been recorded at certain times of the year and in certain locations (Bird et al. 2019). High levels of genetic dominance and a probable metabolic mechanism of indoxacarb resistance in *H. armigera* (Bird 2017, Chen et al. 2017) suggest there is a high risk that resistance will develop rapidly under certain conditions of selection.

An important part of building a risk assessment profile of indoxacarb resistance is to determine any associated implications for fitness. Fitness costs are considered to be important for influencing the development of resistance because they can select against individuals that carry resistance genes in the absence of insecticide (Carrière and Tabashnik 2001). The dominance and magnitude of fitness costs influence the intensity of selection in

93 the absence of insecticide and the presence of such costs could be valuable for informing
94 resistance management strategies (Gassmann et al. 2009, Carrière et al. 2010).
95
96 Deleterious pleiotropic effects of resistance such as reduced survival, increased
97 development time and decreased reproductive performance are well documented in a
98 range of insect species with resistance to *B. thuringiensis* toxins and chemical insecticides
99 (Gassmann et al. 2009, Kliot and Ghanim 2012). Ideally, fitness comparisons should be made
100 between near-isogenic strains which are genetically identical except for the small region of
101 the genome that includes the locus of interest. This can be achieved by a program of serial
102 backcrossing where the locus of interest is introgressed into a susceptible genomic
103 background over multiple generations. This ensures a high probability that observed
104 differences between near-isogenic strains are associated with the resistance allele(s) and
105 not due to heterosis as a result of genetic variance (Hartl and Clark 1997).
106
107 Experimental verification of an association between mutations that underlie resistance and
108 the presence of fitness costs typically relies on two methodologies: (i) direct comparison of
109 single-generation life-history traits (e.g. survival, development rate or weight) between
110 insecticide resistant and susceptible strains in the absence of insecticide; (ii) evaluating
111 stability of resistance to insecticides in heterogeneous strains reared in the absence of
112 insecticide which takes into account the cumulative effects of all fitness components over
113 multiple generations. Fitness costs are detected by the first method when one or more
114 fitness components are significantly lower in the resistant strain than in the susceptible
115 strain and by the second method when the frequency or level of resistance declines

significantly over time in the absence of selection (Gassmann et al. 2009). The objective of this study was to use both methods to determine the presence of differential fitness between near-isogenic indoxacarb resistant and susceptible strains. Firstly, comparisons of fitness components in life-history trait analyses were conducted on glasshouse-grown cotton and artificial diet in the laboratory. Secondly, population cage experiments were conducted in the laboratory in which strains with known resistance frequencies were maintained with and without selection for seven generations and screened each generation to test for stability of resistance.

Materials and methods

Insect Strains

The susceptible strain (New GR) was established from a cohort of a general laboratory strain GR sourced during the mid-1980s from cotton fields in the Namoi Valley, northern New South Wales, Australia. Bioassays were performed on four non-synchronous cohorts of New GR, and results were pooled in the final analysis because there were no significant differences between cohorts tested. The New GR strain was used as the susceptible control in all bioassays and in crosses to the resistant strain. The resistant strain GY7-39 was established from a single *H. armigera* moth collected near Moree, New South Wales, Australia in 2013. The strain was selected from F_2 offspring that survived the diagnostic dose of indoxacarb (12 µg per ml in a diet incorporation bioassay performed on late second or early third instar larvae) (Bird 2017) that kills 99.9% of susceptible larvae (Bird 2015).

Larval stages were reared using a standard artificial diet based in the methods of Teakle and Jensen (1985) with modifications to remove trypsin inhibitors (Bird 2017). Rearing trays (Tacca Plastics, Sydney, Australia) were covered and heat-sealed with perforated lids (Oliver Products, Grand Rapids, MI). Adults were housed in containers open at the top and covered with cloth liners secured around the lip of the containers to provide an oviposition substrate. Moths were fed a 4% honey/sugar solution through a cotton wick. Eggs were harvested by washing cloth liners in 1% bleach. Vacuum filtration was used to collect eggs onto filter papers which were air dried and sealed in plastic bags until neonates hatched. Neonates were transferred to trays containing artificial diet, one larva per well. All insect strains were maintained in a laboratory environment of 25 ± 2°C with 14:10 (L: D) h photoperiod and ambient RH.

Creation of near-isogenic strains by backcrossing

The GY7-39 strain initially had a restricted gene pool because it had originated from a single isofemale line. To reduce the risk of genetic divergence as a result of founder effect and/or genetic drift, and to maintain the indoxacarb resistant and susceptible strains in a common genetic background, the GY7-39 strain was introgressed into the New GR strain by repeated rounds of recombination by backcrossing (at generations 5, 7 and 12) and reselection to produce a near-isogenic resistant strain of *H. armigera*. After the first cross (BC1) the F_1 strain was maintained without selection for one generation and reselected in the subsequent generation with the diagnostic dose of indoxacarb. As there was significant survival of F_1 progeny at this concentration the F_1 progeny from the second (BC2) and third

(BC3) backcrosses were directly subjected to selection. The fitness comparisons reported herein were performed on the near-isogenic fourth backcross of GY7-39BC4.

Insect bioassays and selection procedure

The dose response to indoxacarb was measured by the diet incorporation method of bioassay previously described by Bird (2015) using a commercial insecticide formulation of indoxacarb (Steward EC [15% active ingredient], Du Pont Australia Ltd., Macquarie Park, Australia).

Insects used in bioassays were reared on untreated diet to the late second or early third larval instar then introduced to trays containing bioassay diet (one larva per well) and covered with heat-sealed, perforated lids. The use of late second or early third instar larvae ensured that only strains with sufficient vigour were utilized as bioassay subjects. Each bioassay was performed in triplicate with individual treatments (insecticide concentrations) in replicates consisting of a minimum of 20 individuals; untreated diet was used as the control. Bioassays were maintained for 7 d under the same conditions described above for larval rearing. Larvae were considered dead if one or more of the following criteria were demonstrated: larvae unable to perform coordinated movement when prodded; paralysis of prolegs; larvae very slow to right themselves (time exceeding 3 seconds).

Life table construction

The relative performance of strains reared to pupation on whole cotton plants and artificial diet was tested by comparing the near-isogenic fourth serial backcross of the resistant strain

GY7-39BC4, the susceptible strain New GR, and F_1 progeny from the reciprocal crosses of the two strains. Adults were reared, as described above, and progeny allocated to either a plant diet in the glasshouse, or an artificial diet in the laboratory. The laboratory cohorts were apportioned to either standard rearing conditions or simulated overwintering conditions (see below).

Cotton seed used in glasshouse experiments was the variety Sicot 71RRF provided by Cotton Seed Distributors Australia Ltd (Wee Waa, New South Wales, Australia). Seeds were planted into 20 cm pots containing a soil mix consisting of 60% garden soil and 40% perlite with approx. 20g per pot, of Osmocote slow-release fertilizer (Scotts, Bella Vista, New South Wales, Australia). Plants were grown in a clear-roofed glasshouse. Maximum and minimum temperatures were recorded daily and ranged between 23 and 31°C. Larvae were introduced onto the terminals of plants 10 weeks after germination when plants were at the 10-12 leaf stage of development with fruiting structures ranging from squares to flowers.

Insect survival and development. Neonate larvae were fed on artificial diet for 24 hours before being introduced to cotton plants. The use of fed neonates improved larval establishment and reduced control mortality on cotton plants compared with unfed neonates. Fifty fed larvae from each genotype were transferred onto the terminal leaves of 10-week old cotton plants (one larva per plant) in glasshouse experiments. Larvae were contained on plants using fabric mesh cages and plants arranged in a randomized design in the glasshouse. In diet experiments 180 neonates from the New GR, GY7-39BC4 and GY7-39 female x New GR male strains and 125 neonates from the GY7-39 male x New GR female

strain were fed on artificial diet and maintained under the rearing conditions, described above. Larval growth was measured using a development index where a numerical value was assigned to each larval stage (Bird and Akhurst 2004). Growth and mortality was recorded every two or three days until pupation. Pupation was calculated as the number of larvae that failed to reach the pupal stage divided by the total number of larvae tested in each strain.

Pupal weights and adult emergence. Fifth instar larvae were removed from the glasshouse after they had ceased feeding and had taken on a characteristic pre-pupal appearance. Pre-pupae were placed on artificial diet and observed daily until pupation. After 72 hours pupae from cotton and diet experiments were sorted by gender and weighed. Adult emergence was recorded daily and the percentage of emergence was calculated in each strain as the number of moths that had successfully emerged as a proportion of the number of pupae. Survival from neonate to reproductive adult was calculated as the number of fertile adults as a proportion of the total number of larvae within each strain.

Reproductive potential. Upon emergence, each adult was paired with a single moth of opposite gender from the diet-reared New GR colony. The paired moths were provided with a 4% honey/sucrose solution and each pair maintained in a 500 mL plastic container lined with paper towels. A layer of fine cloth was placed between the container and the lid covering the aeration hole. Both the cloth and the paper served as oviposition substrates and were replaced every two days. Cage liners were incubated at 25°C overnight. Eggs that did not reach the characteristic 'brown ring' stage within 24 hours were considered infertile.

229 Liners containing at least some eggs that showed signs of development were placed at 4°C
230 to prevent hatching. The total numbers of fertile and infertile eggs laid by each were
231 counted. Realized fecundity was calculated for each strain as the mean number of fertile
232 eggs laid per female. Egg viability was calculated as the number of fertile eggs divided by the
233 total number of eggs laid.
234
235 The net replacement rate (R_o), representing the average number of female offspring
236 produced by each female during its lifetime was calculated for each strain as $R_o = (n \times I_e \times I_a)$
237 x p, where n is the mean number of eggs per female, I_e was the proportion of fertile eggs, I_a
238 was the proportion of reproductive adults and p was the proportion of fertile females in
239 each strain (Birch 1948). The net replacement rate was used to calculate intrinsic rate of
240 population increase as $r_m = (\ln R_o)/T$, where T was the development time from egg to adult
241 eclosion (Birch 1948). The relative fitness of the resistant and F_1 strains was calculated as
242 the ratio of R_o of the resistant and F_1 strains to the R_o of the susceptible strain.
243
244 **Survival of diapaused adults.** Resistant, susceptible and F_1 strains were reared from
245 neonate to the early fifth instar on artificial diet. The number of larvae used to initiate
246 diapause were 1170, 2020, 1980 and 855 in the New GR, F_1 (GY7-39 male x New GR female),
247 F_1 (GY7-39 female x New GR male) and GY7-39BC4 strains, respectively. Diapause was
248 induced in each strain by incubating early fifth instar larvae in a diapause chamber at 18°C
249 with the photoperiod of 11:13 (L:D) (Murray and Wilson 1990). Pupae were kept under
250 simulated overwintering conditions for another three to four weeks before being removed
251 from the incubator and examined for eyespot migration. Linear retention of eyespots across

252 the postgenal eye of pupae was used as confirmation of diapause (Cullen and Browning
253 1978). Diapaused pupae were sorted by gender and weighed. Pupal mortality and the
254 emergence of non-diapaused adults were recorded. Surviving pupae were incubated at 4°C
255 for 14 d to break diapause. Pupae were returned to normal adult rearing conditions and
256 adult emergence was recorded. Survival of larvae to adult and pupae to adult was adjusted
257 to exclude the small number of moths that emerged under diapausing conditions.
258
259 **Stability of resistance**
260 After 12 generations of selection of the fourth serial backcross resistance had stabilised at
261 approx. 150-fold compared with the New GR strain. At this point selection was discontinued
262 in a sub-population of the GY7-39BC4 strain, designated the GY7-39BC4 Unsel strain, and
263 was reared for a further seven generations on untreated diet with no further exposure to
264 insecticide. The selected GY7-39BC4 strain was exposed to a diagnostic dose in bioassays
265 using diet-incorporated indoxacarb in each subsequent generation. After seven days larvae
266 of the GY7-39BC4 strain were transferred to untreated diet to complete development.
267 Selected and unselected strains were reared under the conditions described above.
268 Approximately 380 pupae of uniform size and age were used to initiate each generation and
269 the response of selected and unselected strains was measured and compared in each
270 generation by diet incorporation bioassay.
271
272 **Data Analyses**
273 Where necessary, bioassay data were corrected for control mortality (Abbott 1925). Probit
274 regressions to estimate LC_{50}, ET_{50} (median effective time to taken to reach 50% pupation

and emergence) and associated slope values, and 95% fiducial limits were calculated using stand-alone software (Barchia 2001) where source codes were developed by implementing previously described procedures (Finney 1971, Robertson et al. 2007). Resistance ratios (RR) were calculated by dividing the LC_{50} value of the resistant strain by the LC_{50} value of the laboratory strain. Significant differences (P = 0.05) between LC_{50} values were determined by the lethal concentration ratio test described by Wheeler et al. (2006) where if the 95% confidence interval (CI) includes 1 then the LC_{50}s are not significantly different. Mean pupal weights, realized fecundity and moth longevity were compared by one-way analysis of variance (ANOVA) and Tukey-Kramer honestly significant difference multiple comparison tests. Proportional data of survival, fertility and sex ratio were analysed using χ^2 distribution. All analyses were performed using SPSS statistical software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp., 2013)

Results

Response to selection

After three generations of selection the field-derived GY7-39 strain (F_5) had 167-fold resistance to indoxacarb compared with the laboratory strain New GR (Table 1). The GY7-39 strain was then backcrossed with New GR and reselected three times with the resistance ratio reaching 101-, 152- and 151-fold on the first, second and third occasions, respectively (Table 1). Selection of the fourth backcross resulted in a resistance ratio of 149-fold after 13 generations (Table 1). It was estimated by the principle of Mendelian segregation that the fourth backcross of the GY7-39 strain shared > 96% of its genome with that of the parental strain New GR.

298

299 **Comparison of life history traits**

300 *Larval survival and development*

301 There were no significant differences in survival from first instar larvae to pupation between
302 susceptible, resistant or F_1 strains on cotton (χ^2 = 1.572; df = 3; *P* = 0.666) or diet (χ^2 = 4.935;
303 df = 3; *P* = 0.177). There were no significant differences in survival to emergence on cotton
304 (χ^2 = 0.745; df = 3; *P* = 0.863) or diet (χ^2 = 0.147; df = 3; *P* = 0.986) or the number of
305 reproductive adults produced from each strain on cotton (χ^2 = 1.061; df = 3; *P* = 0.786) or
306 diet (χ^2 = 4.601; df = 3; *P* = 0.203). Larvae developed to pupae 2-3 days faster on diet
307 compared with cotton (Table 1). There were small (<1 day) but significant delays in pupation
308 rate in the resistant strain compared with the susceptible strain on cotton and a similar
309 delay in larval development between the two F_1 strains.

310

311 *Pupal weight and moth emergence*

312 Pupal weight on cotton was similar in males between strains (*F* = 2.66, df = 3, 7; *P* = 0.055)
313 and significantly different in females between strains (*F* = 3.57; df = 3, 80; *P* = 0.018) due to
314 significantly smaller (*P* < 0.05) pupae in the GY7-39BC4 compared with the F_1 (GY7-39 male x
315 New GR female) strain (Table 2). The pupal weights of larvae reared on diet were
316 significantly different between strains for both males (*F* = 11.44; df = 3, 301; *P* < 0.01) and
317 females (*F* = 5.57; df = 3, 270; *P* = 0.001). This was because both male and female pupae of
318 the New GR strain were significantly larger than pupae from all other strains on diet (*P* <
319 0.05) (Table 3).

320

Higher pupal mortality in the GY7-39BC4 strain eliminated individuals with slower larval development and resulted in a significantly shorter mean generation time compared with the New GR strain on cotton (Table 2). Pupation rate of larvae reared on diet was similar between strains (pupation not recorded in F_1 (GY7-39 male x New GR female)). Generation time was also similar between GY7-39BC4, New GR and the F_1 (GY7-39 female x New GR male) strain, and significantly ($P < 0.05$) faster in the F_1 (GY7-39 male x New GR female) strain (Table 3).

Reproductive potential

The proportion of females present in each strain was similar on cotton ($\chi^2 = 2.854$; df = 3; $P = 0.415$) (Table 2) and diet ($\chi^2 = 1.751$; df = 3; $P = 0.626$) (Table 3). There was no significant between-strain differences in female fertility on cotton ($\chi^2 = 3.322$; df = 3; $P = 0.345$) (Table 2) or diet ($\chi^2 = 3.488$; df = 3; $P = 0.322$) (Table 3). Egg viability was also similar between strains on cotton ($\chi^2 = 0.434$; df = 3; $P = 0.933$) (Table 2) and diet ($\chi^2 = 2.374$; df = 3; $P = 0.663$) (Table 3). Although realised fecundity was lower in females from the F_1 (GY7-39 male x New GR female) strain reared on cotton the difference was not significant ($F = 2.21$; df = 3, 58; $P = 0.096$) (Table 2). This contributed to a 28% reduction in net replacement rate (R_o) and a reduction in intrinsic rate of natural increase in this strain ($r_m = 0.134$) compared with New GR ($r_m = 0.146$). Reproductive fitness was highest in the F_1 reciprocal cross ($r_m = 0.169$) and GY7-39BC4 ($r_m = 0.167$) (Table 2). Relative values of r_m where the susceptible strain was compared with GY7-9BC4 and reciprocal crosses (GY7-39BC4 male x New GR, GY7-39BC4 female x New GR) were 1.1, 0.9 and 1.1 on cotton, respectively.

344 Significant differences in fecundity on diet (F = 4.04; df = 3, 101; P = 0.009) was the result of
345 reduced egg laying capacity in the GY7-39BC4 strain and led to a reduction in R_o (Table 3).
346 However, r_m was only slightly reduced compared with the susceptible strain due to similar
347 rates of development on diet. A comparison of r_m of the susceptible strain showed relative
348 fitness of GY7-9BC4 and reciprocal crosses (GY7-39BC4 male x New GR, GY7-39BC4 female x
349 New GR) were 0.9, 1.1 and 1.0 on diet, respectively.
350
351 Male fertility was significantly lower (P < 0.05) in the GY7-39BC4 and F_1 (GY7-39 male x New
352 GR female) strains compared with the New GR and F_1 (GY7-39 female x New GR male)
353 strains on cotton (Table 2) and diet (Table 3).
354
355 There was no significant difference in longevity of female moths when reared on cotton (F =
356 1.89; df = 3, 75; P = 0.138). On diet female longevity was significantly different (F = 3.68; df =
357 3, 121; P = 0.014) because the F_1 (GY7-39 male x New GR female) longevity was reduced.
358 However, longevity in the GY7-39BC4 and New GR strains was similar (Table 3). In contrast,
359 the longevity of male moths was significantly different on cotton (F = 4.57; df = 3, 72; P =
360 0.006) and diet (F = 27.4; df = 3, 152; P < 0.001). Male longevity in the GY7-39BC4 strain was
361 significantly (P < 0.05) reduced by 2.8 days on cotton (Table 2) and on diet by 5.4 days (Table
362 3), compared with the New GR strain.
363
364 *Survival following an artificially induced diapause*
365 The percentage of larvae entering diapause ranged between 95.8% and 99.7% was not
366 significantly different between strains (χ^2 = 3.727; df = 3; P = 0.992) (Table 4). Survival of

larvae to pupation when reared in overwintering conditions was lowest in the GY7-39BC4 strain followed by the New GR strain and the F_1 strains but these differences were not significant ($\chi^2 = 1.312$; df = 3; $P = 0.726$) (Table 4). Survival of larvae to adult was significantly different between strains ($\chi^2 = 21.242$; df = 3; $P = 0.0001$). The highest proportion of larvae that entered diapause and then survived to adult was in the F_1 (GY7-39 male x New GR female) strain at 63.9% (Table 4). Although this was higher than survival in the New GR strain (44.5%), the difference was not significant ($\chi^2 = 3.472$; df = 1; $P = 0.062$). Larval survival to adult in the resistant strain (22.9%) was significantly lower than in the F_1 (GY7-39 male x New GR female) strain ($\chi^2 = 19.366$; df = 1; $P < 0.001$) and the New GR strain ($\chi^2 = 6.922$; df = 1; $P = 0.008$), with larval survival significantly different between the F_1 strains ($\chi^2 = 7.666$; df = 1; $P = 0.006$) (Table 4). The proportion of adults that emerged from diapaused pupae was similar between strains ($\chi^2 = 3.727$; df = 3; $P = 0.292$) (Table 4).

Differences between pupal weight of strains in diapause were highly significant for males ($F = 74.15$; df = 3, 2419; $P < 0.001$) and females ($F = 62.80$; df = 3, 2107; $P < 0.001$). Male pupae of the F_1 (GY7-39 male x New GR female) strain were significantly larger ($P < 0.01$) than male pupae of New GR strain. Similarly, female pupae of the F_1 (GY7-39 male x New GR female) strain were significantly larger ($P < 0.01$) than female pupae of New GR strain. Pupae of the F_1 (GY7-39 female x New GR male) and GY7-39BC4 strains were similar. Male pupae from these two strains were significantly smaller compared with the reciprocal F_1 and New GR strain. However, the difference was only significant between the two F_1 strains ($P < 0.01$) (Table 4).

The proportion of females present after diapause in each strain was similar (χ^2 = 0.406; df = 3; *P* = 0.940) (Table 4). Longevity of moths that survived diapause was significantly different between strains in both males (*F* = 16.86; df = 3,611; *P* < 0.001) and females (*F* = 12.60; df = 3,543; *P* < 0.001) with male and female moths of the F_1 (GY7-39 female x New GR male) strain living significantly (*P* < 0.05) longer than all other strains (Table 4).

Stability of resistance

In the first generation following relaxation of selection there was a significant difference between the LC_{50} of the selected and unselected strain (CI 0.549, 0.839) (Table 5). Nevertheless, the resistance ratio of the unselected strain remained at 147-fold. Over the following six generations without exposure to indoxacarb resistance in the GY7-39BC4 Unsel strain was stable with no further significant decline in LC_{50} based on comparison with the selected strain of GY7-39BC4 (Table 5).

Discussion

Evaluation of fitness and stability is important for determining resistance risk associated with the use of insecticides and can be useful for formulating insecticide resistance management strategies (Gould 1998, Carrière and Tabashnik 2001). Relative fitness can be measured directly by comparing life history parameters between resistant and susceptible strains in the absence of insecticide, and indirectly by measuring stability of resistance in strains containing a mixture of resistant and susceptible alleles when not exposed to insecticide which effectively creates inter-genotype competition over multiple generations. Fitness should be compared in strains with known resistance genotypes and with similar

genetic background to conclude causal links between resistance alleles and observed differences in population growth dynamics (Raymond et al. 2011, ffrench-Constant and Bass 2017). Fitness costs are generally larger on host plants than on artificial insect diet suggesting that assessment of fitness on plants rather than artificial diet provides more relevance to field scenarios (Gassmann et al. 2009) as does the use of field-derived strains rather than chronically selected laboratory strains (ffrench-Constant and Bass 2017). The study reported herein investigated relative fitness of near-isogenic indoxacarb resistant, susceptible and F_1 hybrid strains of *H. armigera* previously isolated from a field population of survivors from an F_2 screen (Bird 2017) using both comparison of fitness components and selection experiments to determine stability of resistance.

We found no evidence of a major cost associated with survival, development time or female reproductive potential in indoxacarb resistant *H. armigera*. Similarly, pyrethroid resistant phenotypes of *H. armigera* did not demonstrate deleterious fitness effects on development rate (Glenn et al. 1994). In the present study, smaller female pupae in the resistant strain did not result in reduced fecundity or female longevity compared with the susceptible or hybrid F_1 strains when reared on cotton. On diet, lower pupal weight was correlated with lower fecundity in the resistant strain. However, pupal weight was not correlated with fecundity in the hybrid F_1 strains. Although insect size can be associated with fecundity (Honěk 1993) this relationship is less well defined in Lepidoptera in which fecundity is more strongly influenced by longevity (Leather 1988).

435 Male fertility was lower in the resistant strain compared with the susceptible strain. This
436 combined with a significant reduction in fertility in one of the F_1 hybrid strains reared on
437 cotton and diet suggests that a non-recessive fitness cost may be associated with
438 reproductive capacity in males with alleles for indoxacarb resistance. However, because net
439 reproductive rate was not reduced in the resistant strain or F_1 hybrid strains male
440 reproductive costs alone may not contribute to a large overall decline in resistance
441 frequency. In contrast, strains of *Heliothis virescens* (F.) and *H. armigera* selected for
442 indoxacarb resistance in the laboratory had lower growth rate, lower fecundity and reduced
443 relative fitness (0.06 and 0.67, respectively) than unselected strains (Sayyed et al. 2008a, Cui
444 et al. 2018). However, unlike in the present study these fitness comparisons were not made
445 in near-isogenic strains.
446
447 Typically the degree of fitness costs are magnified by a plant diet compared with artificial
448 diet (Gassmann et al. 2009). Therefore, our finding that individual fitness components in
449 resistant and heterozygous strains of *H. armigera* was not substantially reduced on cotton
450 further suggests that there was no cost associated with indoxacarb resistance in this strain.
451 Moreover the relative overall fitness of the resistant strain evaluated in population cage
452 experiments, which takes into account the cumulative effect of individual fitness
453 parameters, was slightly higher in the GY7-39BC4 strain and one of the reciprocal crosses
454 compared with the New GR strain, providing further evidence that strains with resistance
455 alleles were not at a significant disadvantage on cotton. However, it is possible that fitness
456 in resistant individuals may be impacted to a greater degree under more challenging
457 environmental conditions such as host plant (Carrière et al. 2004, Bird and Akhurst 2007,

458 Raymond et al. 2011), interspecific competition (Raymond et al. 2005), presence of
459 entomopathogens (Gassmann et al. 2006) and in locations where cool winters induce
460 diapause (Foster et al. 2000).
461
462 The gene(s) for indoxacarb resistance in the GY7-39BC4 strain is inherited as an
463 incompletely dominant trait (Bird 2017). Because the level of resistance in the GY7-39BC4
464 strain was stable when the strain was maintained under selection we have assumed that, by
465 the principle of Hardy-Weinberg equilibrium, the gene for indoxacarb resistance was fixed in
466 the GY7-39BC4 strain and the frequency of *rr*, *rs* and *ss* was constant. If the rate of reversion
467 to susceptibility in heterogeneous populations is correlated with a reduction in fitness of
468 resistant phenotypes, then stability of resistance indicates there is no deleterious effect
469 from the presence of a resistance gene (Roush and McKenzie 1987, Tabashnik 1994).
470 Stability of resistance has been reported in Bt resistant strains of *P. xylostella* and *S.*
471 *frugiperda* and *H. armigera* (Tang et al. 1997, Santos-Amaya et al. 2017, Mahon and Young
472 2010). The finding that there was no significant decline in indoxacarb resistance when the
473 GY7-39BC4 strain was reared for seven generations in the absence of insecticide compared
474 with a selected strain, indicates a high level of stability of resistance and is consistent with a
475 lack of fitness cost demonstrated in the life-history traits studied.
476
477 Winter diapause is an adaptive strategy which allows survival of *H. armigera* populations in
478 temperate regions of eastern Australia when host plants are scarce and temperatures are
479 generally too low to allow successful development. A 52% reduction in survival of larvae to
480 post-diapaused adults and significantly reduced male pupal size in the GY7-39BC4 strain

481 compared with the New GR strain suggests indoxacarb resistance may be associated with a
482 reduced physiological capability of indoxacarb resistant *H. armigera* to survive in
483 overwintering conditions. However, the cost appears to be largely recessive and would
484 provide limited benefits from a resistance management perspective. This is because
485 recessive costs are a relatively weak force for driving a decrease in resistance allele
486 frequency in early-stage of resistance when heterozygotes are the main carriers of
487 resistance alleles (Carrière and Tabashnik 2001, Carrière et al. 2004b). Reduced fitness as a
488 result of larval exposure to overwintering conditions may explain a shift in phenotypic
489 frequency of pyrethroid resistance in *H. armigera* observed following an artificially induced
490 diapause in the laboratory (Daly and Fisk 1995) and reduced pyrethroid resistance frequency
491 in overwintering field populations (Daly and Fitt 1990). Survival costs associated with
492 overwintering also selected against dieldrin and diazinon resistance in *Lucilia cuprina*
493 (Wiedemann) (McKenzie 1990, 1994), and resistance to Bt toxins in *Pectinophera gossypiella*
494 (Saunders) (Carrière et al. 2001) and *Leptinotarsa decemlineata* (Say) (Alyokhin and Ferro
495 1999).
496
497 The magnitude of pleiotropic effects associated with resistance to synthetic insecticides is
498 dependent on the mechanism of resistance (Roush and Daly 1990). Considerable
499 disadvantages are usually associated with energetically costly processes associated with
500 gene amplification or up-regulation of metabolic enzymes (Devonshire and Field 1991,
501 Rivero et al. 2011) or with target site resistance mechanisms involving point mutations
502 which result in structural and/or functional constraints at the modified target site (Taylor
503 and Feyereisen 1996, Gassmann et al. 2009, ffrench-Constant and Bass 2017). The

504 involvement of metabolic mechanisms in indoxacarb resistance has been reported in several
505 lepidopteran species based on synergistic effects of metabolic inhibitors on indoxacarb
506 toxicity. Esterases and glutathione-S-transferase (GST) are thought to have a role in
507 indoxacarb resistance in *P. xylostella* (Sayyed and Wright 2006, Nehare et al. 2010) and
508 *Spodoptera exigua* (Hübner) (Gao et al. 2014) while indoxacarb resistance is associated with
509 enhanced metabolism by P450 monooxygenases in *Spodoptera litura* (Fabricius) (Sayyed et
510 al. 2008b), *Choristoneura rosaceana* (Harris) (Ahmad and Hollingworth 2004), *Musca*
511 *domestica* (L.) (Shono et al. 2004) and *H. armigera* (Bird 2017, Cui et al. 2018). The putative
512 mechanism of indoxacarb resistance in a precursor strain of GY7-39BC4 was associated with
513 the CYP6AE gene cluster (Chen et al. 2017) which is consistent with findings from inhibition
514 experiments (Bird 2017) and from CYP6AE gene cluster knockout experiments which
515 restored susceptibility to indoxacarb in *H. armigera* (Wang et al. 2018). The lack of a fitness
516 cost in GY7-39BC4 suggests that the either detoxification of indoxacarb is not energetically
517 costly or the involvement of fitness modifiers that ameliorate the cost of resistance
518 (McKenzie 1996).
519
520 Susceptibility to indoxacarb in Australian populations of *H. armigera* is relatively high (Bird
521 et al. 2017) which is likely due to a 90% reduction in sprays to control *Helicoverpa* species
522 following the introduction of dual toxin transgenic cotton in 2003 (Fitt 2008). An insecticide
523 resistance management strategy (IRMS) primarily aimed at the cotton industry has been in
524 place for over three decades. However, use of selective products such as indoxacarb for
525 control of *H. armigera* has increased over the past decade due to expansion of the
526 Australian pulse industry (Brier et al. 2008, ABARES 2018) and the cotton IRMS is not

sufficiently effective for managing resistance risk in insecticides utilized to target *H. armigera* in other commodities (Bird 2018). Moreover, because of high levels of genetic dominance and probable metabolic mechanism of indoxacarb resistance in *H. armigera* (Bird 2017, Chen et al. 2017) it is likely that resistance will develop rapidly under certain conditions of selection.

The presence of fitness costs associated with resistance could delay the spread of resistance alleles under certain conditions (Carrière et al. 2010). However, the fitness cost of indoxacarb resistance in the GY7-39BC4 strain of *H. armigera* is, at best, small and transient. Dominant inheritance of a metabolic mechanism exacerbates resistance risk because as dominance of resistance increases the effect of the dominance of the fitness cost diminishes (Tabashnik et al. 2004) and management tactics for delaying resistance under these circumstances will be more difficult to implement (Roush 1989, ffrench-Constant and Bass 2017). Therefore, it is imperative that effective resistance management strategies that rely on chemical rotations are adopted to prolong the efficacy of indoxacarb in all farming systems that play host to *H. armigera*. The finding that there may be a partially non-recessive fitness cost associated with survival through a winter diapause suggests that there is a greater imperative for adoption of management strategies in northern regions of Australia where a winter diapause does not occur. Further studies are warranted to model possible impacts of fitness costs on stability of indoxacarb resistance in the field where diapause occurs.

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Compliance with Ethical Standards

Conflict of Interest

The authors declare they have no conflict of interest.

Ethical Approval

This article does not contain any studies with human participants or animals (vertebrates) performed by any of the authors.

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Table 1. Response of the resistant strain (GY7-39) during selection of near-isogenic lines compared with the susceptible New GR strain.

Genotype	Generations †	LC_{50} [µg/ml diet] (95% FL) ‡	Fit of probit line			RR
			Slope ± SE	X^2 (df)	*P*	
New GR		0.15 (0.144, 0.165)	3.1 ± 0.1	6.66 (4)	0.155	-
GY7-39 (F_5) §	3	25.02 (21.418, 29.251)	2.6 ± 0.2	6.47 (3)	0.091	167
GY7-39 (BC1)	2	15.21 (7.593, 25.631)	2.6 ± 0.5	14.02 (3)	0.003	101
GY7-39 (BC2)	5	22.86 (19.547, 26.698)	2.6 ± 0.2	2.96 (3)	0.398	152
GY7-39 (BC3)	8	22.68 (19.426, 26.447)	2.6 ± 0.2	2.68 (3)	0.444	151
GY7-39 (BC4)	13	22.31 (5.555, 67.154)	2.6 ± 0.8	12.59 (3)	0.006	149

† Number of generations selected between each backcross.

‡ Response to indoxacarb at the time of each backcross.

§ GY7-39 strain was isolated by F_2 screening and the survivors selected for three generations.

Table 2. Mean fitness parameters for New GR, GY7-39BC4 and F_1 progeny from a reciprocal backcross of the two strains on 10 week-old glasshouse-grown cotton.

Life History Trait	New GR	F_1 strains		GY7-39
		GY7-39 male x New GR female	GY7-39 female x New GR male	
% Survival				
Pupation	97.8[a]	84.2[a]	100[a]	95.6[a]
Adult emergence	93.3[a]	100[a]	95.7[a]	88.4[a]
Reproductive adult	91.3[a]	84.2[a]	95.7[a]	84.4[a]
Development time (days)				
ET_{50} pupation (FL)	18.6 (18.38, 18.91)[bc]	19.2 (18.50, 19.84)[ab]	18.3 (17.86, 18.64)[c]	19.5 (18.99, 20.03)[a]
ET_{50} emergence (FL)	36.7 (35.99, 37.31)[a]	37.0 (36.09, 39.04)[a]	34.4 (33.92, 34.97)[b]	34.3 (33.53, 35.09)[b]
Mean pupal weight (mg)				
Female (*n*)	319.1 ± 8.3[ab] (20)	299.7 ± 12.0[ab] (18)	324.9 ± 6.7[a] (25)	292.5 ± 8.0[b] (22)
Male (*n*)	330.1 ± 5.8[a] (25)	314.7 ± 8.2[a] (16)	301.0 ± 9.1[a] (19)	307.2 ± 10.3[a] (17)
Reproductive potential				
% female fertility	73.3[a]	66.7[a]	88.0[a]	81.0[a]
% male fertility	80.0[a]	50.0[b]	84.2[a]	56.3[b]
% egg viability	86.8[a]	86.7[a]	94.0[a]	91.5[a]
Realized fecundity	939 ± 154[a]	602 ± 81[a]	979 ± 108[a]	953 ± 84[a]
Proportion of females	0.43[a]	0.56[a]	0.53[a]	0.56[a]
Net replacement rate (R_o)	243	165	332	312
Intrinsic rate of population increase (r_m)	0.146	0.134	0.169	0.167
Relative fitness †	1.00	0.92	1.16	1.14
Adult longevity (days)				
Female	14.4 ± 0.6[a]	14.9 ± 1.0[a]	16.1 ± 0.8[a]	13.7 ± 0.8[a]
Male	13.6 ± 0.6[a]	14.5 ± 1.1[a]	13.9 ± 0.5[a]	10.8 ± 0.7[b]

Superscript letters after means within life history traits indicate significant differences ($P < 0.05$) between the means.

†Relative fitness = r_m (GY7-39 or F_1 progeny) / r_m (New GR).

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Table 3. Mean fitness parameters for New GR, GY7-39BC4 and F_1 progeny from a reciprocal backcross of the two strains, on artificial diet.

Life History Trait	New GR	F_1 strains		GY7-39
		GY7-39 male x New GR female	GY7-39 female x New GR male	
% Survival				
Pupation	96.6[a]	73.6[a]	97.7[a]	80.4[a]
Adult emergence	100[a]	97.8[a]	95.4[a]	100[a]
Reproductive adult	96.6[a]	71.9[a]	93.2[a]	80.4[a]
Development time (days)				
ET_{50} pupation (FL)	16.3 (16.08, 16.52)[a]	NR	16.1 (15.59,16.64)[a]	16.2 (16.12, 16.35)[a]
ET_{50} emergence (FL)	32.1 (31.85, 32.29)[a]	30.4 (28.64, 31.98)[b]	31.7 (31.04, 32.30)[ab]	32.2 (31.91, 32.44)[a]
Mean pupal weight (mg)				
Female (*n*)	417.1 ± 3.4[a] (72)	398.3 ± 5.8[b] (46)	397.9 ± 4.0[b] (81)	397.2 ± 4.0[b] (75)
Male (*n*)	416.3 ± 2.9[a] (101)	388.2 ± 5.7[b] (43)	392.5 ± 3.5[b] (92)	399.7 ± 4.1[b] (69)
Reproductive potential				
% female fertility	75.0[a]	63.3[a]	78.9[a]	85.7[a]
% male fertility	91.7[a]	60.0[b]	97.6[a]	60.5[b]
% egg viability	84.5[a]	85.9[a]	89.5[a]	74.0[a]
Realized fecundity	1009 ± 100[ab]	1367 ± 151[a]	1087 ± 91[ab]	810 ± 96[b]
Proportion of females	0.42[a]	0.52[a]	0.47[a]	0.52[a]
Net replacement rate (R_o)	189	298	221	155
Intrinsic rate of population increase (r_m)	0.158	0.181	0.165	0.152
Relative fitness †	1.00	1.15	1.04	0.96
Adult longevity (days)				
Female	17.7 ± 0.7[ab]	15.0 ± 1.0[a]	17.4 ± 0.6[ab]	18.4 ± 0.7[b]
Male	16.2 ± 0.4[a]	16.0 ± 0.5[a]	18.2 ± 1.2[a]	10.8 ± 0.5[b]

Superscript letters after means within life history traits indicate significant differences ($P < 0.05$) between the means.

†Relative fitness = r_m (GY7-39 or F_1 progeny) / r_m (New GR).

Table 4. Effect of artificially induced diapause on survival of New GR, GY7-39BC4 and F_1 progeny from a reciprocal cross of New GR and GY7-39BC4, reared on artificial diet.

Life History Trait	New GR	F_1 strains		GY7-39
		GY7-39 male x New GR female	GY7-39 female x New GR male	
% pupae that entered diapause	97.2[a]	99.7[a]	95.8[a]	99.1[a]
Survival				
% survival of larvae to pupae	83.4[a]	80.8[a]	79.7[a]	70.0[a]
% survival of larvae to adult†	44.5[ab]	63.9[a]	36.2[bc]	22.9[c]
% emergence from diapaused pupae	83.7[a]	92.5[a]	70.0[a]	74.6[a]
Mean pupal weight (mg)				
Female (*n*)	378.9 ± 2.6[b] (414)	420.5 ± 3.4[a] (347)	369.5 ± 2.5[bc] (702)	367.4 ± 2.6[c] (648)
Male (*n*)	388.4 ± 2.3[b] (517)	430.1 ± 3.3[a] (342)	379.1 ± 2.0[c] (828)	376.2 ± 2.3[c] (736)
Proportion of females	0.45[a]	0.50[a]	0.46[a]	0.47[a]
Adult longevity (days)				
Female	17.1 ± 0.4[bc]	16.0 ± 0.6[c]	19.7 ± 0.4[a]	18.4 ± 0.4[ab]
Male	15.6 ± 0.3[c]	17.9 ± 0.5[ab]	19.0 ± 0.3[a]	17.3 ± 0.4[b]

Superscript letters after means within life history traits indicate significant differences ($P < 0.05$) between the means.

† Survival excludes the number of moths that emerged under diapausing conditions.

Table 5. Response of the GY-39BC4 strain to indoxacarb in the absence of insecticide selection.

Generation of GY7-39BC4	LC_{50} [µg/ml diet] (95% FL)	Fit of probit line			Lethal dose ratio (95% CI)	RR
		Slope ± SE	X^2 (df)	*P*		
F14 Sel	32.56 (27.859, 37.948)	2.8 ± 0.3	5.68 (2)	0.058		217
F14 Unsel	22.08 (19.111, 25.561)	2.8 ± 0.2	2.35 (4)	0.572	0.678 (0.549, 0.839)	147
F15 Sel	27.73 (11.044, 53.634)	3.2 ± 0.6	6.33 (2)	0.042		185
F15 Unsel	31.40 (27.761, 35.467)	3.2 ± 0.3	2.39 (2)	0.303	1.133 (0.854, 1.501)	209
F16 Sel	23.86 (13.175, 42.658)	3.6 ± 0.7	14.34 (3)	0.003		159
F16 Unsel	24.17 (21.169, 27.526)	3.6 ± 0.3	6.47 (3)	0.091	1.013 (0.739, 1.388)	163
F17 Sel	19.89 (17.208, 23.022)	2.8 ± 0.2	3.43 (4)	0.489		133
F17 Unsel	23.42 (20.256, 27.138)	2.8 ± 0.2	9.16 (4)	0.057	1.177 (0.956, 1.449)	156
F18 Sel	21.54 (18.649, 24.929)	2.8 ± 0.2	7.11 (4)	0.130		144
F18 Unsel	16.33 (11.002, 24.515)	2.8 ± 0.4	12.56 (4)	0.014	0.758 (0.563, 1.021)	109
F19 Sel	19.32 (16.672, 22.401)	2.7 ± 0.2	2.29 (4)	0.683		130
F19 Unsel	19.55 (16.876, 22.676)	2.7 ± 0.2	9.02 (4)	0.061	1.012 (0.820, 1.249)	130
F20 Sel	20.29 (17.880, 23.018)	3.7 ± 0.4	5.49 (3)	0.139		135
F20 Unsel	23.06 (20.327, 26.159)	3.7 ± 0.4	5.39 (3)	0.145	1.136 (0.950, 1.359)	154

† Survival at the diagnostic dose (DD) of indoxacarb, 12µg / ml artificial diet.

Linkage mapping an indoxacarb resistance locus in *Helicoverpa armigera* (Lepidoptera: Noctuidae) by genotype-by-sequencing

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Abstract:

BACKGROUND: A major challenge to sustainable agricultural pest control is the rapid evolution of insecticide resistance. This is caused by mechanisms that reduce insecticide efficacy. Understanding the genetic mechanisms of resistance is essential for DNA based monitoring of resistance in field populations. One such insecticide is indoxacarb, an important selective control option for *Helicoverpa armigera* in a range of crops including grain, horticulture, and cotton. Recently, a strain of *H. armigera* (GY7-39) resistant to indoxacarb (198 fold) was isolated from field collected moth.

RESULTS: To identify the indoxacarb resistance locus, GY7-39 was backcrossed for 6 generations to susceptible strain New GR. In each generation, only resistant males were used to cross back to New GR. Genotype-by-sequencing (GBS) was carried out on 95 *H. armigera* samples. In total 13203 tags with 8697 unique locations on the *H. armigera* genome were obtained. The indoxacarb resistance locus in strain GY7-39 was mapped to a 2.5Mbp region on chromosome 16. In this region, two close linked loci (IndoR1 and IndoR2) were found to be associated with indoxacarb resistant GY7-39.

CONCLUSIONS: We mapped indoxacarb resistance in GY7-39 to two closely linked loci IndoR1 and IndoR2 in a narrowed 2.6Mb region of *H. armigera* chromosome 16. The results provide essential background data for future genetic investigations including fine mapping of the indoxacarb resistance gene and the eventual development of an effective DNA based diagnostic to support resistance management.

Keyword:

Helicoverpa armigera; linkage mapping; indoxacarb resistance; genotype-by sequencing.

Introduction

Linkage analysis was the predominant statistical genetic mapping tool used in the latter half of the twentieth century to identify genes and mutations associated with a particular phenotype[1]. It has been particularly useful in identifying genes associated with genetic mediated disease in humans [2] and for identifying the genetic basis of mutants in model organisms [1, 3]. Additionally, the same basic principle has been used to map insecticide resistance genes in insects [4-7]. Linkage analysis starts with a linkage or genome-wide association to identify which narrow region may harbour the mutant gene. This is then followed by positional cloning and sequencing to define the causal mutation(s). Once a candidate causal mutation is identified, final confirmation can be carried out via additional molecular and cellular function studies [2].

Most target site insecticide resistances were first identified by linkage studies or simply looking for changes in the target gene itself (e.g. acetylcholinesterase or sodium channel)[8]. Understanding detoxification based resistance mechanisms (e.g. esterases, cytochromes P450 or glutathione-S-transferases) is more complex due to the number of genes within these detoxification families. Here a candidate gene or gene family approach has been used to identify differential expression or mutations in these genes associated with resistance. Linkage mapping of insecticide loci was widely used in the model organism *Drosophila melanogaster* [9, 10]. However, the recent explosion in the availability of insect genomes enables us to use more sophisticated, high throughput genetic mapping tools, such as genotyping-by-sequencing (GBS) to identify linkages between phenotype and genotype.

The cotton bollworm, *Helicoverpa armigera*, is a highly polyphagous insect pest found in Australia, Asia, Africa, and more recently, South America [6, 7]. Worldwide, *H. armigera* is known to attack at least 60 cultivated and 67 wild host plants including 72 known host species in Australia [11]. In Australia, *H. armigera* is a significant pest of broad acre cropping including grain, horticulture, and cotton with great economic cost due to direct yield reduction and control costs [12, 13].

Before the introduction of transgenic cotton with insecticidal traits, Australian cotton was heavily sprayed for *H. armigera* control [6]. As a consequence, populations of *H. armigera* developed resistance to pyrethroids, organochlorines and carbamates [12, 13]. In the 1980s, an insecticide resistance management strategy that included chemical use restrictions (URL: https://apvma.gov.au/node/988) was introduced that was underpinned by resistance monitoring [12, 13] and Integrated Pest Management (IPM) [14]. Management including IPM caused Australian cotton growers to become increasingly reliant on newer selective insecticides such as emamectin benzoate, chlorantraniliprole, and indoxacarb due to their high efficacy and low toxicities to beneficial insects [15-18].

Monitoring insecticide resistance in *H. armigera* was originally achieved via the topical application of a discriminating insecticide concentration [13]. The discriminating concentration was determined from multiple susceptible baseline dose-response mortality data. A discriminating concentration should be lethal to susceptible but allow resistant individuals to survive and are often set at an $LC_{99.9}$ level, which is an estimate of concentration required to kill 99.9% of the population tested [19]. Simply, if an insect survived the test it was *prima facia* resistant and such techniques have been useful for measuring resistance mediated by dominant alleles or when the resistance allele frequency is high. However, it is less effective when the resistance is due to a recessive allele or the resistance allele frequency is particularly low [20].

To detect recessive resistance an alternative F_2 screen method is used (Andow and Alstad 1998). Such methodology was introduced in 2002 for monitoring toxins produced by the bacterium *Bacillus*

thuringiensis including Cry1Ac, Cry2Ab and Vip3A [21]. In 2013 an F_2 screen was introduced to monitor chemical resistance to emamectin benzoate, chlorantraniliprole, and indoxacarb [22]. The methodology identified the first case of indoxacarb resistance isolated from a field population of *H. armigera* in Australia [23]. From this screening, a near-isogenic resistant strain of *H. armigera* (known as GY7-39) was established from a single egg and was subsequently shown to have 168-fold indoxacarb resistance when compared to a known susceptible strain. The GY7-39 strain did not confer cross-resistance to chlorantraniliprole and had negative cross-resistance to emamectin benzoate and fenvalerate [23]. Indoxacarb resistance in GY7-39 was significantly reduced when exposed to the metabolic inhibitor PBO suggesting a metabolic mediated detoxification. It has been reported mutations in the voltage-gated sodium channel (VGSC) were involved in indoxacarb and metaflumizone resistance in diamondback moth, *Plutella xylostella* [24] and in tomato leafminer, *Tuta absoluta* [25].

The recent availability of the *H. armigera* genome [26] enables the application of high throughput genetic mapping approaches to identify resistance mechanisms in this species. Here we report such mapping to identify the region containing the major gene conferring indoxacarb resistance in *H. armigera* strain GY7-39 via advanced crossing and genotype-by–sequencing (GBS).

Materials and methods

Insect strains, crosses, and bioassays.

We used two strains of *H. armigera*. A known laboratory strain, New GR was established from a series of collections from cotton fields in the Namoi Valley, northern New South Wales in the mid-1980s and is known to be susceptible to indoxacarb (LC_{50} at 0.147 $\mu g mL^{-1}$ indoxacarb in diet-incorporation bioassays conducted on a late second or early third instar larvae) [23, 27]. The resistant strain GY7-39 was established from a single *H. armigera* egg from maize near Moree, New South Wales in November 2013 [23]. The first generation of GY7-39 survived a discriminating concentration of indoxacarb (12 $\mu g\ mL^{-1}$ indoxacarb in diet-incorporation bioassays conducted on late second or early third instar larvae) from an F_2 screen. During the development of the GY7-39 strain, it was backcrossed three times to susceptible New GR at generations 5, 7 and 12. The GY7-39 strain used in this experiment is a near-isogenic strain of indoxacarb resistant *H. armigera* with 198 fold resistance compared to susceptible strain New GR [23].

Strains were maintained as described by [28]. Moths were provided a 4% honey/sugar solution and housed in containers with cloth liners secured around the lip. Adults were maintained at 25±2 °C, 14:10 (L: D) and relative humidity was maintained at 70–80% for the duration of the dark cycle via a steam humidifier. Larvae were maintained under similar conditions of light and temperature but with relative humidity maintained at 45–55%. The susceptible strain New GR and resistant strain GY7-39 were monitored regularly to confirm susceptibility and resistance. Commercial insecticide as Steward® EC Insecticide (150 g/L indoxacarb) (DuPont Australia Ltd, Macquarie Park, Australia) was used for all testing as described by Bird *et al.* [23].

Indoxacarb resistance in strain GY7-39 was revealed by genetic analysis to be autosomal and incompletely dominant [23]. Males from the resistant strain GY7-39 males were crossed with susceptible strain New GR females to produce progeny (Figure 1). We then selected resistant male progeny (discriminating concentration survivors) to cross back to susceptible 5 times to generate F_6 progeny. Three independent crossing lineages were established, with each lineage producing more

than 100 F_6 resistant progeny. The F_6 resistant larvae were collected and stored in RNAlater® (Sigma-Aldrich, USA Lithuania) at -20 ºC.

[insert Figure 1]

Figure 1 Schematic diagram of the advanced crossing for gene mapping. In F_0, the resistant male was crossed to susceptible female. In the following generations, only the resistant male was selected to cross back to susceptible females. In generation F_6, only the resistant individuals were collected for genotype-by-sequencing.

DNA extraction and Genotype-by-sequencing

DNA was extracted with the DNeasy® Blood & Tissue kit (Qiagen, Australia) according to the manufacturer's instructions. DNA was extracted from the head of adult or larvae [29]. Sample tissue was homogenized using a disposable microtube pestle in 180 µL sterile 1 × phosphate buffered saline (PBS) (Sigma-Aldrich, USA), and lysed at 56 °C for 10 min in the presence of 20 µL proteinase K and 200 µL Buffer AL. DNA was precipitated with 200 µL of pure ethyl alcohol (Sigma-Aldrich, USA) and the mixture pipetted directly onto a silica-based membrane of a DNeasy® Mini spin column placed in a 2 mL collection tube. DNA was bound to the membrane by centrifugation at 16,300 x g for 1min. The column was washed with 500 µL of Buffer AW1 followed by 500 µL of Buffer AW2. To ensure any residual ethyl alcohol was removed from the column, a final centrifugation step was performed at 16,300×*g* for 3 min. DNA was then eluted in 200 µL Buffer AE at room temperature.

Ninety-five DNA samples plus one negative control (no DNA) were placed in a 96-well plate. Samples included eight individuals from susceptible New GR (including two susceptible F_0 females), fourteen resistant GY7-39 (including two resistant F_0 males), and seventy-three resistant F_6 (one family with 35 and a second family with 38 F_6). All the DNA samples were quantified by the Nanodrop™ 2000 Spectrophotometer (Thermo Scientific) and further analysed by gel electrophoresis on a 1% agarose gel in 1× Tris/Acetic Acid/Ethylenediaminetetraacetic Acid (TAE) buffer (Bio-Rad, USA) and visualized using GelRed™ stain (Jomar Diagnostics, USA).

GBS library construction and sequencing were performed at the Cornell genomic diversity facility (http://www.biotech.cornell.edu) using restriction enzyme *Pst*I [30]. Here individual DNA samples were digested with *Pst*I and adapters ligated. Individual ligations were pooled and amplified by PCR. The pooled *Pst*I associated DNA fragments were sequenced in one lane of the Illumina HiSeq® 2000 (Illumina, Inc) with 1x 100bp at the Cornell genomic diversity facility.

Processing sequenced reads

Unique sequence tags (64 bp) were obtained by processing raw sequence reads using "sequence to Tag/TaxaDB" of GBSv2 in the TASSEL 5.0 [31]. The unique sequence tags were exported as fastQ with Tag-toFastQ and aligned to the *H. armigera* genome with HISAT2 [26, 32].

To obtain individual tag read counts, one lane of Illumina sequence were deindexed into a single fastq file per sample by using the process *radtags* in Stacks 1.4 with default parameters [33] The read counts for each tag and sample were extracted by tagdigger_interactive.py in TagDigger [34].

To determine tag presence or absence, the read counts were normalized for each tag to a read count per million reads thereby adjusting the variation of the sequencing depth for each sample. We only used the tags with at least 10 sequence reads (normalized). For all the samples, each tag was ranked

based on the normalized sequence reads and we consider the tag to be absent if the normalized read count is less than 0.1 of the maximum of the normalized read counts.

Tag mapping in a *Helicoverpa armigera* genome

The filtered Tags were first aligned to a *H. armigera* reference genome using HISAT2 [32] with default parameters. A Basic Local Alignment Search Tool (BLAST) [35] was used to query tags that were not aligned by HISAT2.

The genome-wide association analysis between the tag and indoxacarb resistance for both resistant and susceptible individuals was carried out using plink 2.0 [36]. We treated each tag as a dominant marker and assumed it to be fixed in either the resistant or susceptible strains. To achieve this, a hypothetical resistant tag was added, which is present in all resistant individuals and absent from susceptible individuals. We calculated the theoretical *p*-value for the association between the hypothetical resistance tag and indoxacarb resistance at 1.0×10^{-11} based on 14 resistant and 8 susceptible individuals.

Searching for candidate haplotype block for indoxacarb resistance locus

The full sequence of the tags that are significantly associated with indoxacarb resistance from GWAS were extracted from the raw sequence file. These sequences were re-aligned to a *H. armigera* reference genome using a HISAT2 and BLAST program. The alignments were manually examined via IGV[37]. Tags that overlapped on the chromosome location or from the complementary strand were merged into a haplotype block. The resultant candidate haplotype block for indoxacarb resistance should satisfy the following criteria, 1) The full tag sequence is uniquely mapped in the *H. armigera* genome references sequence via HISAT2 or BLAST. 2) The haplotype should be uniquely present in all GY7-39 resistance individuals (total 14) and absent from the 8 New GR individuals. 3) Importantly, the haplotype should be present in majority of F_6 resistant progeny from Family 1 or Family 2.

Validation of haplotypes by Sanger sequencing

For each resistance associated haplotype, tag sequences were imported into SEQUENCHER 5.4.6 (Gene Codes, Ann Arbor, MI, USA) and aligned with the *H. armigera* reference genome. Primer pairs were designed to amplify the identified region using Primer 3 [38].

PCR was performed in a 25µl reaction containing 1 x MyFi™ Mix (Bioline, Australia) master mix, ~20ng DNA and 1µM each primer. Thermal cycling conditions were as follows: 95°C for 10 min followed by 35 cycles of 95°C for 30 s, 53°C for 30 s, 72°C for 60 s, with a final extension step for 5 min at 72°C. PCR products were gel extracted and purified using the Wizard SV PCR and Gel Clean-Up system (Promega, Australia). DNA sequencing was performed with ABI PRISM BigDye terminator cycle sequencing Version 2.0 at the Australian Genome Research Facility (AGRF). For each haplotype, two resistant and two susceptible individuals were sequenced to confirm the haplotype.

Genotyping of HaChr16:6101072 polymorphism

A TaqMan SNP assay was designed based on the aligned sequences for susceptible and resistant strains at position HaChr16:6101072. The assay uses forward primer 5'-AACCAATATACTCATGGGTAGTAACTC-3' and the reverse primer 5'-AACCGCCGCATCTGCATT-3' with a dual-labelled probe, 5'-Fam - CGAAGAGGGTTACTATTCAA-3'- BHQ2 for the susceptible allele and probe 5'-Hex-

CGAAGAGGGTTACTATTTTA-3'-BHQ1 matching the resistance allele. All primers and probes were synthesized by Biosearch Technologies Inc. (Biosearch Technologies Inc, Novato USA).

PCR contained 2.5 µM forward primer and reverse primer, 0.5 µM susceptible probe and 1.0 µM resistant probe, in a MyFi™ Mix (Bioline, Australia) comprising a 25 µl reaction volume. Real-time PCR was performed in an ABI7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with 10 min at 95°C followed by 45 cycles of 15 seconds at 95°C and 1 min at 60°C.

Results

Indoxacarb resistance phenotype segregation in backcrosses

GY7-39 was crossed with New GR and 68%-79.9% of progeny survived a discriminating dose. For F_2 progeny, the survival rate dropped to about 45% (40.5% to 47.2) and by generation F_6, the F_6 progeny survival continued to be stable at about 43% (Table 1). Previous study data indicated the GY7-39 survival at the discriminating dose of indoxacarb is 78.3% indoxacarb and that resistance was inherited as a partially dominant trait[23]. We expect an F1 survival rate of F1 between 50 to 100% , and an F2 and F6 rate <50%.

[***Insert Table 1***]

Sequencing and identification of informative tags

We obtained 258,100,460 reads from 95 DNA samples. These sequence reads start with the barcode + *Pst*I cut site and do not contain any N bases. The mean number of reads per sample is about 2.7 million (sd 1,474,376) (Supplement Figure S1). The TASSEL GBS pipeline identified 189,697 unique sequence tags. 115,848 unique sequence tags mapped to the reference genome sequence. The read count for each sequence tag ranged from 1 to 87,248. In total, 22,679 tags were obtained with normalised read count > 10 reads/million sequence reads and 13,202 tags were mapped to the *H. armigera* genome. These mapped tags were distributed across 31 chromosomes on the *H. armigera* genome with 8,697 unique locations (Figure 2).

[Insert Figure 2]

Figure 2 Distribution of tags on the *Helicoverpa armigera* chromosomes produced by Phenogram [39].

Genome-wide association for indoxacarb resistance

We identified 131 tags significantly associated with indoxacarb resistance using a genome-wide threshold of $p < 7.57 \times 10^{-8}$. Importantly, 51 tags showed high association producing a *p*-value of 3.28×10^{-11}. These tags are present in all 14 resistant individuals and absent from the 8 susceptible individuals (Table 2). This is equivalent to a completed linkage to hypothetical resistance indoR. Forty-two out of 53 tags are located in a 2.6 MB region on chromosome 16 (Figure 3). In addition, we also performed an association analysis with the tags which do not have a unique mapped position or no mapped position. We found 32 tags that are highly associated with indoxacarb resistance (Table 3) and more than 50% of those tags (17/32) have a map position in the 2.6 Mb region but eleven tags did not map to the *H. armigera* genome.

[Insert Table 2]

[Insert Figure 2]

[Insert Table 3]

Figure 3 Manhattan plot showing genome-wide *p*-values of association on chromosome 16. The y-axis shows the −log10 *p* values of SNP, and the x-axis shows their chromosomal positions. The horizontal red line represents the thresholds of $p = 10^{-10}$. Green dots show tags that exceed the genome-wide threshold of $p < 7.57 \times 10^{-8}$ located in the 3.6 MB region

Fine mapping of the indoxacarb resistance gene

We treated each Tag sequence as a haplotype and grouped tags into a haplotype if the tag-sequence overlapped on the chromosome or the complementary strand. We included only tags with a unique map position. We also excluded the tags which were only presented in susceptible New GR individuals, but absent from resistant GY7-39 (RP.64799, RP.46673, RP.188639, RP.9281 and RP.145682), as we cannot detect the segregation of the haplotypes in F_6 progeny. In total, fifteen haplotypes were identified on chromosome 16 spanning 2.6 Mb (5922895 - 8558542). All these haplotypes are represented in the 14 resistant GY7-39 individuals and co-segregated with F_6 resistance progeny (Table 4). In Family 1, 8 haplotypes in the region HaChr16:6294157-8558542 are 100% co-segregated with the resistance phenotype, which means all 35 F_6 resistant progeny carried these 8 haplotypes. At least 32 out 35 F_6 resistant progeny carried the haplotypes in the region HaChr16:5922778-6229552. In Family 2, only one haplotype (H3 HaChr16:6101017) was co-segregated with the resistant phenotype and all 38 F_6 resistant progeny carried this haplotype.

The first candidate gene region (Region 1) is between HaChr16:6083884-6023558 and the second candidate region (Region 2) is between HaChr16: 6294314-8558542. Table S1 lists all the genes in these two regions. There were only 15 genes in Region 1, but 171 genes in region 2.

Validation of the haplotype by Sanger sequencing

As shown in Figure 4, we confirm that these susceptible individuals have the same susceptible haplotype with allele ATTTT at position HaChr16: 6101017. The absence of tag sequence in strain New GR is due to it lacking the *Pst*I restriction cutting site.

[Insert Figure 4]

Figure 4 Haplotypes at HaChr16:6101072. 1 shows the susceptible haplotype lacks the *Pst*I restriction cutting site. 2 shows the resistance haplotype has one 'T' deletion**.**

Validation of the presence of resistant haplotype in the third family

Table 5 lists the genotypes of SNP at HaChr16:6101072 using a TaqMan assay for the F_0 GY7-39 resistant strain including the founder F_0 resistant male of the crosses, F_0 New GR susceptible strain and F_6 resistant progeny. These genotypes included third family (Family 3) which was not used for the GBS analysis but validation purpose. All F_0 GY7-39 individual were homozygous for allele ATTT and the susceptible individuals from the New GR strain were homozygous for ATTTT. The F_1 resistant males for all three families were heterozygous at this locus. F_6 resistance progeny were 50% heterozygous in Family 1, 90% heterozygous in Family 2 and 100% heterozygous in Family 3.

[Insert Table 5]

Discussion

Bioassay using the discriminating indoxacarb concentration verified resistance at each backcross and so confirmed the mode of inheritance in strain GY7-39 to be incompletely dominant [23]. The genetic model is also similar to indoxacarb resistance in oriental leafworm moth, *Spodoptera litur*a that was associated with cytochrome P450 monooxygenases [40]. Interestingly, the Indoxa-SEL strain of Sayyed

et al. (2008) was cross-resistant to spinosad and abamectin while the *H. armigera* indoxacarb resistant GY7-39 did not show cross-resistance to chlorantraniliprole, emamectin benzoate, fenvalerate, Cry1Ac, and Cry2Ab [23]. However, the mode of indoxacarb inheritance found in GY7-39 is different from the field collected housefly strain NYINDR (*Musca domestica*) that was found to be autosomal and recessive [41].

We carried out gene mapping of indoxacarb resistance in *H. armigera* by performing a genome-wide association using advanced backcross and genotype-by-sequencing. Here we used only resistant males to generate more recombinants when backcrossed to indoxacarb susceptible females. For that reason, after 5 backcrosses the F_6 progeny produced had less than 2% original founder genome. Given that the strain GY7-39 originated from a single female moth and was three times backcrossed to susceptible New GR before we started, the F_6 progeny had less than 0.1% of the founder genome. Therefore, we were able to map the indoxacarb resistance gene within 1 centimorgan. A similar approach has been used for mapping fenvalerate resistance in *H. armigera* [42] and pyrethroid resistance in the bed bug *Cimex lectularius* [43].

Our use of normalized tag count is the first attempt to take into account variation in the sequencing depth of each sample for obtaining accurate allele data from the GBS. GBS provides a valuable tool for reducing genome complexity and can simultaneously generate thousands of genetic markers for any organism with or without a known linkage map (Baxter et al. 2011; Elshire et al. 2011). The two most used program for GBS data analysis TASSEL's GBS version 2 pipeline (Glaubitz et al. 2014; Clark & Sacks 2016) and Stacks (Catchen et al. 2011; Catchen et al. 2013) use the tag count without considering any variation of the sequencing depth among the samples. Our normalized tag count is calculated as the normalized tag count /million sequence reads. Sequence read normalisation with sequencing depth is used for quantification of mRNA and microRNA expression in next-generation sequencing (Trapnell et al. 2012; Al-Husseini et al. 2016).

Our results suggest that the locus, which confers indoxacarb resistance, is located on Hachr16:6083884-8558542. There are two indoxacarb resistance loci in this region, IndoR1 and IndoR2. The first, IndoR1, is in the region HaChr16: 6083947-6023558 and the second, IndoR2 is located on HaChr16: 6229379-8558542. It appears that the strain GY7-39, F_0 individuals carry both IndoR1 an IndoR2 in a ~ 2.6 Mbp segment that has likely originated from single F_0 female moth (Table 4). In Family1, the majority of F_6 resistant progeny still carried IndoR1 and IndoR2. However in Family 2, the F_6 resistant progeny have lost IndoR2. This confirms the initial genetic analysis of indoxacarb resistance by Bird (2016) which suggest that one (or a few closely linked) loci conferred indoxacarb resistance in strain GY7-39. However, IndoR1 and R2 did not overlap with the known selective sweep identified on HaChr16:3944012-4404093 (90kb) by Anderson et al[44].

The mechanism of GY7-39 indoxacarb resistance studied here is consistent with cytochrome P450 monooxygenases determined from inhibition studies using piperonyl butoxide (Bird 2016). Mutations at the voltage-gated sodium channel (VGSC) were involved in indoxacarb and metaflumizone resistant in diamondback moth, *P. xylostella* [24] and in tomato leafminer, *T. absoluta* [25], Our results suggest that the VGSC could be excluded as the cause of indoxacarb resistant in GY7-39. The VGSC locus is located on HaChr15 in *H. armigera* genome. There is no tag near the VGSC locus associated with the indoxacarb resistance phenotype found in this study.

Further, the enzyme that catalyses the conversion of indoxacarb to a N-decarbomethoxyllated metabolite is clear, although there is still some suggestion of an esterase/amidase subclass of hydrolases[45, 46]. Interestingly, using a BLAST search (www.ncbi.nlm.nih.gov) we did not find any

genes in Region 1 that possess hydrolase activity (Table 1S). Also, the result of a PBO synergism test from other experiments suggested a likely oxidative mechanism[23]. We noticed HaOG207481 cytochrome b-c1 complex subunit 6, mitochondrial-like isoform X2 belongs to the group redox genes. HaOG207487 (gephyrin-like isoform X2) has the domain that is involved in biosynthesis of the molybdenum cofactor (MoCF), an essential cofactor of a diverse group of redox enzymes. There are 5 uncharacterized proteins in this region, the exact of mode of action of IndoR1 is yet to be determined.

Based on the current genome annotation, IndoR2 is located in a region HaChr16: 6229379-8558542-8558542 containing 171 genes. Here there is a cluster of nine P450 genes located on Hachr16, namely *CYP6AE14, CYP6AE20, CYP6AE19, CYP6AE18, CYP6AE17, CYP6AE16, CYP6AE11, CYP6AE15 and CYP6AE12,* spaning about 85kb (HaChr16: 8451146-8531656). Wang et al[47] used CRISPR gene editing to knockout the 9 *CYP6AE* gene cluster in *H. armigera* and found that the knock-out strain had increased susceptibility to esfenvalerate and indoxacarb. In particular, *CYP6AE17* and *CYP6AE18* are able to metabolise indoxacarb by recombinant P450s in vitro[48]. Therefore, we consider *CYP6AE17* and *CYP6AE18* to be the primary candidate detoxifiers for IndoR2, give their function and position in the candidate region. However, a number of gaps in the current *Helicoverpa* spp. genome assembly still remain, particularly on HaChr16, so we cannot exclude the possibility of a chromosomal rearrangement or gene duplication. like the case of CYP337B3, a chimeric P450 enzyme resulted from unequal crossing-over between two parental P450 genes CYP337B1 and CYP337B2[49]. GY7-39 had weak negative cross resistance to fenvalerate[23], while the knock-out the CYP6AE cluster increases susceptibility to fenvalerate. Given that CYP6AE17 can metabolize fenvalerate, it may suggest that IndoR2 is caused by some chromosome structure change affecting the CYP6AE17 function.

H. armigera have evolved mechanisms of insecticide resistance including detoxification and/or reduced sensitivity to insecticides via target site mutations [50, 51]. Increased detoxification can occur by gene duplication of carboxylesterase and increased transcription of cytochrome P450 monooxygenase and glutathione S-transferase (GST) [52, 53] or chimeric P450 enzymes [49]. Despite our relatively high-resolution mapping, the current study was not able to determine if GY7-39 indoxacarb resistance is caused by gene duplication or single nucleotide mutation. For this reason, it is important to pursue further studies such as this to understand the genetic resistance mechanism(s) associated with resistance. This understanding should improve insecticide resistance monitoring in field populations and ultimately support insecticide resistance management strategies.

Conclusion

By multi-generation backcrossing combined with genotype-by-sequencing, we mapped the indoxacarb resistance gene in *H. armigera* to chromosome 16 within a 2.6MB narrowed region (HaChr16:6083884- 6223558) and identified two indoxacarb resistance loci IndoR1 and IndoR2. These results provide essential background data for future genetic investigations including fine mapping of the indoxacarb resistance gene and the eventual development of an effective DNA based diagnostic. These strategies can also be applied to map insecticide resistance genes in other insect species that are lacking detailed linkage information.

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Table 1 Number of larvae surviving a discriminating concentration of indoxacarb (12 µg mL−1 indoxacarb in a diet-incorporation bioassay) in the GY7-39 strain and backcrosses F_1, F_2 and F_6

Family	No. tested	No. dead	% survival
GY7-39	60	13	78.3
F_1 (♂ GY7-39 x ♀ New GR)			
4	657	183	72.1
6	658	215	67.3
9	199	46	76.9
F_2 (♂ F_1 x ♀ New GR)			
4	2208	1032	46.7
6	2523	1022	40.5
9	1146	541	47.2
F_6 (♂ F_5 x ♀ New GR)			
4	2032	882	43.4
6	2878	1224	42.5
9	2155	951	44.1

Table 2 The mapped tags highly associated with indoxacarb resistant *Helicoverpa armigera* via GWAS analysis

HaChr	SNP	BP	Resistant (14)	Susceptible (8)	CHISQ	*P*
-	IndoR		14	0	44	3.28E-11
4	RP.64799	4940323	0	8	44	3.28E-11
8	RP.162794	7668981	14	0	44	3.28E-11
10	RP.46673	4747513	0	8	44	3.28E-11
10	RP.188639	4747738	0	8	44	3.28E-11
14	RP.44166	7656505	14	0	44	3.28E-11
15	RP.9281	8860119	0	8	44	3.28E-11
15	RP.145682	8860212	0	8	44	3.28E-11
15	RP.4874	12289216	14	0	44	3.28E-11
16	RP.1979	711728	14	0	44	3.28E-11
16	RP.45008	5922835	14	0	44	3.28E-11
16	RP.125314	6005065	14	0	44	3.28E-11
16	RP.11984	6005110	14	0	44	3.28E-11
16	RP.167836	6083947	14	0	44	3.28E-11
16	RP.33234	6101017	14	0	44	3.28E-11
16	RP.46883	6168317	14	0	44	3.28E-11
16	RP.150575	6223558	14	0	44	3.28E-11
16	RP.150582	6223558	14	0	44	3.28E-11
16	RP.81110	6223561	14	0	44	3.28E-11
16	RP.82642	6223561	14	0	44	3.28E-11
16	RP.82382	6224282	14	0	44	3.28E-11
16	RP.161613	6229379	14	0	44	3.28E-11
16	RP.121567	6229552	14	0	44	3.28E-11
16	RP.178869	6294314	14	0	44	3.28E-11
16	RP.56749	6294372	14	0	44	3.28E-11
16	RP.191053	7115384	14	0	44	3.28E-11
16	RP.86169	7134345	14	0	44	3.28E-11
16	RP.58565	7244562	14	0	44	3.28E-11
16	RP.98117	7261347	14	0	44	3.28E-11
16	RP.39327	7261598	14	0	44	3.28E-11
16	RP.7320	7271353	14	0	44	3.28E-11
16	RP.157494	7271400	14	0	44	3.28E-11
16	RP.30390	7331142	14	0	44	3.28E-11
16	RP.141663	7381512	14	0	44	3.28E-11
16	RP.163022	7561428	14	0	44	3.28E-11
16	RP.191902	7561588	14	0	44	3.28E-11
16	RP.156916	7792959	14	0	44	3.28E-11
16	RP.181582	7793204	14	0	44	3.28E-11
16	RP.21247	8257054	14	0	44	3.28E-11
16	RP.167913	8558542	14	0	44	3.28E-11
16	RP.180228	8708522	14	0	44	3.28E-11
16	RP.15275	8708559	14	0	44	3.28E-11
16	RP.156407	9528852	14	0	44	3.28E-11
16	RP.168132	9528995	14	0	44	3.28E-11

16	RP.12351	9531961	14	0	44	3.28E-11
16	RP.20867	9532004	14	0	44	3.28E-11
16	RP.167345	9773135	14	0	44	3.28E-11
16	RP.121821	9773182	14	0	44	3.28E-11
16	RP.2313	9802908	14	0	44	3.28E-11
16	RP.35847	9802965	14	0	44	3.28E-11
16	RP.39316	9802967	14	0	44	3.28E-11
20	RP.172235	64631	14	0	44	3.28E-11

HaChr: Chromosome
SNP: Tag_ID
BP: Start nucleotide position of the Tag
Resistant (14): Number of individuals has the tag out of 14 resistance individuals.
Susceptible (8): Number of individuals has the tag out 8 susceptible individuals.
CHISQ : The chi-squared statistic for this test (1 df)
P: The asymptotic significance value for this test

Table 3 The tags highly associated with indoxacarb resistance in *Helicoverpa armigera* by GWAS analysis without unique map position

HaChr	SNP	BP	Resistance(14)	Susceptible(8)	CHISQ	*P*
17	RP.98630	1754859	14	0	44	3.28E-11
16	RP.44906	6083791	14	0	44	3.28E-11
16	RP.19913	6101129	14	0	44	3.28E-11
16	RP.151138	6153872	14	0	44	3.28E-11
16	RP.8757	6153872	14	0	44	3.28E-11
16	RP.92529	7134117	14	0	44	3.28E-11
16	RP.98544	7244919	14	0	44	3.28E-11
16	RP.8623	7330999	14	0	44	3.28E-11
16	RP.189986	7334774	14	0	44	3.28E-11
16	RP.81428	7445302	14	0	44	3.28E-11
16	RP.150151	8549292	14	0	44	3.28E-11
16	RP.3695	9228121	14	0	44	3.28E-11
16	RP.43455	9228121	14	0	44	3.28E-11
16	RP.87273	9528789	14	0	44	3.28E-11
16	RP.108662	9580601	14	0	44	3.28E-11
16	RP.75328	9580632	14	0	44	3.28E-11
16	RP.182290	9803049	14	0	44	3.28E-11
16	RP.105600	9917226	14	0	44	3.28E-11
	RP.157141		14	0	44	3.28E-11
	RP.182154		14	0	44	3.28E-11
	RP.120995		14	0	44	3.28E-11
	RP.123153		14	0	44	3.28E-11
	RP.130697		14	0	44	3.28E-11
	RP.136766		14	0	44	3.28E-11
	RP.150772		14	0	44	3.28E-11
	RP.154900		14	0	44	3.28E-11
	RP.155945		14	0	44	3.28E-11
	RP.158188		14	0	44	3.28E-11
	RP.173450		14	0	44	3.28E-11
	RP.174048		14	0	44	3.28E-11
	RP.180820		14	0	44	3.28E-11
	RP.34381		14	0	44	3.28E-11

HaChr: Chromosome or scaffold number
SNP: Tag_ID
BP: Start nucleotide position of the Tag
Resistant (14): Number of individuals has the tag out of 14 resistance individuals.
Susceptible (8): Number of individuals has the tag out 8 susceptible individuals.
CHISQ : The chi-squared statistic for this test (1 df)
P: The asymptotic significance value for this test

Table 4 Candidate haplotype blocks for indoxacarb resistant *Helicoverpa armigera* on chromosome 16

No	Tags	Chr	Position	Sus (8)	Res (14)	Family 1 F_6 (35)	Family 2 F_6 (38)
H1	**RP45008**	**16**	**5922778-5922895**	**0**	**14**	**33**	**0**
H2	**RP.167836**	**16**	**6083855-6083947**	**0**	**14**	**33**	**0**
H3	**RP.33234**	**16**	**6101017-6101106**	**0**	**14**	**33**	**38**
	(IndoR1)		**6083855-6223558**				
H4	**RP.150582**	**16**	**6223558-6223640**	**0**	**14**	**34**	**0**
	(IndoR2)		**6223558-8558542**				
H5	**RP.82382**	**16**	**6224279-6224371**	**0**	**14**	**33**	**0**
H6	**RP.121567**	**16**	**6229379-6229552**	**0**	**14**	**32**	**0**
H7	**RP.56749**	**16**	**6294157-6294317**	**0**	**14**	**35**	**0**
H8	**RP.191053**	**16**	**7115296-7115384**	**0**	**14**	**34**	**0**
H9	**RP.98117**	**16**	**7261347-7261439**	**0**	**14**	**35**	**0**
H10	**RP.39327**	**16**	**7261506-7261598**	**0**	**14**	**35**	**0**
H11	**RP.7320**	**16**	**7271397-7271489**	**0**	**14**	**35**	**0**
H12	**RP.141663**	**16**	**7381511-7384575**	**0**	**14**	**35**	**0**
H13	**RP.163022**	**16**	**7561428-7561517**	**0**	**14**	**35**	
H15	**RP.181582**	**16**	**7793112-7793204**	**0**	**14**	**35**	**0**
H15	**RP.167913**	**16**	**8558467-8558542**	**0**	**14**	**35**	**0**

Chr: Chromosome number,
Sus: number of haplotype present in New GR strain (total of 8).
Res: number of the haplotype present in GY7-39 (total 14).
Family 1 F_6 (35): number of haplotype present in family 1 F_6 resistant progeny (total 35).
Family 2 F_6 (38): number of haplotype present in family 2 F_6 resistant progeny (total 38).

Table 5 Genotype HaChr16:6101072 in *Helicoverpa armigera* resistant and susceptible individuals

Sample	**Homozygote (ATTT)***	**Heterozygote (ATTT/ATTTT)***	**Homozygote (ATTTT/ATTTT)***
GY7-39 indoxacarb resistant[1]	6	0	0
New GR indoxacarb susceptible[2]	0	0	12
F_1 resistant male[3]	0	5	0
F_2 resistant progeny[4]	0	5	1
F_6 resistant progeny (Family 1)	0	5	5
F_6 resistant progeny (Family 2)	0	9	1
F_6 resistant progeny (Family 3)	0	10	0

*ATTT allele from GY7-39 and ATTTT allele from New GR.

[1] Including 3 F_0 founder males for Family 1, Family 2 and Family 3.

[2] Including 3 F_0 founder females for Family 1, Family 2 and Family 3.

[3] F_1 resistant males used for the subsequent backcrossing (2 for Family 1, 1 from Family 2 and 2 for Family 3).

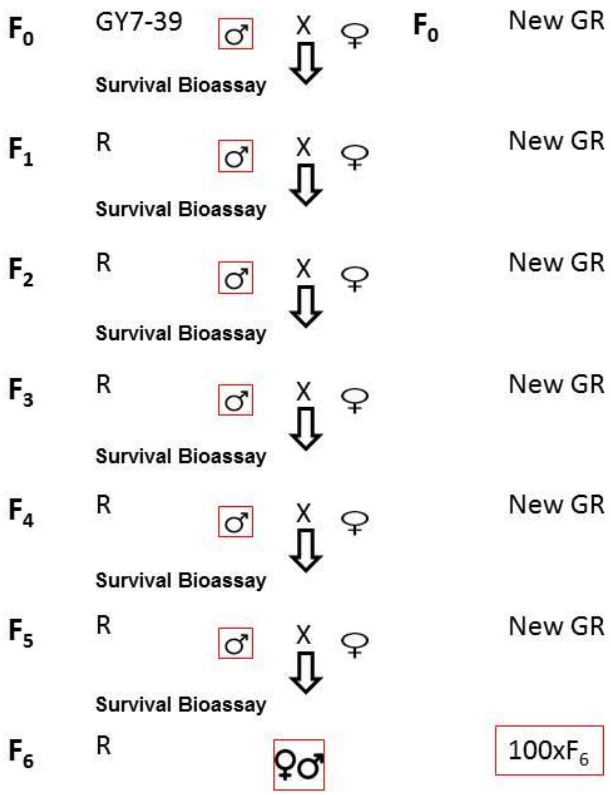
[4] F_2 resistant progeny (3 from Family 1 and 3 from Family 3).

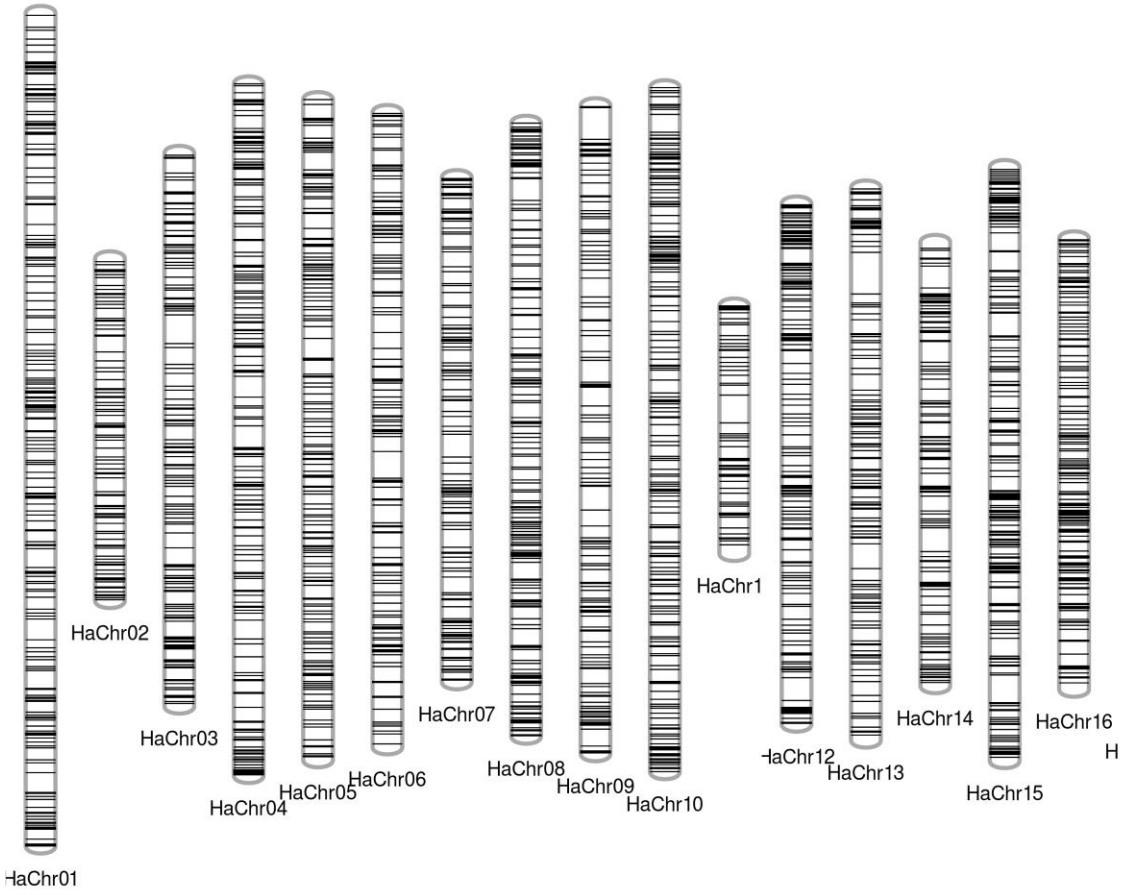
Figure 1 Schematic diagram of the advanced crossing for gene mapping. In F_0, the resistant male was crossed to susceptible female. In the following generations, only the resistant male was selected to cross back to susceptible females. In generation F_6, only the resistant individuals were collected for genotype-by-sequencing.

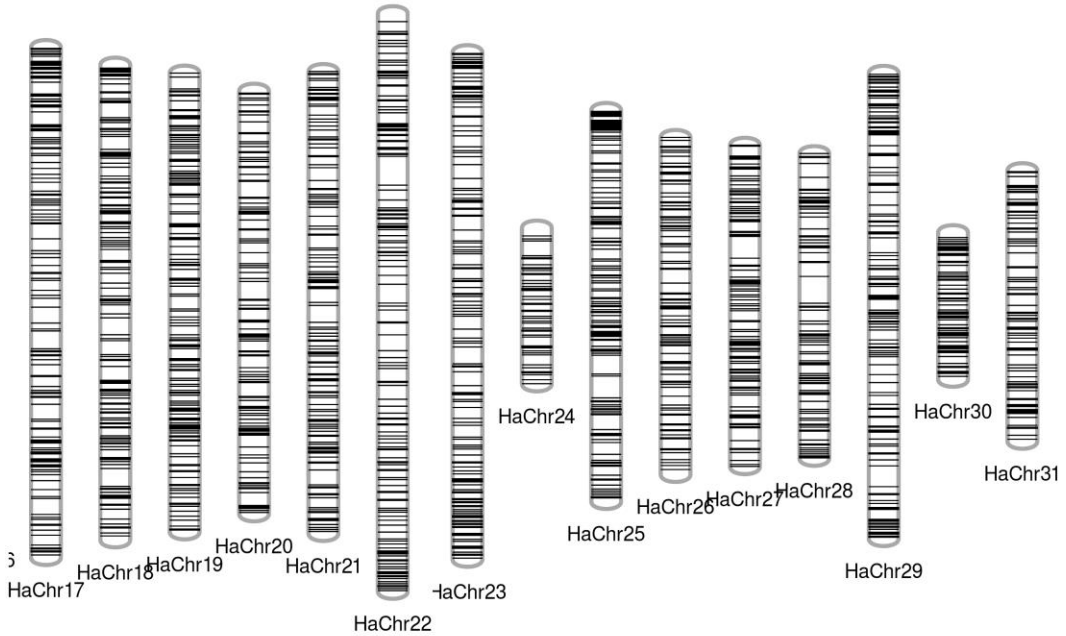
Figure 2 Distribution of tags on the *Helicoverpa armigera* chromosomes produced by Phenogram [39].

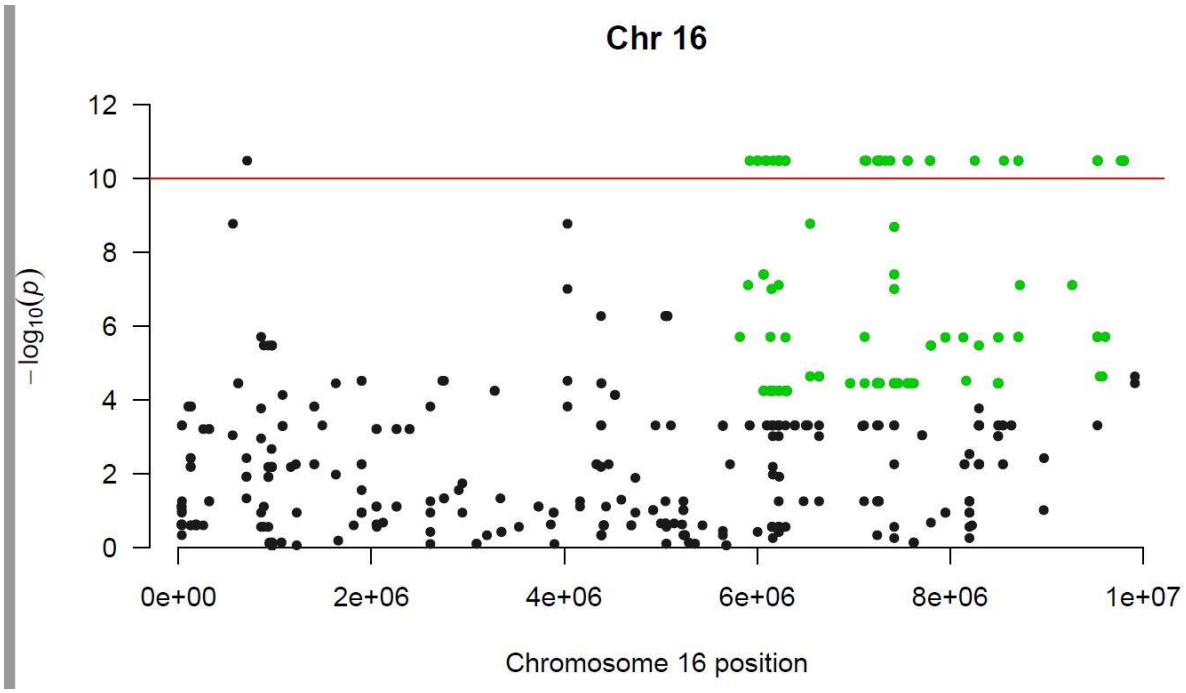
Figure 3 Manhattan plot showing genome-wide *p*-values of association on chromosome 16. The y-axis shows the −log10 *p* values of SNP, and the x-axis shows their chromosomal positions. The horizontal red line represents the thresholds of $p = 10^{-10}$. Green dots show tags that exceed the genome-wide threshold of $p < 7.57 \times 10^{-8}$ located in the 2.6 MB region

Figure 4 Haplotypes at HaChr16:6101072. 1 showed the susceptible haplotype lacks the PstI restriction cutting site. 2 showed the resistance haplotype has one 'T' deletion**.**







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H3_haplotype_R          -------------------------AAGAGCAAAGAAACGCGGTACAAAAGCCGAAAGAG
HaChr16                 CATTTAAACGAGTTCGACCAAAGCAAAGAGCAAAGAAACGCGGTACAAAAGCCGAAAGAG
H3_haplotype_S2         -------------------------AAGAGCAAAGAAACGCGGTACAAAAGCCGAAAGAG
                                                  ************************************
                                    1

H3_haplotype_R          TTTCAACATTCGGCTGGCTGCAGTATTGCGTATGGGCACTGATTCAGTGTTCAACCATGA
HaChr16                 TTTCAACATTCGGCTGGCG--GGTATTGCGTATGGGCACTGATTCAGTGTTCAACCATGA
H3_haplotype_S2         TTTCAACATTCGGCTGGCG--GGTATTGCGTATGGGCACTGATTCAGTGTTCAACCATGA
                        ******************   ************************************
                                    2
H3_haplotype_R          AATGGAATTATCAGAACATT-TGTCTCACCATTTCGAAATAAGACAACGACTGAATTGCT
HaChr16                 AATGGAATTATCAGAACATTTTGTCTCACCATTTCGAAATAAGACAACGACTGAATTGCT
H3_haplotype_S2         AATGGAATTATCAGAACATTTTGTCTCACCATTTCGAAATAAGACAACGACTGAATTGCT
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H3_haplotype_R          TTTC-TCTTACTCTTGATTCTACTTTGAATGAATGTCTTTATCAAAATCAAATTACATAG
HaChr16                 TTTCTCTTTACTCTTGATTCTACTTTGAATGAATGTTTTTATCAAAAGCAAGTTACATAG
H3_haplotype_S2         TTTCTCTTTACTCTTGATTCTACTTTGAATGAATGTTTTTATCAAAAGCAAGTTACATAG
                        ****   **************************** ********** *** ********

H3_haplotype_R          GTATACCAAAGCACTGCACTTCAAGACGTCCTGCAAA
HaChr16                 GTGCACCAAAGTACTGCACTTCAAGACGTCCTGCAAG
H3_haplotype_S2         GTGCACCAAAGTACTGCACTTCAAGACGTCCTGCAAA
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Research

Baseline Susceptibility of *Helicoverpa punctigera* (Lepidoptera: Noctuidae) to Indoxacarb, Emamectin Benzoate, and Chlorantraniliprole

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Abstract

Susceptibility in *Helicoverpa punctigera* (Wallengren) to emamectin benzoate, chlorantraniliprole, and indoxacarb was established from feeding assays on insecticide-incorporated artificial diet in the laboratory. The variation in dose responses was examined in *H. punctigera* field populations collected in eastern Australia between September 2013 and January 2016 and compared with a laboratory strain. Chlorantraniliprole was the most toxic insecticide with an average LC_{50} of 3.7 µg of insecticide per liter of diet (n = 12 field strains). The average LC_{50} for emamectin benzoate was 5.6 µg of insecticide per liter of diet (n = 11 field strains), whereas indoxacarb had the lowest toxicity with an average LC_{50} of 172 µg of insecticide per liter of diet (n = 14 field strains). Variation in susceptibility between field strains was low at 1.9-, 2.4-, and 2-fold for chlorantraniliprole, emamectin benzoate, and indoxacarb, respectively. Narrow ranges of intra-specific tolerance, high slopes, and goodness-of-fit to a probit binomial model suggested feeding bioassays using insecticide-incorporated diet were a more effective laboratory method for measuring dose responses of these insecticides in *H. punctigera* than traditional topical bioassays. We propose discriminating concentrations of 0.032, 0.026, and 4 µg of insecticide/ml of diet for chlorantraniliprole, emamectin benzoate, and indoxacarb, respectively, to monitor insecticide resistance in *H. punctigera*. Although the potential for *H. punctigera* to develop insecticide resistance is considered low based on historical records, recent changes in population dynamics of this species in eastern Australia may have increased the risk of resistance development.

Key words: insecticide, resistance, monitoring, management

The *Helicoverpa* genus in Australia comprises two important pests of field and horticultural crops: the native species *Helicoverpa punctigera* (Wallengren) and the cosmopolitan species *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). The larval feeding behavior of both species is targeted on the reproductive structures of host plants. The host range of *H. punctigera* is restricted to dicotyledonous plants, whereas *H. armigera* attacks both dicotyledonous and monocotyledonous plants (Zalucki et al. 1986). Another difference between the two species is the differential response to synthetic insecticide selection. *Helicoverpa armigera* has developed resistance to many insecticidal classes, including pyrethroids, carbamates, cyclodienes, and organophosphates (McCaffery 1998), spinosad (Gunning 2002), indoxacarb (Bird 2017), and toxins derived from *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) produced by transgenic cotton (Mahon et al. 2007). In contrast, there has only been a single incidence of metabolic resistance in field populations of *H. punctigera* to a synthetic insecticide reported (Gunning et al. 1997) and incipient resistance to toxins from *B. thuringiensis* (Downes et al. 2009, Mahon et al. 2012). Notwithstanding detectable resistance in *H. punctigera* to these insecticides, they continue to provide effective field control of this pest (Bird and Downes 2014, Downes et al. 2016).

Differences in population dynamics and migratory behavior are thought to be the most likely explanations for the differential resistance profile of the two species. Typically, *H. armigera* populations establish in cropping areas following a period of spring emergence of adults from locally overwintering pupae (Murray and Zalucki 1994). Despite experiencing similar selection pressures, a lack of winter diapause and regular immigration of *H. punctigera* populations from the vast Australian inland are presumed to contribute to the low incidence of phenotypic resistance in this species (Gregg et al. 1995).

In Australia, a prolonged period of drought between 2001 and 2009 reduced the prevalence of *H. punctigera* native hosts in inland

Australia (Gregg et al. 2016, Gregg 2017). The subsequent decline in *H. punctigera* populations migrating from the inland to cropping areas (Baker and Tann 2017) may have reduced the capacity for resistance to be diluted, which, in turn, could be a driver of resistance to insecticides used in the management of *H. punctigera* such as chlorantraniliprole, emamectin benzoate, and indoxacarb.

These insecticidal classes are important rotational options in *Helicoverpa* spp. control in a range of pulse and oilseed crops in Australia because of their selectivity in comparison to broad-spectrum insecticides (Torres and Bueno 2018) and low-resistance allele frequency (Bird et al. 2017). Resistance to selective insecticides was initially monitored in *Helicoverpa* spp. by topical application (Rossiter et al. 2008). Although these insecticides have some contact activity in Lepidoptera, ingestion is considered to be the primary route by which insects accumulate a lethal dose of insecticide (Lasota and Dybas 1991, Wing et al. 2004, Temple et al. 2009). Previous findings in *H. armigera* were that delivery of these insecticides by contact may not be the optimal method for measuring dose response of insecticides where a lethal dose of insecticide is accumulated more efficiently by ingestion (Bird 2015).

In the light of reported recent changes in population dynamics of this species in Australia, we readdress the potential for *H. punctigera* to develop insecticide resistance and assess the suitability of methods for discriminating between resistant and susceptible phenotypes as a preemptive step in resistance management in this species. The objectives of this study were to 1) evaluate insecticide-incorporated diet feeding bioassay method for determining the toxicity of the selective insecticides to *H. punctigera* as an alternative to the traditional topical method of bioassay; 2) accumulate baseline susceptibility data to determine the range of intra-specific tolerance in field populations of *H. punctigera* collected across cropping areas of eastern Australia; 3) use the baseline data generated from these bioassays to establish discriminating concentrations for emamectin benzoate, chlorantraniliprole, and indoxacarb; and 4) compare susceptibility of *H. punctigera* and *H. armigera* to these insecticides.

Materials and Methods

Insect Strains

A laboratory-susceptible strain of *H. punctigera*, designated LHP, was used to check for consistency of bioassay results during this study. This strain was originally established from a population collected from uncultivated hosts in southwest Queensland in the spring of 1999.

Field populations of *H. punctigera* were sampled from major cropping areas located across New South Wales and in southern and central Queensland, Australia. They were collected as eggs and larvae between September 2013 and January 2016 from cotton, pigeon pea, and a range of pulse crops; a minimum of 50 field-collected individuals constituted each geographically distinct strain. Each strain of *H. punctigera* was tested within two generations of establishment in the laboratory. Rearing methods were the same as described for *H. armigera* in Bird (2015). Strains were reared under laboratory conditions of 25 ± 2°C with a photoperiod of 14:10 (L:D) h and ambient RH.

Insect Bioassays

The dose responses of laboratory and field strains were determined from bioassays on artificial diet into which formulated insecticide was incorporated, as described in Bird (2015). This involved addition of twofold serial dilutions of insecticide to artificial diet which was dispensed into 45-well bioassays trays (Tacca Plastics, Sydney, NSW, Australia). The dose range for each insecticide spanned six or seven concentrations, expressed as micrograms per liter of diet, hereafter referred to as microgram per liter. Larvae reared on untreated diet were then introduced to trays containing bioassay diet at the late second or early third instar (one larva per well) and covered with heat-sealed, perforated lids. Each bioassay was performed in triplicate with individual treatments (insecticide concentrations) in replicates consisting of a minimum of 20 individuals; untreated diet was used as the control.

Each insecticide was tested on nonsynchronous cohorts of laboratory LHP strain on two occasions between November 2013 and December 2014. As there were no significant differences between cohorts for any of the insecticides tested, the bioassay results were pooled to generate an average LC_{50} for the LHP strain.

All bioassays were maintained under the same conditions used for insect rearing and were assessed for mortality at 7 d. Exposure of larvae to insecticide-incorporated diet for 7 d was found to be the optimal time for assessment of mortality because this provided a high level of discrimination between larvae that survived and larvae that were killed at each insecticide concentration, hence ensuring mortality would not be underestimated if insects recovered from initial knockdown effects of the insecticide. The criteria for mortality included one or more of the following: larvae unable to perform coordinated movement when prodded; paralysis of prolegs; larvae very slow to right themselves (time exceeding 3 s).

Insecticides

Commercial insecticide formulations were used in all bioassays: indoxacarb (Steward EC [15% active ingredient], Du Pont Australia Ltd., Macquarie Park, NSW, Australia), emamectin benzoate (Affirm [1.9% active ingredient], Syngenta Australia Pty. Ltd., Macquarie Park, NSW, Australia), and chlorantraniliprole (Altacor [35% active ingredient], Du Pont Australia Ltd.).

Data Analysis

The dose responses of larvae were corrected for control mortality using the formula of Abbott (1925). Probit regressions including slope, LC_{50}, $LC_{99.9}$, and associated 95% fiducial limits were calculated using software developed by Barchia (2001). Toxicity ratio for field strains was calculated by dividing the LC_{50} of each field population by the LC_{50} of the laboratory strain LHP (pooled result from bioassays of two nonsynchronous cohorts). Significant differences ($P < 0.05$) between LC_{50} values were determined by the lethal concentration ratio test, where if the 95% confidence interval includes 1, then the difference between LC_{50}s was not significant (Wheeler et al. 2006).

Results

Toxicity of Insecticides in Laboratory Strains

Chlorantraniliprole had the highest toxicity when tested by diet incorporation bioassay (LC_{50} = 4.2 µg/l; Table 1). Emamectin benzoate had a similar level of toxicity to the laboratory strain using this method of insecticide delivery (LC_{50} = 4.8 µg/l; Table 2), and indoxacarb had the lowest toxicity (LC_{50} = 147.2 µg/l; Table 3). There was a goodness-of-fit of the response in diet incorporation bioassays to the probit binomial model for emamectin benzoate ($P = 0.9976$) and indoxacarb ($P = 0.0681$), whereas there was a slight deviation from the model for chlorantraniliprole ($P = 0.0347$).

Table 1. Bioassay on 12 field strains and one laboratory-susceptible strain of *Helicoverpa punctigera* tested as late second/early third instars on diet-incorporated chlorantraniliprole (350 g/kg) and assessed for mortality at 7 d

Collection data							Fit of probit line			% Mortality (n)[c]	
Origin of field strains (G[a])	Host	Collection date	LC_{50} (µg/l diet)	(95% FL)	$LC_{99.9}$ (µg/l diet)	Toxicity ratio[b]	Slope ± SE	χ^2 (df)	P	15.6 µg/l diet	31.2 µg/l diet
Emerald QLD (F_1)	Chickpea	Sept. 2016	2.9	(2.56–3.52)	13.0	0.7	4.7 ± 0.6	12.87 (9)	0.1686	100 (60)	100 (59)
Breeza NSW (F_1)	Canola	Sept. 2013	2.9	(2.46–3.35)	28.7	0.7	3.1 ± 0.4	17.35 (10)	0.0670	—	—
Goondiwindi QLD (F_2)	Pigeon pea	Jan. 2016	3.0	(2.67–3.42)	15.3	0.7	4.4 ± 0.5	4.37 (9)	0.8854	100 (60)	100 (59)
Kingaroy QLD (F_1)	Cotton	Dec. 2015	3.2	(2.83–3.68)	19.4	0.8	4.0 ± 0.5	6.42 (9)	0.6973	100 (60)	100 (60)
Mungindi NSW (F_1)	Cotton	Dec. 2015	3.6	(3.16–4.21)	24.7	0.9	3.7 ± 0.4	13.76 (12)	0.3163	96.7 (60)	100 (60)
Griffith NSW (F_1)	Cotton	Dec. 2015	3.8	(3.42–4.23)	14.0	0.9	5.5 ± 0.6	5.11 (9)	0.8246	100 (60)	100 (60)
St George QLD (F_1)	Cotton	Nov. 2015	3.8	(2.82–4.91)	31.3	0.9	3.4 ± 0.6	18.10 (8)	0.0205	98.3 (60)	100 (60)
Emerald QLD (F_1)	Cotton	Nov. 2015	3.9	(3.48–4.44)	20.7	0.9	4.3 ± 0.5	8.40 (8)	0.3954	96.7 (60)	100 (60)
Wee Waa NSW (F_2)	Cotton	Jan. 2014	4.0	(3.53–4.44)	19.1	1.0	4.6 ± 0.5	5.15 (10)	0.8809	100 (60)	100 (60)
Goondiwindi QLD (F_1)	Cotton	Dec. 2015	4.1	(3.34–4.95)	20.6	1.0	4.4 ± 0.7	19.69 (10)	0.0323	100 (60)	100 (60)
Trangie NSW (F_2)	Cotton	Jan. 2016	4.2	(3.66–4.79)	32.3	1.0	3.5 ± 0.4	4.03 (11)	0.9690	98.3 (60)	100 (60)
Bellata NSW (F_2)	Pigeon pea	Jan. 2016	5.6	(5.02–6.27)	24.8	1.3	4.8 ± 0.5	6.18 (11)	0.8611	98.3 (60)	100 (60)
Pooled			3.7	(3.46–3.92)	22.8		3.9 ± 0.2			98.9 (660)	100 (658)
Laboratory strain LHP			4.2	(3.72–4.71)	26.4		3.9 ± 0.4	36.73 (23)	0.0347	100 (120)	100 (120)

FL (fiducial limit).

[a]Generation tested.

[b]Toxicity ratio = LC_{50} of field population/LC_{50} of LHP strain (average of two nonsynchronous LHP cohorts).

[c]Mortality at highest concentration tested.

Table 2. Bioassay on 11 field strains and one laboratory-susceptible strain of *Helicoverpa punctigera* tested as late second/early third instars on diet-incorporated emamectin benzoate (19 g/l) and assessed for mortality at 7 d

Collection data							Fit of probit line			% Mortality (n)[c]	
Origin of field strains (G[a])	Host	Collection date	LC_{50} (µg/l diet)	(95% FL)	$LC_{99.9}$ (µg/l diet)	Toxicity ratio[b]	Slope ± SE	χ^2 (df)	P	11.9 µg/l diet	23.7 µg/l diet
Bellata NSW (F_2)	Pigeon pea	Jan. 2016	3.6	(3.20–3.95)	13.3	0.7	5.1 ± 0.6	12.22 (10)	0.2706	100 (60)	100 (60)
Trangie NSW (F_2)	Cotton	Jan. 2016	3.7	(3.32–4.14)	16.0	0.8	4.9 ± 0.6	17.05 (11)	0.1064	98.3 (60)	100 (60)
Wee Waa NSW (F_1)	Chickpea	Oct. 2013	5.2	(4.80–5.69)	11.9	0.9	8.7 ± 1.4	4.89 (8)	0.7693	100 (60)	100 (60)
Emerald QLD (F_1)	Chickpea	Sept. 2016	5.5	(5.03–6.04)	14.6	1.2	7.3 ± 1.0	2.72 (7)	0.9096	100 (60)	100 (60)
Goondiwindi QLD (F_2)	Pigeon pea	Jan. 2016	5.6	(5.07–6.22)	19.2	1.2	5.8 ± 0.7	13.90 (9)	0.1259	95.0 (60)	100 (60)
St George QLD (F_1)	Cotton	Nov. 2015	5.7	(5.15–6.24)	16.7	1.2	6.6 ± 0.8	8.38 (9)	0.4964	100 (59)	100 (60)
Griffith NSW (F_1)	Cotton	Dec. 2015	5.8	(5.24–6.40)	18.6	1.2	6.1 ± 0.7	5.20 (9)	0.8165	98.3 (60)	100 (60)
Wee Waa NSW (F_2)	Cotton	Jan. 2014	5.9	(5.31–6.65)	26.1	1.2	4.8 ± 0.6	12.04 (8)	0.1494	93.3 (60)	100 (60)
Kingaroy QLD (F_1)	Cotton	Dec. 2015	6.4	(5.77–7.11)	23.3	1.3	5.5 ± 0.6	12.53 (11)	0.3252	96.7 (60)	100 (60)
Breeza NSW (F_1)	Canola	Sept. 2013	8.5	(7.85–9.25)	16.9	1.8	10.4 ± 1.2	5.89 (7)	0.5527	93.3 (60)	100 (60)
Moree NSW (F_1)	Chickpea/faba bean	Sept. 2013	8.7	(7.99–9.41)	16.5	1.8	11.1 ± 1.3	1.65 (7)	0.9767	93.3 (60)	100 (60)
Pooled			5.6	(5.04–6.25)	23.0		5.0 ± 0.5			97.1 (659)	100 (660)
Laboratory strain LHP			4.8	(4.47–5.06)	12.0		7.7 ± 0.7	6.67 (20)	0.9976	100 (120)	100 (120)

FL (fiducial limit).

[a]Generation tested.

[b]Toxicity ratio = LC_{50} of field population/LC_{50} of LHP strain (average of two nonsynchronous LHP cohorts).

[c]Mortality at highest concentration tested.

Table 3. Bioassay on 14 field strains and one laboratory-susceptible strain of *Helicoverpa punctigera* tested as late second/early third instars on diet-incorporated indoxacarb (150 g/l) and assessed for mortality at 7 d

Collection data							Fit of probit line			% Mortality (n)[c]	
Origin of field strains (G[a])	Host	Collection date	LC_{50} (µg/l diet)	(95% FL)	$LC_{99.9}$ (µg/L diet)	Toxicity ratio[b]	Slope ± SE	χ^2 (df)	P	1,500 µg/l diet	3,000 µg/l diet
Bellata NSW (F_2)	Pigeon pea	Jan. 2016	133.2	(114.6–153.9)	1,560	0.9	2.9 ± 0.3	12.33 (13)	0.5008	100 (60)	100 (60)
Goondiwindi QLD (F_1)	Cotton	Dec. 2015	135.4	(109.6–167.5)	935	0.9	3.7 ± 0.6	19.70 (10)	0.0322	100 (60)	100 (60)
Emerald QLD (F_1)	Cotton	Nov. 2015	142.5	(124.0–163.2)	1,306	1.0	3.2 ± 0.3	11.48 (13)	0.5707	98.3 (60)	100 (60)
Wee Waa NSW (F_2)	Cotton	Jan. 2014	148.2	(127.6–171.5)	1,823	1.0	2.8 ± 0.3	23.21 (14)	0.0569	100 (59)	100 (60)
Breeza NSW (F_1)	Canola	Sept. 2013	148.7	(110.8–195.8)	1,083	1.0	3.6 ± 0.7	34.83 (11)	0.0003	100 (60)	100 (60)
Kingaroy QLD (F_1)	Cotton	Dec. 2015	148.8	(127.5–172.6)	2,032	1.0	2.7 ± 0.2	13.83 (16)	0.6114	100 (60)	100 (60)
St George QLD (F_1)	Cotton	Nov. 2015	152.2	(134.6–172.5)	946	1.0	3.9 ± 0.4	12.48 (11)	0.3287	100 (60)	100 (60)
Goondiwindi QLD (F_2)	Pigeon pea	Jan. 2016	153.3	(130. 5–178.9)	2,396	1.0	2.6 ± 0.2	8.31 (15)	0.9108	100 (59)	100 (60)
Mungindi NSW (F_1)	Cotton	Dec. 2015	163.9	(121.4–224.1)	1,013	1.1	3.9 ± 0.8	48.85 (12)	0.0001	100 (60)	100 (60)
Emerald QLD (F_1)	Chickpea	Sept. 2016	193.0	(168. 1–221.5)	1,885	1.3	3.1 ± 0.3	17.75 (15)	0.2760	100 (60)	100 (60)
Trangie NSW (F_2)	Cotton	Jan. 2016	214.1	(184.2–248.8)	3,008	1.5	2.7 ± 0.2	16.11 (15)	0.3748	100 (59)	100 (60)
Griffith NSW (F_1)	Cotton	Dec. 2015	220.8	(189.8–256.9)	3,229	1.5	2.7 ± 0.2	17.73 (15)	0.2771	98.3 (60)	100 (60)
Moree NSW (F_1)	Chickpea/faba bean	Sept. 2013	243.9	(212.0–280.8)	2,585	1.7	3.0 ± 0.3	21.51 (15)	0.1213	96.7 (60)	100 (60)
Wee Waa NSW (F_1)	Chickpea	Oct. 2013	248.7	(213.3–289.6)	3,991	1.9	2.6 ± 0.2	18.43 (17)	0.3622	96.7 (60)	100 (60)
Pooled			172.2	(160.6–184.6)	2,159		2.8 ± 0.1			99.3 (837)	100 (840)
Laboratory strain LHP			147.2	(135.3–160.1)	825		4.1 ± 0.3	33.80 (23)	0.0681	99.2 (120)	100 (120)

FL (fiducial limit).

[a]Generation tested.

[b]Toxicity ratio = LC_{50} of field population/LC_{50} of LHP strain (average of two nonsynchronous LHP cohorts).

[c]Mortality at highest concentrations tested.

Toxicity of Insecticides in Field Strains

There was a narrow variation in response to chlorantraniliprole between the 12 field strains of *H. punctigera* tested (1.9-fold) with LC_{50} values ranging from 2.9 to 5.6 µg/l (Table 1). The dose responses to this insecticide were accompanied by high slopes (3.9 ± 0.2 from the pooled data of field strains). High susceptibility to chlorantraniliprole was indicated by low toxicity ratios ranging between 0.7 and 1.3. The most tolerant strains included a population collected from pigeon pea at Bellata (NSW) was with an LC_{50} value of 5.6 µg/l and a strain from St George (QLD) which had the highest $LC_{99.9}$ of 31.3 µg/l (Table 1).

The response *H. punctigera* field populations to emamectin benzoate also showed a narrow range of intra-specific variation between the 11 field populations tested (2.4-fold) with LC_{50} values ranging from 3.6 to 8.7 µg/l (Table 2). The pooled slope value from emamectin benzoate bioassays of field strains was 5.0 ± 0.5. Toxicity ratios, ranging between 0.7 and 1.8, indicate high susceptibility of field populations to this insecticide. A population established from chickpea and faba bean fields at Moree (NSW) had the highest level of tolerance with an LC_{50} value of 8.7 µg/l, whereas a strain from originating from cotton fields at Wee Waa (NSW) had the highest $LC_{99.9}$ value of 26.1 µg/l (Table 2).

The response of *H. punctigera* to indoxacarb showed a narrow (twofold) intra-specific variation in the 14 field strains tested, with LC_{50} values ranging from 133.2 to 248.7 µg/l (Table 3). High susceptibility of field strains was shown by low toxicity ratios ranging between 0.9 and 1.9. The least sensitive strains had $LC_{99.9}$ values > 3,000 µg/l. In the case of a strain established from chickpea fields near Wee Waa (NSW), the $LC_{99.9}$ was 3,991 µg/l (Table 3).

General goodness-of-fit to the probit binomial model was indicated by a significant deviation from the model in only 2 of the 12 field strains tested with chlorantraniliprole (St George F_1 cotton, $P = 0.0205$; Goondiwindi F_1 cotton $P = 0.0323$) and the laboratory strain LHP ($P = 0.0347$; Table 1), and three of the 14 field strains tested with indoxacarb (Goondiwindi F_1 cotton $P = 0.0322$; Breeza F_1 canola $P = 0.0003$; Mungindi F_1 cotton $P < 0.0001$; Table 3). The dose response of emamectin benzoate demonstrated a goodness-of-fit to the probit model in all 11 strains tested (Table 2).

Establishment of Discriminating Doses

To validate the suitability of candidate diagnostic concentrations for use in resistance monitoring programs, strains were assessed at two doses at the extreme upper end of the range expected to cause 90–100% mortality. For chlorantraniliprole, mortality at the two highest concentrations tested of 15.6 and 31.2 µg/l was 98.9 and 100%, respectively (Table 1). The empirical result from bioassays was consistent with the predicted $LC_{99.9}$ response of the most highly tolerant strains, and only slightly exceeded the highest dose tested in two strains collected; one from cotton at Trangie (NSW) and the other from St George (QLD; Table 1). Therefore, the recommended diagnostic dose for monitoring resistance to chlorantraniliprole in *H. punctigera* is 0.032 µg/ml. The two highest concentrations of emamectin benzoate tested were 11.9 and 23.7 µg/l and resulted in 97.1 and 100% mortality, respectively (Table 2). Predicted $LC_{99.9}$ values exceeded the highest dose tested in two strains originating from chickpea and cotton fields at locations near Wee Waa (NSW; Table 2). Based on both the empirical and theoretical data, the recommended diagnostic dose for monitoring resistance to emamectin benzoate in *H. punctigera* is 0.026 µg/ml. The highest concentrations of indoxacarb tested were 1,500 and 3,000 µg/l, which produced 99.3 and 100%, respectively. The most tolerant strains exceeded the highest concentration tested in three cases from NSW: Trangie cotton, Griffith cotton, and Wee Waa chickpeas (Table 3). Based on the $LC_{99.9}$ estimates of mortality in the most tolerant strains and, considering empirical mortality, the recommended discriminating dose for indoxacarb is 4 µg/ml.

Discussion

The present study demonstrates that when insecticides were administered orally to larvae from a laboratory strain of *H. punctigera* in insecticide-incorporated diet, the toxicity of chlorantraniliprole and emamectin benzoate was similar, whereas indoxacarb was approximately 30-fold less toxic. However, when insecticides were administered by contact in topical bioassays to the same laboratory strain of *H. punctigera* emamectin benzoate had an LC_{50} of 6.96 µg/ml (L.J.B., unpublished data), which was 21-fold less toxic than chlorantraniliprole (LC_{50} 0.33 µg/ml; Bird and Downes 2014) and 5-fold less toxic than indoxacarb (LC_{50} 1.46 µg/ml; Bird and Downes 2014). Emamectin benzoate and analogues of this compound such as abamectin were also shown to have relatively weak contact activity compared with stomach toxicity in other noctuid larvae (Anderson et al. 1986, Venkateswari et al. 2008, Bengochea et al. 2014) possibly due to poor cuticular permeability to these insecticides in some species (Deecher et al. 1990).

The finding that indoxacarb, chlorantraniliprole, and emamectin benzoate were 10-, 80-, and 1,450-fold, respectively, more toxic by ingestion than by contact in the laboratory *H. punctigera* strain, combined with high slope values and goodness-of-fit to a probit model, suggests delivery of these insecticides by ingestion using a diet incorporation bioassay technique could increase discrimination between susceptible and resistant genotypes and provide a more suitable method for monitoring insecticide resistance in these species compared with traditional topical bioassays used for determining toxicity of broad-spectrum insecticides that are primarily active by contact.

A comparison of this study on *H. punctigera* with a previous study on *H. armigera* by Bird (2015) using the same method of bioassay to test activity of chlorantraniliprole and emamectin benzoate showed that these insecticides had 6.2- and 1.7-fold higher toxicity, respectively, in *H. punctigera* compared with *H. armigera* field strains. Although field strains of *H. punctigera* were also more susceptible than field strains of *H. armigera* to indoxacarb, the response to indoxacarb in laboratory strains of *H. armigera* and *H. punctigera* was similar, suggesting that field strains of *H. punctigera* may have acquired additional metabolic pathways that increase bioactivation of the parent compound to the active N-decarbomethoxyllated metabolite. Similar inter-specific variability was also found with broad-spectrum insecticides by Forrester et al. (1993). Although fenvalerate was found to be twofold more efficacious on *H. punctigera* than on *H. armigera*, endosulfan had similar toxicity in both species. Variability in efficacy of *B. thuringiensis* toxins between species was also found by Bird and Akhurst (2007). Neonates from laboratory and field strains of *H. punctigera* were significantly more sensitive to Cry1Ac in mortality bioassays than *H. armigera*. In contrast, field strains of *H. armigera* were significantly more sensitive to Cry2Ab than field strains of *H. punctigera*.

The present study demonstrated that *H. punctigera* had high susceptibility to chlorantraniliprole, emamectin benzoate, and indoxacarb, based on comparisons with the laboratory strain, and narrow intra-specific variation between the dose responses of geographically diverse populations sampled from cotton growing areas of eastern

Australia. Low intra-specific variation in susceptibility to emamectin benzoate and indoxacarb was also found among populations of *H. armigera* sampled from similar regions of Australia (Bird 2015). Low variation in susceptibility to chlorantraniliprole was reported for geographically diverse strains of *H. armigera* (Bird 2015) and field populations of other noctuid species including *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) (Su et al. 2012) and *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Temple et al. 2009).

Forrester et al. (1993) reported narrow variation in the response of *H. punctigera* field strains to fenvalerate and endosulfan (1.9- and 2.6-fold, respectively). Similarly, Gunning and Easton (1994) found that the range of variation in susceptibility of geographically diverse populations of *H. punctigera* to fenvalerate, endosulfan, and DDT was narrow at 2-, 3.4-, and 1.9-fold, respectively. Bird and Akhurst (2007) also found small differences in the response of *H. punctigera* neonates to *B. thuringiensis* toxins Cry1Ac (3.2-fold) and Cry2Ab (3.5-fold) in surface treatment bioassays. However, a narrow range of susceptibility among intra-specific populations may not prevent populations responding to selection and developing resistance that results in the failure of insecticides to provide field control (Tabashnik 1994). For example, pyrethroid resistance was detected in field populations of *H. punctigera* at low frequencies (<5%) during periods of high usage of these products in consecutive years during the mid to late 1980s, presumably due to intensive selection of both larval and adult populations from insecticide use in the cotton industry (Forrester et al. 1993). In a subsequent study, pyrethroid resistance in *H. punctigera* was detected at elevated levels (17-fold) in a field population established from cotton fields in the Macquarie Valley in central NSW in 1994 (Gunning et al. 1997).

Findings from metabolic studies suggest that *H. punctigera* and *H. armigera* have a similar capacity to develop metabolic detoxification systems (Collins and Hooper 1984a; Gunning et al. 1994, 1997). This was further supported by laboratory selection of *H. punctigera*, which resulted in a strain with 10.5-fold resistance to fenvalerate and 17.4-fold resistance to deltamethrin (Forrester et al. 1993). Resistance in this strain was partially suppressible by the metabolic inhibitor piperonyl butoxide, suggesting the involvement of a metabolic resistance mechanism. Thus, differential levels of resistance between the two species may not have resulted from divergent physiology or biochemistry, and some other factor is likely to be involved. However, another comparative study of enzyme systems in larval midgut microsomes indicated *H. armigera* larvae had a higher binding affinity with DDT and permethrin and higher specific activity of cytochrome P450 compared with *H. punctigera*, suggesting a greater potential for *H. armigera* to develop resistance to insecticides (Collins and Hooper 1984b).

Notwithstanding the similar metabolic profiles in the two species, the induction of metabolic enzymes in *H. punctigera* may not necessarily be associated with specific functions associated with detoxification (Collins 1985). This is because elevated activity of enzymes commonly associated with resistance (such as esterases, mixed-function oxidases, or glutathione transferases) using model substrates are often used to infer the presence of causal mechanisms. This can be misleading because only specific enzymes within each class are involved in resistance to different insecticides in the same chemical group (Sawicki 1987). Results from the present study showed that indoxacarb was equally toxic to laboratory strains of both *H. armigera* and *H. punctigera*. This suggests that both species may have similar metabolic capacities for bioactivation of the parent compound, but this is not necessarily indicative of a differential ability to develop detoxification pathways involving specific enzyme systems.

The most common explanation for the nonpersistent nature of insecticide resistance in *H. punctigera* in the field is dispersal ecology associated with migratory behavior in this species (Forrester et al. 1993). In addition, *H. punctigera* has a complex diapause (Murray 1992) and cohorts that avoid exposure to insecticides through quiescence could be an important source of dilution for resistance. However, as noted in early work by Gunning and Easton (1994), changes in environment and habitat may influence the population ecology of *H. punctigera*, which could in turn favor resistance development in this species.

Helicoverpa punctigera is a highly migratory species and large populations frequently develop on native hosts in the normally arid regions of southwestern Queensland after summer–autumn rain (Zalucki et al. 1994, Gregg et al. 2016). The ephemeral nature of host plants stimulates migration from inland Australia, resulting in a large annual influx of moths into the cropping landscapes of eastern Australia, usually in early spring (Duffield and Steer 2006, Baker et al. 2011, Baker and Tann 2017). These immigrants are thought to mix with resident *H. punctigera* populations within cropping areas, thus diluting any resistance alleles that may have been selected for through exposure to insecticide (Forrester et al. 1993, Gregg et al. 1995).

In contrast, *H. armigera* is generally more abundant in regions where host plant availability is more predictable such as agricultural landscapes (Fitt 1989). Populations are characterized by a period of spring emergence of adults from overwintering pupae within cropping regions, and regular influxes of large populations from outside cropping areas where exposure to insecticides is minimal may not occur to any large extent. Therefore, any insecticide resistance that develops in *H. armigera* populations is more likely to persist locally if selection pressure remains high. However, there is evidence that the annual spring migration of *H. punctigera* from inland regions into cropping areas of eastern Australia has considerably decreased over the last two decades (Baker and Tann 2017). The millennium drought (2001–2009) severely affected the availability of *H. punctigera* native hosts in inland Australia (Gregg et al. 2016, Gregg 2017), and the impact on *H. punctigera* breeding habitat consequently reduced the number of moths migrating into eastern Australia, as shown by pheromone trapping (Baker and Tann 2017). Since the end of the millennium drought, irregular summer and autumn rainfall has fallen in parts of inland Australia, but the abundance of primary *H. punctigera* host plants has not yet recovered to predrought levels (Le Mottee 2015, Gregg 2017). Consequently, the size of the spring *H. punctigera* immigrant population originating from inland Australia remains low, and this could have serious implications for resistance management.

A first step toward preemptive management of resistance to pivotal selective insecticides used in the management of *H. punctigera* requires the determination of discriminating concentrations of insecticide for use in resistance monitoring. It is debatable whether standard laboratory-susceptible strains are appropriate reference points for determining changes in susceptibility in field populations over time (ffrench-Constant and Roush 1990). Of greater importance is the comparison with natural tolerance of field populations that are controlled by the field dose, rather than by reference to an unrepresentative response of a laboratory-susceptible strain (Sawicki 1987). Therefore, the determination of diagnostic concentrations of insecticide should be informed by the limits of tolerance from a range of geographically diverse field populations to ensure a realistic and field-relevant dose is assigned to discriminate between resistant and susceptible individuals. Using these principles, and basing our estimates on the response of strains sourced from a wide range

of cropping systems and geographical locations, we propose the use of concentrations of 0.032, 0.026, and 4 µg of insecticide/ml of diet for chlorantraniliprole, emamectin benzoate, and indoxacarb, respectively, to discriminate between resistant and susceptible *H. punctigera*.

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