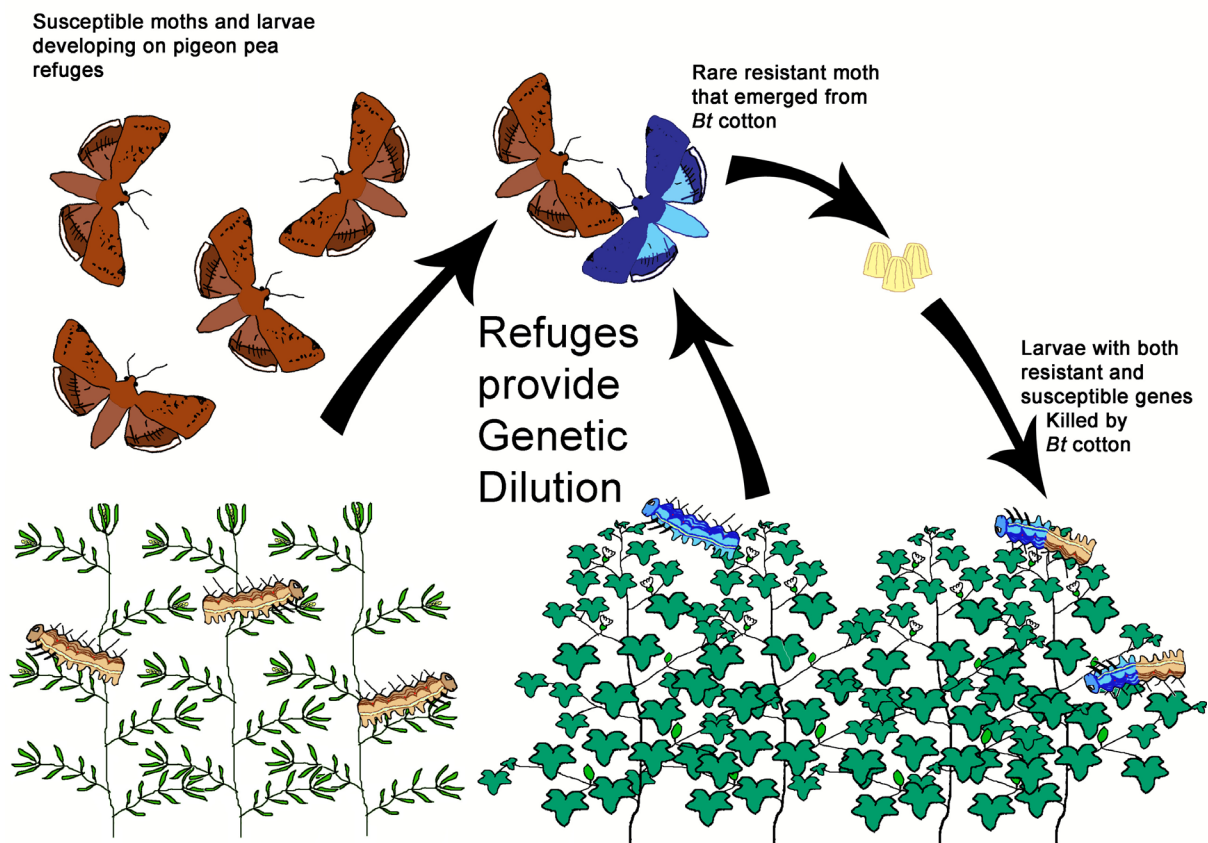




# FINAL REPORT 2018

For Public Release

## Managing Bt resistance and induced tolerance in Bollgard 3 using refuge crops



Dr. Mary E.A. Whitehouse

***Part 1 - Summary Details***

---

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

CRDC Project Number: CSE1601

---

**Project Title:** Managing Bt resistance and induced tolerance in Bollgard 3 using refuge crops

---

**Project Commencement Date:** 1/07/2015      **Project Completion Date:** 30/06/2018

CRDC Research Program: 2 Industry

---

***Part 2 – Contact Details***

---

**Administrator:** Jo Cain  
**Organisation:** CSIRO Agriculture and food  
**Postal Address:** Locked Bag 59, Narrabri NSW 2390  
**Ph:** 02 6799 1513      **Fax:** 02 6793 1186      **E-mail:** jo.cain@csiro.au

---

**Principal Researcher:** Mary Whitehouse  
**Organisation:** CSIRO Agriculture and food  
**Postal Address:** Locked Bag 59, Narrabri NSW 2390  
**Ph:** 02 6799 1538      **Fax:** 02 6793 1186      **E-mail:** mary.whitehouse@csiro.au

---

**Supervisor:** Sharon Downes  
**Organisation:** CSIRO Agriculture and food  
**Postal Address:** Locked Bag 59, Narrabri NSW 2390  
**Ph:** 02 6799 1576      **Fax:** 02 6793 1186      **E-mail:** Sharon.downes@csiro.au

---

**Signature of Research Provider Representative:** \_\_\_\_\_  \_\_\_\_\_

**Date Submitted:** \_\_\_\_\_ 30/04/2019 \_\_\_\_\_

## ***Part 3 – Final Report***

---

(The points below are to be used as a guideline when completing your final report.)

### ***Background***

#### **1. Outline the background to the project**

The objectives of this project were addressed by dividing the work into three sections, which is how the Methods, Results, and Discussions will be presented. Consequently, the background of the project is also presented in three sections.

#### **Section 1. Comparing moth emergences between refuges and crops**

Bt cotton has enabled the cotton industry to reduce its use of insecticides and improve the lifestyles of cotton growers by producing a more reliable and sustainable crop through controlling the major pests, *Helicoverpa armigera* and *H. punctigera*. To maintain the efficacy of Bt cotton by limiting the development of resistance by *Helicoverpa* spp (here after “*Helicoverpa*”) to Bt toxins, the industry has in place a Resistance Management Plan (RMP).

Refuges are an important tool in the RMP. Their role is to produce enough moths so that the genetic contribution to the next generation of any moths emerging from Bt is strongly diluted. As such, they work as Genetic Diluters (Whitehouse et al 2017). The moths driving the genetic dilution can originate from refuges specifically planted to produce moths (structured refuges) or from other crops or vegetation not designed for this purpose (unstructured refuges).

The RMP for Bollgard II required growers to grow 10% of their cotton crop as a structured refuge. If they used pigeon pea as their structured refuge, this could be reduced to 5%, because it produces twice as many moths as cotton (Baker et al 2008).

However field tests in CSE 1304 revealed unexpectedly high numbers of *H. armigera* and *H. punctigera* moths emerging from Bollgard II cotton. When combined with their associated refuges metre for metre, between 4% to 22% of all moths emerging originated from Bollgard II cotton. However these moths were almost exclusively non-resistant (of the 433 tested, only 2 may have been carrying resistant genes to Cry2Ab, and both of these had originated from pigeon pea). Nevertheless, if these findings are extrapolated to the relative proportion of land planted in Bollgard II (90%/95%) compared to that planted in structured refuges (10%/5%), they indicate that from 2012 to 2015 about half the moths emerging from the Bollgard II cotton/refuge system in the Namoi could have originated from Bt cotton, putting pressure on the development of resistance (CSE 1304).

Bollgard 3 varieties (first grown commercially in 2016) introduced a new toxin, Vip3A, in order to reduce the resistance risk. After consultation, the TIMS committee accepted that the resistance risk was reduced, and allowed the refuge requirement for Bollgard 3 to be reduced to 5% of the cotton crop (or 2.5% if the refuge was pigeon pea).

However, the expression of Vip3A in cotton plants and the amount of resistance to Vip3A already in *Helicoverpa* populations was less than ideal (Mahon et al 2012) with an *r* frequency of 0.034 and 0.01 in *H. armigera* and *H. punctigera* respectively (Downes et al 2016). Consequently control of *Helicoverpa* via the original toxins in Bollgard II (Cry1Ac & Cry2Ab) remained crucial. Of these two toxins, Cry2Ab in Bt cotton has the greatest efficacy against *Helicoverpa*, but the level of resistance by *Helicoverpa* to this toxin is similar to that of Vip3A. Cry1Ac resistance is rare (*r* frequency <0.001), but its expression in Bt cotton can be poor (Downes et al 2016). Given the relative efficacy and levels of resistance to Vip3A, Cry1Ac and Cry2Ab and the findings of CSE1304, an aim of this project was to compare moth emergences from Bollgard 3 crops, Bollgard II crops and their structured refuges to ensure that the current RMP strategies in relation to refuges are appropriate.

While refuges play a major role in resistance management through genetic dilution, refuge moth productivity is highly variable (CRC 1.01.52). CRC1.01.35 undertook glasshouse experiments which indicated that this variability in pigeon pea refuges could be reduced if plants have adequate nutrients & water. We compared the effect of reduced water & nutrients on pigeon pea refuge productivity under field conditions.

The pigeon pea used as a structural refuge in cotton was originally the variety Quest, but as this variety has not been maintained; the seed used now may not be a true form of Quest, and can be unreliable in terms of flowering timing and quantity. Paul Grundy (DAF Qld, Toowoomba) identified a novel variety (“Sunrise”) that appeared to produce more flowers over a longer period of time, therefore attracting and producing more *Helicoverpa*. While he conducted trials in the north (Grundy 2018), we undertook trials at ACRI (Lower Namoi valley) to compare the moth productivity of Sunrise with the currently used “Quest”.

## Section 2. C<sub>4</sub> moths in cotton systems.

Unstructured refuges are any plants that were not planted as cotton refuges, but still contribute moths to the cotton environment. They can be other crops, weeds or natural vegetation. Identifying moths in the cotton environment from unstructured refuges is difficult, but can be achieved using stable isotope analyses. There are two main isotopes of carbon: <sup>12</sup>C which is the most abundant and <sup>13</sup>C. During photosynthesis, plants fix Carbon using either the C<sub>3</sub>, C<sub>4</sub> or CAM pathways. Plants that use the C<sub>3</sub> pathway discriminate more against the heavier <sup>13</sup>C isotope than those that use the C<sub>4</sub> and CAM pathways. Therefore, it's possible to discriminate between C<sub>3</sub>, C<sub>4</sub> and CAM plants by comparing the proportion of <sup>13</sup>C to <sup>12</sup>C in their tissues. To make this comparison, the proportion of <sup>13</sup>C to <sup>12</sup>C is compared to a standard known proportion, subtracted from 1 and multiplied by 1000 to give us its delta <sup>13</sup>C value (δ <sup>13</sup>C) in parts per thousand. C<sub>4</sub> plants consist of only 3% of all plants, but include important cropping plants such as sorghum, maize and sugar cane. They have delta carbon values of -16 to -10. The CAM pathway is less common, and is predominantly associated with plants from the succulent family, Crassulaceae; although other plants, such as pineapple, also use this pathway. CAM plants have delta carbon values of -20 to -10. C<sub>3</sub> plants, which are the most common and include woody plants such as cotton and pigeon pea, have delta carbon values of -33 to -24. If moths in cotton fields developed on C<sub>4</sub> plants, then they can be identified as originating from unstructured refuges.

The delta <sup>13</sup>C values of plants are passed onto the animals that feed on these plants, enabling them to be identified as consuming C<sub>3</sub> or C<sub>4</sub> plants. Because both cotton and pigeon pea are C<sub>3</sub> plants, if a carbon isotope analysis reveals that a moth has delta carbon values of a C<sub>4</sub> plant, we can be confident that it did not develop as a larvae on cotton or its pigeon pea refuge crop (Baker and Tann 2013). Previous work (CSE1306; UNE1301) using a stable isotope analysis, showed that a large proportion of *Helicoverpa* moth populations found in the vicinity of Bt cotton crops had developed as larvae on C<sub>4</sub> plants, and therefore on crops outside the cotton production system (*H. armigera* were more likely than *H. punctigera* to originate on C<sub>4</sub> plants). This held true for diverse regions such as the Namoi, St George, and the Darling Downs, from three seasons indicating that C<sub>4</sub> moths were entering Bt cotton systems from unknown unstructured refuges, which could have a major diluting (positive) effect on resistance management (Baker et al 2019). In this project we again sampled moths from the Namoi valley to see if the influx of C<sub>4</sub> moths into Bt cotton systems was constant over seasons.

In Central Queensland (CQ) cotton can be planted early in July or late in February. Therefore cotton can be in the ground all year around (see Fig 1.4). As *Helicoverpa* is active all year round, all generations of *Helicoverpa* in CQ could be exposed to Bt cotton crops, increasing the pressure on *Helicoverpa* in CQ to develop resistance to Bt toxins. However, an influx of C<sub>4</sub> moths from non-structured refuges could counter this vulnerability. An aim of this project

was to test if C<sub>4</sub>, and, at the southern end of the cotton range, Griffith, included a number of moths carrying a C<sub>4</sub> signature, to establish if moths from unstructured refuges may be common in cotton systems industry wide.

Nitrogen also forms 2 stable isotopes; <sup>14</sup>N which is the most common, and the heavier <sup>15</sup>N. Ambika et al (2005) argued that the relative proportion of these isotopes in *Helicoverpa* could reflect whether these animals as larvae fed on plants that used bacteria to fix atmospheric nitrogen (in which case their isotope ratios would reflect that of air: 0 ‰), or whether they obtained nitrogen from the soil. As pigeon pea is a nitrogen fixer (but a poor nitrogen fixer) and cotton is not, the delta Nitrogen readings could be used to distinguish between these C<sub>3</sub> *Helicoverpa* food sources. However Baker and Tann (2013, CSE 1306) found that nitrogen isotopes did not distinguish between *Helicoverpa* that consumed pigeon pea or cotton as juveniles. Nevertheless nitrogen isotopes could be used to identify different food sources in unstructured refuges as different Australian C<sub>4</sub> grasses vary in their Delta <sup>15</sup>N values (Murphy and Bowman 2009) although there is a lot of variability. As it is possible to measure <sup>13</sup>C/<sup>12</sup>C and <sup>15</sup>N/<sup>14</sup>N ratios on a single sample (Fry et al. 1992) for the same amount of work and money, we also examined the Nitrogen isotope ratios when measuring those on Carbon. We measured Delta <sup>15</sup>N values to look for differences among C<sub>4</sub> plants and to confirm Baker and Tann's findings.

### **Section 3: *Helicoverpa* survival on cotton and pigeon pea refuges.**

If pigeon pea is able to produce moths at twice the rate of cotton only when it is flowering, then the timing of its flowering becomes critical for it to support the RMP through genetic dilution. Previous studies have shown that non-flowering pigeon pea is less attractive than flowering pigeon pea (CRDC final report CSE 1304). In addition, it is common for cotton to start flowering before pigeon pea. Therefore, for pigeon pea to be effective, larvae need to be able to survive equally well on both non-flowering pigeon pea and flowering cotton. In the previous study (CSE 1304) we found that *H. punctigera* was the species that was most likely to overcome Bt toxins under field conditions. We also found that *H. punctigera* maintained tolerance to Bt toxins across generations. Consequently, pigeon pea needs to be particularly effective at producing *H. punctigera*. Therefore we tested if *H. punctigera* larvae differed in their ability to survive on flowering and non-flowering pigeon pea in relation to flowering cotton.

Heavy early season egg lays in 2016/17 challenged many growers' IPM programs. In Central Queensland (CQ), consultants reported up to 200 *Helicoverpa* eggs per metre while in Southern New South Wales smaller egg lays were seen; although few larvae were seen in crops. With the release of new cotton varieties and unusually high egg lays, many growers were concerned about 1) gene expression and whether neonates could survive for long enough to cause damage, and 2) the plant's ability to compensate for early season insect damage. These concerns prompted some growers to apply insecticide early. We addressed these concerns in collaboration with Sharna Holman (Development Extension Officer, DAF Qld) in Emerald by testing the ability of neonates to damage Bollgard 3 crops in Emerald and Narrabri.

### ***Objectives***

**List the project objectives and the extent to which these have been achieved, with reference to the Milestones and Performance indicators.**

- i. **Record moth emergence from Bollgard crops & associated refuges** in the Lower Namoi & Emerald to quantify the proportion of moths in this system that have been exposed to Bt toxins. This objective was achieved. In the Lower Namoi at ACRI we sampled 10,575; 19,880 and 20,160 cage days (the number of cages in the crop during the season multiplied by the number of days they were sampling moths) over the three

seasons (2015/16, 2016/17, 2017/18 respectively); while at Emerald we sampled in the 2016/17 season for 3360 cage days, which were spread evenly between early and late season planted cotton. During the course of this study no moths emerged from Bollgard 3 cotton in the lower Namoi, indicating it is very effective. However, at Emerald, four moths did emerge from Bollgard 3, indicating that the industry needs to be especially vigilant against the development of resistance in this area. For more information see **Section 1**.

- ii. **Quantify differences in moth emergences from Bollgard II and Bollgard 3 crops.** Over the three years in the lower Namoi we sampled 11,540 cage days in Bollgard II, and 11,880 cage days in Bollgard 3. Very few moths were recorded from Bt cotton crops during this project. In the lower Namoi, the only moth that emerged from Bt cotton emerged from a Bollgard II crop, indicating that Bollgard 3 has higher efficacy; but this object could not be achieved because the difference could not be quantified. For more information see **Section 1**.
- iii. **Record moth emergences from unstructured refuges.** This objective was achieved. Over the three years we sampled two unstructured refuges: a crop of Sorghum and the stock route next to the Namoi river, to better estimate the proportion of moths from these refuges in cotton. In total we sampled for 4275 days in the stock route, and 2520 days in Sorghum. Very few moths emerged from the stock route or sorghum (one *H. punctigera* from the Stock route, and one *H. armigera* from the sorghum). Nevertheless, because NSW stock routes cover about 2 million Hectares (DPI crown land: <https://www.industry.nsw.gov.au/lands/access/travelling-stock>), and sorghum can be 396,000 ha in a good season, they could significantly contribute to populations of non-Bt cotton exposed *Helicoverpa*. For more information see **Section 1**
- iv. **Test if the presence of C<sub>4</sub> moths occurs throughout the industry and is consistent over many seasons.** This objective was achieved. We sampled moths from Emerald (December) and Griffith (January) over 2 years to test for the presence of C<sub>4</sub> moths at the two latitudinal extremes of the commercial industry. We sampled moths weekly at 6 sites in the lower Namoi for 2 years. The findings, particularly when combined with those found in the project CSE1306, demonstrated that C<sub>4</sub> moths are consistently found in the lower Namoi over many seasons. In total we processed 663 *H. armigera* and 425 *H. punctigera*. We found that while C<sub>4</sub> moths are present, and do potentially contribute to the Genetic Dilution strategy of the RMP throughout the industry, they may be particularly effective in *H. armigera* in the lower Namoi. C<sub>4</sub> moths made up less than 10% of the moths caught in Griffith, and their numbers were significantly variable between the two seasons sampled in Emerald. For more information see **Section 2**.
- v. **Promote and quantify good refuge management practices.** This objective was partially achieved. In the 2015/16 season we compared non-irrigated pigeon pea (1650 cage days) with irrigated pigeon pea (1815 cage days), but we did not get a large difference in moth productivity because of significant rainfall. In the 2016/17 and 2017/18 seasons we compared “Quest” pigeon pea (5040 cage days, the original pigeon pea variety for refuges) with “Sunrise” pigeon pea (5040 cage days, the variety developed by Paul Grundy). Sunrise significantly out-performed Quest, but it still began flowering much latter than cotton. Non-flowering pigeon pea is not attractive to laying moths (CSE 1304). In addition, we found that *H. punctigera* larvae do not survive well on non-flowering pigeon pea. Consequently, the flowering delay is a problem for the RMP. For more information see **Section 1 and 3**
- vi. **Continue identifying the level of resistance and tolerance to Cry1Ac and Cry2A toxins in the F<sub>2</sub> generation of *Helicoverpa* moths caught emerging from Bollgard II, Bollgard III and pigeon pea.** As we were unable to obtain live *Helicoverpa* from Bollgard 3 crops, we were unable to achieve this objective and compare larval tolerance in Bollgard 3 and refuges. Nevertheless, we tested the effect of neonates on Bollgard 3

cotton both in Emerald (one experiment, 60 samples) and the lower Namoi (3 experiments, 280 samples) and found that the equivalent of heavy egg lays had no effect on fruit production of Bollgard 3 crops either mid-season or at the beginning of flowering. For more information see **Section 3**

## *Methods, Results, Discussion.*

To address the above Objectives, the work was divided into three sections. For clarity, the Methods, Results and Discussion of each section are presented together.

### **Section 1: Comparing moth emergences between refuges and crops**

#### **1.1 Methods**

Over the 2015/16, 2016/17, 2017/18 seasons moths were collected from non-Bt cotton; Bollgard II; Bollgard 3; unstructured refuges (stock route, sorghum); and Pigeon pea that was either poorly managed (unirrigated) or well managed (irrigated). Irrigated pigeon pea was either the Quest (the current variety) or Sunrise (the newly proposed variety). Most work was undertaken at Myall Vale in the lower Namoi, although in the 2016-17 season, comparisons between Bollgard 3 cotton and Quest pigeon pea, were also undertaken in Emerald.

Comparing moth emergences between crops address Objectives i, ii, iii, and v. In particular, to address Objective (i), Bollgard 3 cotton was compared to refuges, including non-Bt cotton and the Pigeon pea variety “Quest” (the most common refuge used). For Objective (ii) Bollgard II cotton was compared to Bollgard 3 cotton. For Objective (iii) we sampled emergences from unstructured refuges, such as the stock route or sorghum. To address Objective (v), irrigated Quest pigeon pea was compared to unirrigated Quest pigeon pea, and irrigated Quest and Sunrise pigeon pea varieties were compared. Non-Bt cotton, Bollgard II, Bollgard 3 and irrigated Quest pigeon pea were sampled in all three seasons (Table 1.1); unirrigated Quest and sorghum were sampled in one season, and Sunrise pigeon pea and the unstructured stock route were sampled during two seasons.

Because these comparisons were undertaken concurrently, their methods, results and discussion will be presented together but separated by regions into the lower Namoi and Emerald.

Year	Bollgard II	Bollgard 3	Non-Bt Cotton	PP Quest Unirrigated	PP Quest Irrigated	PP Sunrise Irrigated	Unstructured Stock Route	Sorghum	TOTAL Cages in Crop	TOTAL cage days
2015/16	1740	1800	1815	1650	1815		1755		810	10575
2016/17	4760	5040	2520		2520	2520	2520		1420	19880
2017/18	5040	5040	2520		2520	2520		2520	1440	20160

**Table 1.1** The crops tested at ACRI in each season in terms of cage days (the number of cages in the crop during the season multiplied by the number of days they were sampling moths).

#### ***Lower Namoi.***

As in previous studies (1.01.65 CRC1005, CSE 1304), we used white cages to collect moths emerging from Bt cotton, its associated refuges, and unstructured refuges (Fig. 1.1). Each cage sampled 1m<sup>2</sup> of soil surface at its base (*Helicoverpa* pupate in the soil). The cages were sealed to the ground with pegs and soil (in crops) or pegs and sand-bags (on the stock route where it was too difficult to dig the soil in around the cages, Fig.1.1). The collecting vial at the apex of the dome had an inverted mesh funnel which allowed moths to enter but not leave. The vials were checked daily for moths. For each moth, we recorded its species and the location in the field where it emerged. All *Helicoverpa* were collected and preserved.

Cages in crops were arranged in rows of 15 to 20, with 1 metre between them and an un-sampled row of cotton existed between each row of cages. The placement of the cages in the field was staggered in time, with a new row of 20 cages added every week, and then left in situ for 2 weeks. Crop plants within the cage were removed ensuring that only *Helicoverpa* pupae that existed before the cage was put in place were captured as emerging moths. On the stock route, rather than putting cages in rows, cages were secured in blocks of 15 (5 x 3 rows of cages, one metre apart, Fig. 1.1) so that they could be more easily protected from cattle using the stock route. A new block was set up every 5-7 days. Its location was decided by throwing a stick blindly, and using where it landed as the corner of the next block. Before the cages were sealed, the proportion and species of plants within the cages were recorded. Each cage was left in place for at least 2 weeks but no more than 3 weeks, depending on irrigation schedules and the weather.



**Fig.1.1** White Cages being set up on the stock route (an unstructured refuge). Because the base of the cages could not be dug in, sand-bag sausages were used to secure the base. The second photo shows two blocks of 15 cages in a 5x3 formation.

Because very few moths were expected to emerge from Bt cotton, twice the number of white cages were set up in BG 3 and BGII crops to increase the chance of capturing them. However, the amount of land that could be used in these experiments was restricted, therefore to increase the number of cages, Bt fields were sampled more intensively than non-Bt fields.

In previous years we noticed three peaks in *Helicoverpa* moth numbers during the season at Myall Vale: a low peak in early January, a main peak in early February, and a low peak in late March. Consequently, in this project we focused our sampling efforts to capture the early February moth peak, by sampling from mid-January to late-March.

*Overwintering pupae.* After the 2016/17 season we compared the number of pupae overwintering in Bollgard 3, non-Bt cotton, Quest and Sunrise. Once the crop had been harvested but before it was cultivated (12<sup>th</sup> July 2017) we set up 10 cages, 1 metre between each cage, in one row of each of the four crops. We then left them, uncultivated, over winter, while checking them weekly from the 24<sup>th</sup> of August until 1<sup>st</sup> December 2017.

### ***Emerald.***

Previously (project CSE 1304) we recorded a large proportion of moths emerging from Bt cotton in Emerald. In the 2016/17 season we again had the opportunity to sample in this region, through collaborating with Paul Gundy and Sharna Holman (both DAF, QLD) to compare the emergences of *Helicoverpa* moths from Bollgard 3 cotton and its associated refuge.

Two sets of cages were set up in Emerald to capture *Helicoverpa* emerging from early planted cotton, and late planted cotton. Sampling began two weeks after cotton started

flowering. The first set of cages were set up at Avondale, and ran from the 7<sup>th</sup> of November to the 26<sup>th</sup> of December 2016, while the second set was set up on Deneliza Downs, and ran from the 15<sup>th</sup> Feb to the 5<sup>th</sup> April 2017.

Again the placement of the white cages was staggered, with a new row of 10 cages added weekly for 6 weeks. Cages were left in place for about two weeks before being removed. Each row of 10 cages that were set up in BG3 cotton were paired with a corresponding row of 10 cages in the neighbouring pigeon pea refuge. There was 1 metre between the cages in a row, and an un-sampled row between each row of cages. Cages were set up more than 20 metres from the tail ditch, and 10 metres from the edge of the field.

## 1.2. Results

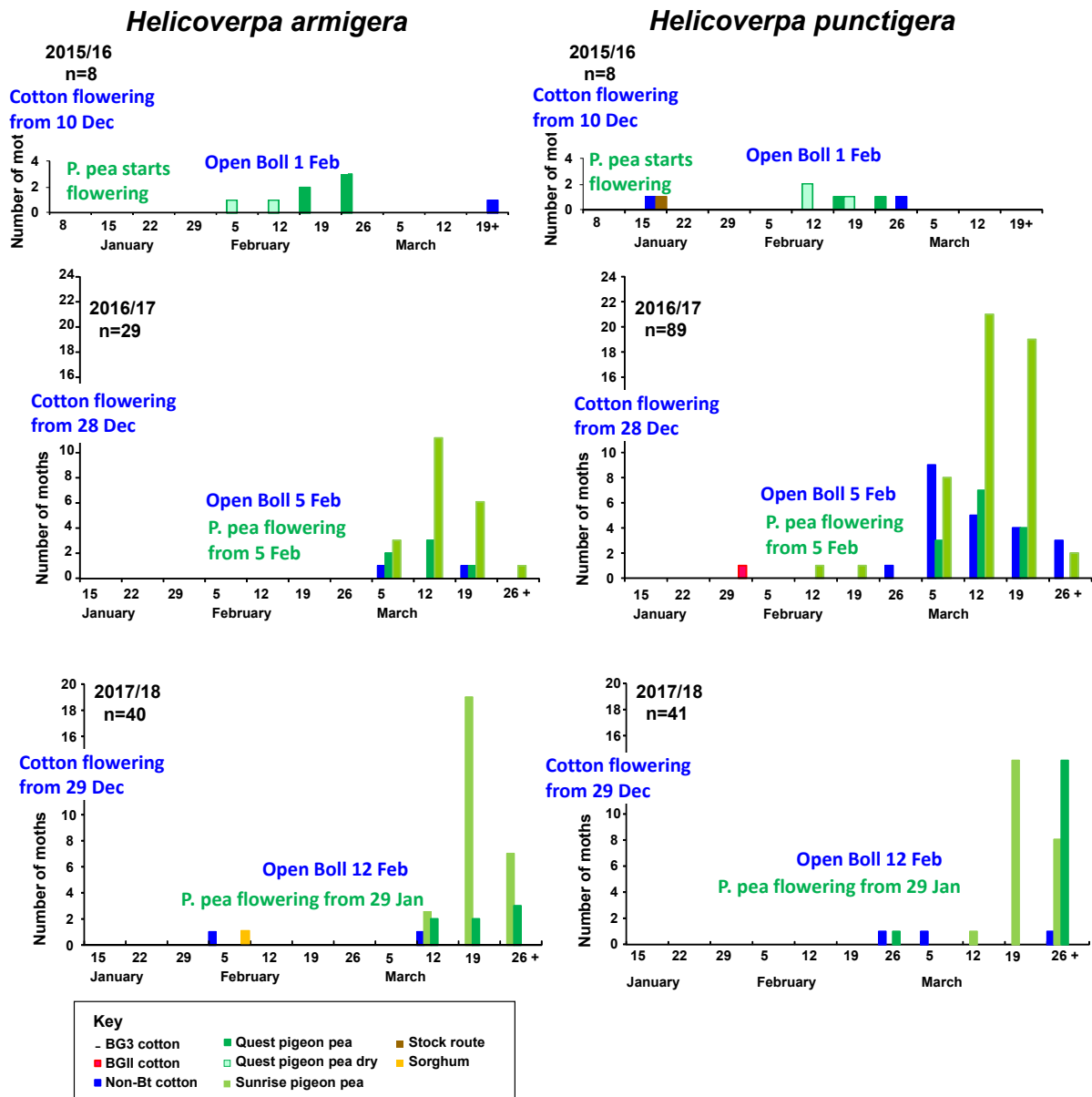
### *Lower Namoi.*

Over the three years, we sampled moth emergences from eight crops (Table 1.1) for over 50 thousand cage days (from 3670 cages). In the first season very few moths were caught, so in subsequent years we were able to increase our sampling effort and also doubled the number of cages in the Bt cotton crops compared to the non-Bt crops.

As in the previous project (CSE 1601), the number of *Helicoverpa* caught per season was highly variable (2015/16: n=16; 2016/16: n=118; 2017/18: n=81), but instead of most moths emerging in early February as they did in the previous project, most moths emerged at the end of the season in mid-March. For example, only 5 moths emerged from both pigeon pea and cotton+unstructured refuges before February 19, but 132 and 68 (pigeon pea, cotton+unstructured refuges respectively) emerged after February 19. That is, *Helicoverpa* in non-Bt cotton also emerged later, despite the cotton flowering from late December. However the numbers of moths retrieved from cotton were so low, particularly for *H. armigera* (1, 2, 3, in 2015/16, 2016/17 and 2017/18 respectively) that it is hard to confirm any emergence pattern.

*Comparing moths emerging from Bt crops.* Over the three years, only one moth was caught at the lower Namoi from Bt cotton, and that was a male *Helicoverpa punctigera* in the 2016/17 season from Bollgard II cotton in late January. We were unable to obtain any offspring from the moth. At the time when this moth emerged, no other moths emerged from any refuges for a further two weeks (Fig. 1.2).

When the number of moths caught in Bollgard II cotton is adjusted to moths per 100 cages (0.02 moths/100 cages, Table 1.2), the ratio is within the lower range of that seen in other seasons (Table 1.2). As previous ratios in Bollgard II have been as low as 0.01 moths/100 cage days, the lack of moths emerging from Bt cotton is not extreme per season, although as the overall yield of *H. punctigera* of Bollgard II cotton from 2015 – 2018 was 0.009 moths/100 cage days, this was an order of magnitude less than that found in the previous project over three seasons (0.089 moths/100 cage days for *H. punctigera*, and 0.041 moths/100 cage days for *H. armigera*). However, the number of moths in non-Bt cotton was also low compared to previous seasons (Tables 1.2, 1.3).



**Fig. 1.2** The number of *Helicoverpa armigera* (histograms on the left) and *H. punctigera* (histograms on the right) caught during the three seasons of sampling in the Namoi. The Cotton flowering and pigeon pea flowering dates are when the first flowers appeared on those crops. In 2015/16 we recorded when pigeon pea was already in flower, and therefore there is no starting date.

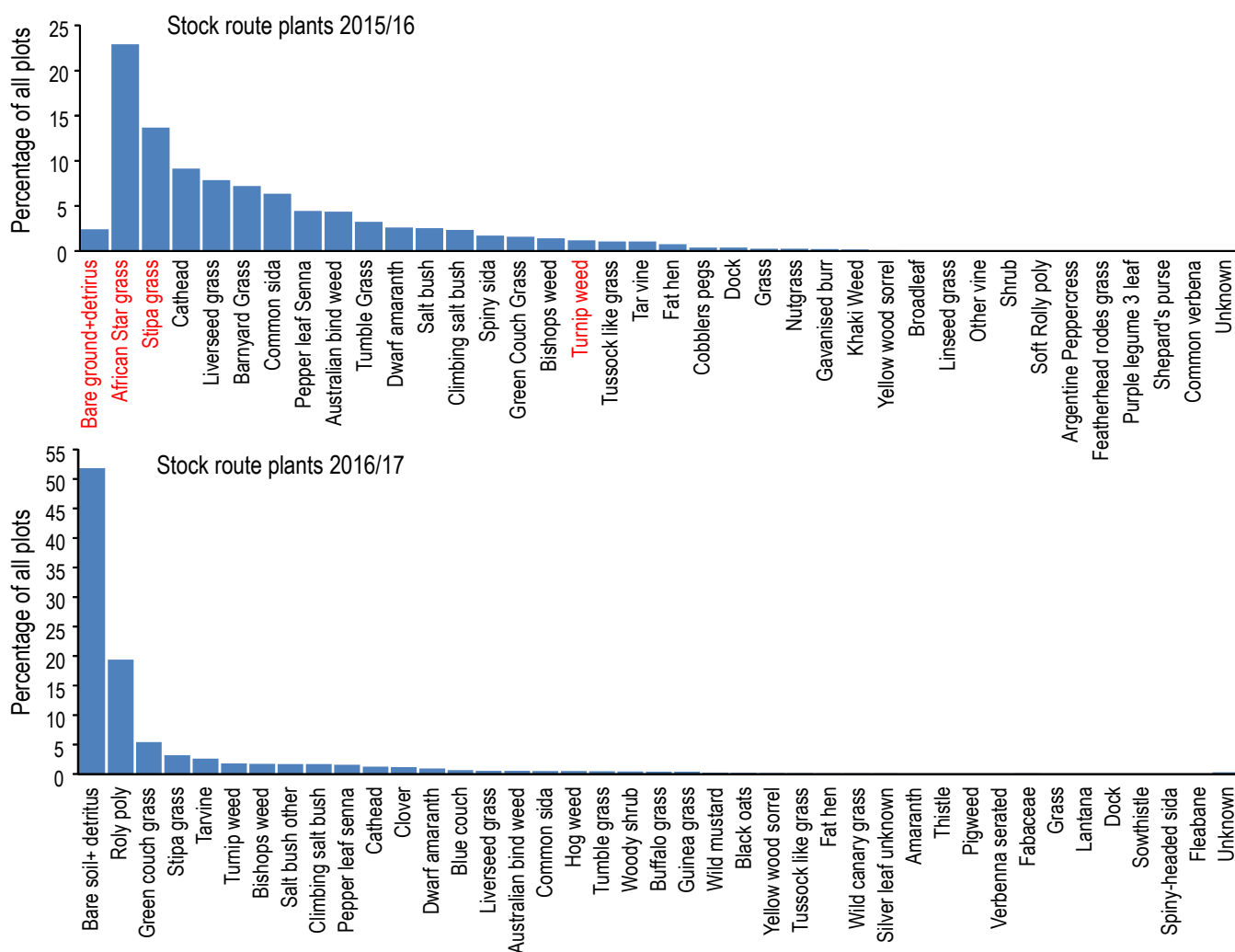
Year	Species	Bollgard II	Bollgard 3	non-Bt cotton	Ppea Quest dry	Ppea Quest irrigated	Ppea sunrise irrigated	Stock route	Sorghum
2015/16	<i>H.armigera</i>	<0.057	<0.056	0.055	0.121	0.275		<0.057	
2016/17	<i>H.armigera</i>	<0.021	<0.020	0.079		0.238	0.833	<0.040	
2017/18	<i>H.armigera</i>	<0.018	<0.018	0.071		0.250	1.036		0.071
<b>Over all seasons</b>	<b><i>H.armigera</i></b>			<b>0.073</b>		<b>0.263</b>	<b>0.992</b>		
2015/16	<i>H.punctigera</i>	<0.057	<0.056	0.110	0.182	0.110		0.057	
2016/17	<i>H.punctigera</i>	0.021	<0.020	0.873		0.556	2.063	<0.040	
2017/18	<i>H.punctigera</i>	<0.018	<0.018	0.107		0.536	0.821		<0.036
<b>Over all seasons</b>	<b><i>H.punctigera</i></b>	<b>0.009</b>		<b>0.394</b>		<b>0.452</b>	<b>1.488</b>	<b>0.023</b>	

**Table 1.2** The number of moths caught per 100 cage days for each species per year per crop. In crops where no moths were caught, the emergence/100 cage days is expressed (in red) as less than that if one moth had been caught.

*Comparing refuge types.* In the 2015/16 season we tested the effect of not irrigating Quest pigeon pea. Although the unirrigated pigeon pea flowered earlier than the irrigated pigeon pea, there was no clear disadvantage, in terms of moth emergences, to not irrigating pigeon pea in the season we tested, which received 143mm of rain during January. *H.armigera* moths emerged 2 weeks earlier from the unirrigated pigeon pea than the irrigated pigeon pea (Fig 1.2) although given the small number of emergences, this difference is not significant. The number of moths/100 cage days were also similar between irrigated and unirrigated Quest pigeon pea in the 2015/16 season (Table 1.2).

The new Sunrise Pigeon pea variety consistently out-performed Quest Pigeon pea in the number of moths it yielded (Fig 1.2; Table 1.2) usually yielding 4-fold more moths.

*Unstructured refuges: Stock route and Sorghum.* Very few moths were collected from the unstructured refuges, the stock route and sorghum (stock route: 1 moth or 0.057 moths /100 cage days; sorghum: 1 moth or 0.071 moths /100 cage days; Fig. 1.2; Table 1.2).



**Fig. 1.3** The proportion of ground covered by different plants in the stock route over the two seasons. The plants highlighted in red were those found in the plot that yielded a *H. punctigera*.

The moth collected from the stock route was a male *H. punctigera* that was caught mid-January in a plot consisting of 50% African Star grass (*Cynodon nlemfuensis*), 40% Stipa grass (*Stipa* spp), 5% turnip weed (*Rapistrum rugosum*) and 5% bare ground. The main components of this sample were the two most common plants sampled in the stock route (Fig. 1.3). In this season the stock route was comparatively lush as bare ground or detritus made up only 2.4% of the total area sampled. In the following season no *Helicoverpa* were

obtained from the stock route, although the same or similar plants were present to those collected from the plot that yielded the male *H. punctigera* (Green couch grass; *Cynodon dactylon*, is very similar to African star grass). However, the site was more barren, with 51.8% of the area sampled either bare ground or detritus (Fig. 1.3).

**Overwintering pupae.** Four moths were harvested from the cages left over winter. Two *H. armigera* females were collected from Pigeon pea Sunrise (emerging on the 21<sup>st</sup> of September and the 11<sup>th</sup> of October 2017). A *H. armigera* female and *H. punctigera* male were collected from non-Bt cotton (emerging on the 11<sup>th</sup> and 31<sup>st</sup> October 2017 respectively).

### **Emerald.**

In the 2016/17 season, four moths were caught (10<sup>th</sup> Nov: *H. armigera* F; 18<sup>th</sup> Nov: *H. punctigera* M; 6<sup>th</sup> Dec: *H. armigera* F; 15<sup>th</sup> Dec: *H. armigera* M). Only the female *H. armigera* caught in Nov was found alive, but she did not mate or produce eggs. All were found in the early planted Bollgard 3 cotton.

No moths were caught in late planted Bollgard 3 cotton or pigeon pea. During the time of the sampling, the cotton in both samples had been flowering for 2 weeks, but the pigeon pea refuges paired with the early and late season cotton crops were not flowering.

## **1.3. Discussion**

### **Lower Namoi.**

Compared to previous seasons, in this project we saw a large reduction in the number of *Helicoverpa* emerging from cotton (Table 1.3). The drop was apparent for both *Helicoverpa* species for both Bollgard II and non-Bt cotton. There were significantly fewer *H. punctigera* moths caught per cage days in 2015-18 compared to 2012-15 season (Chi-square value: 73.15, df=1 P<0.001 \*\*) which would explain the lower rates.

However *H. armigera* was caught at a similar rate in both the 2015-18 and the 2012-15 seasons (Chi-square value: 1.44, df=1 P=0.231 NS). For *H. armigera* it appears that while lower rates were caught in non-Bt cotton (Chi-square value: 6.18, df=1 P=0.013 \*) there was no difference in the rates that moths were caught in all pigeon pea combined (Chi-square value: 0.18, df=1 P=0.674 NS). As *Helicoverpa* moths show preferences between crops as oviposition sites (Zalucki 1986), this may indicate that in to *H. armigera*, the new Sunrise Pigeon pea variety is particularly more attractive than cotton.

Along with the low numbers of moths in cotton, no moths were caught emerging from Bollgard 3 in the Namoi, indicating that Bollgard 3 was very effective at controlling both *Helicoverpa* species. This is a positive finding for the RMP.

Crop	<i>H.armigera</i>		<i>H.punctigera</i>	
	CSE1304	CSE1601	CSE1304	CSE1601
Bollgard II	<b>0.041</b>	<0.009	<b>0.089</b>	0.009
non-Bt cotton	<b>0.222</b>	0.073	<b>0.705</b>	0.394
Ppea Quest irrigated	<b>0.56</b>	0.263	<b>2.31</b>	0.452
Ppea sunrise irrigated		0.999		1.488
TOTAL	<b>0.182</b>	0.152	<b>0.627</b>	0.273

**Table 1.3** compares the number of moths caught per 100 cage days for each species over three years in the 2012-15 study (CSE1304) compared to the 2015-18 study (CSE1601). No *H. armigera* moths were caught on Bollgard II in CSE1601, so that the emergence/100 cage days is expressed (in red) as less than that if one moth had been caught.

In this study we found that unirrigated pigeon pea performed as well as irrigated pigeon pea, in that it produced similar numbers of moths. This is probably because the season in which we tested this assumption was a low pressure year with a high rainfall during the season, so the unirrigated pigeon pea was not water stressed.

In comparison to previous seasons, we also saw changes in the timing of moths emerging from cotton and refuge crops, including pigeon pea. For example, in the 2016/17 season, even though there appeared to be a strong influx of moths into the region in October-November (see section 2, Fig 2.2) this did not cause a large number of *Helicoverpa* to develop in cotton and emerge in January or February. In fact, in both 2016/17 and 2017/18 we saw a shift in emergence patterns from early February to mid-March. In the 2016/17 season, the climate was harsh, starting cold, but then becoming very hot fast. These temperature extremes meant that cotton could not put down fruit, and so most of the fruit was set late in the season.

The aim of refuges is to dilute resistance in any moths emerging out of Bt cotton during the season. In our study pigeon pea, particularly Sunrise, produced large amounts of moths able to dilute the effect of any moths emerging from cotton, but only a month after the pigeon pea starts flowering, which was usually a month after cotton started flowering (Fig. 1.2). Sunrise did not start flowering any sooner than Quest, and in these seasons only produced moths in the last generation before the moths estivate, at a time when cotton is less attractive to *Helicoverpa*. Therefore there was a month when cotton was attractive and pigeon pea was not attractive and not able to perform the role of genetic diluter. These observations are consistent with previous reports demonstrating that pigeon pea attracts egg-layers later than cotton (Whitehouse et al 2017).

In Grundy's (2018) report on the pigeon pea Sunrise, he found that in the 2016/17 season, plants in the Darling Downs flowered in early January, while at two sites flowering was delayed a month (so no pupae were collected until February). In the 2016/17 season our plants also did not flower until February. Grundy suggests that excessive watering (that is, using the same watering practices as that used for cotton) may have caused the delay. In the 2017/18 season, Grundy did not find pupae until March, which matches our experience here in the Namoi. In this case Grundy suggests that mirids may have delayed flowering.

Any delay in pigeon pea flowering is a concern for the RMP, as the role of refuges is to contribute to genetic dilution during the season. However, as pigeon pea refuges continue to be consistently attractive towards the end of the season, they assist the RMP through seasonal quarantining rather than genetic dilution. Pigeon pea refuges assist in seasonal quarantining in Central Queensland, where they can be converted into a trap crop ([http://bollgard3.com.au/prod/media/1641/bollgard-3\\_resistance-management-plan.pdf](http://bollgard3.com.au/prod/media/1641/bollgard-3_resistance-management-plan.pdf)). In other regions pigeon pea refuges cannot be destroyed until cotton is harvested and so act as an end of season trap crop, supporting seasonal quarantining. Seasonal quarantining is clearly beneficial to the RMP. However maintaining a diverse tool kit for the RMP also requires maintaining genetic dilution techniques. It is important to monitor when pigeon pea refuges commence flowering in comparison to Bt cotton crops to be clear on how pigeon pea support the RMP.

In this study, even though cotton was flowering, it too produced very few moths during its attractive period, and the timing of emergences from cotton and pigeon pea did not differ. High temperatures during January could have affected the development of *Helicoverpa*. Zalucki et al (1986) reported earlier laboratory temperature studies that found that few larvae survived at 40°C, and at temperatures consistently over 35°C, larvae failed to complete development. Mironidis et al (2008) reported in another laboratory study that constant high temperatures kill *H. armigera*. While in our study the temperature always dropped below 35°C at night, the extended periods of 40°C highs may have killed many larvae. It would be

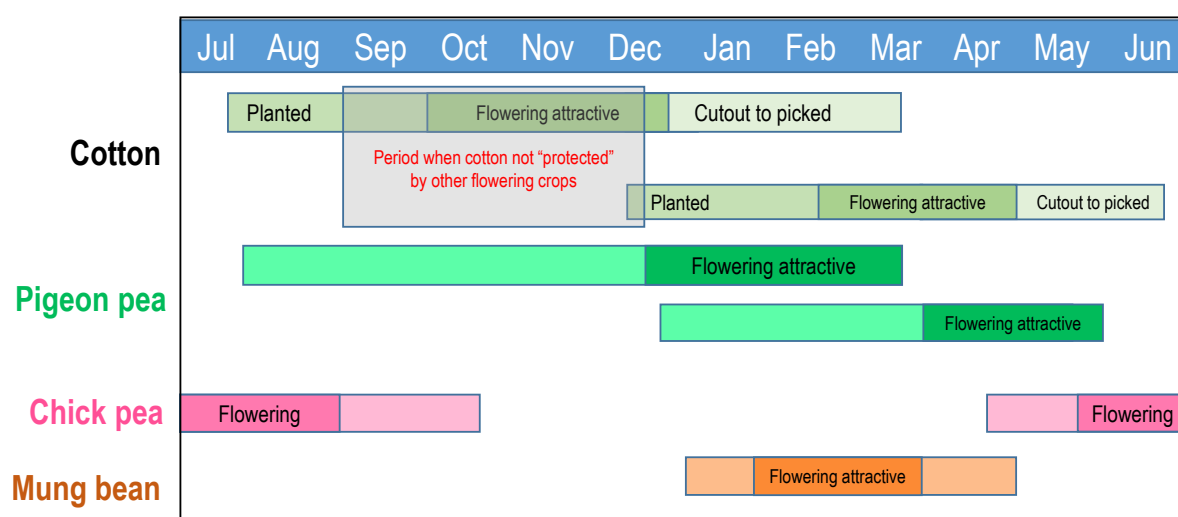
beneficial to understand more about the effect of temperature on *Helicoverpa* in the field, and why there was a universal lack of moth emergences in January and February.

The two unstructured refuges tested in this project both produced moths early in the season and could be a source of early season moths for genetic dilution. This is particularly the case with the stock route, which covers large stretches of land. For example, in NSW the stock route covers 2,000,000 ha (DPI crown land):

<https://www.industry.nsw.gov.au/lands/access/travelling-stock>, In 2015/16, which was a good season with lush early season growth, the stock route yielded 1 *H. punctigera* moth out of 1755 cage days, or 176 moths per ha during January. While it is unclear how much of the stock route is similar country to that we sampled, the stock route would have contributed to early season moth dilution in the 2015/16 season. However, in the dry season (2016/17) we did not measure any moth emergences, indicating that the stock route during this season would have provided little support as genetic diluters to the RMP. Therefore unstructured refuges like the stock route may support the RMP in lush seasons, but are not reliable in poor seasons.

### ***Emerald.***

Although the number of moths caught in Emerald was low, all four moths were caught on early season Bollgard 3 cotton. None were caught on the associated pigeon pea. In both the early and late season tests, the refuge pigeon pea associated with the crop was not flowering. As we have shown, pigeon pea that is not flowering is neither attractive to laying moths, nor conducive to *H. punctigera* larval survival (although work undertaken by us in the 2018/19 season indicates that *H. armigera* can survive on non-flowering pigeon pea; Towns and Whitehouse, unpubl. data). So it is not surprising that no moths emerged from the pigeon pea crops tested in Emerald. However, 4 moths emerging from the early season Bollgard 3 crop is high, and is equivalent to 0.48 moths/100 cage days. It is unwise to put too much emphasis on four moths, and this finding needs further investigation. Nevertheless the finding matches the pattern found in CSE1304 that emergences from Bollgard crops appear to be higher in Emerald than in the Namoi.



**Fig. 1.4** Diagrammatic representation of flowering crops in Emerald in the 2016/17 season, in relation to early planted and late planted cotton (olive bars) as gleaned from discussions with stakeholders. The period during cotton's growth when other flowering plants or crops within the region were not flowering is highlighted. The RMP was potentially compromised at this time because there was no structured or unstructured refuges flowering, and therefore there were no alternative attractive *Helicoverpa* hosts to Bollgard 3 cotton within the region.

With the late and early cotton plantings, cotton in Emerald could be in the ground throughout the year (Fig. 1.4). However, in the 2016/17 season, the timing of pigeon pea flowering in our trials meant it was not an attractive alternative host between September and early December. As this period coincides with early planted cotton, pigeon pea may not be an effective genetic diluter for most of the time that the early planted cotton is attractive (Fig. 1.4).

Nevertheless early season pigeon pea was still flowering when late season cotton was planted and began flowering (Fig. 1.4). Likewise, other prominent crops, such as Chick pea and Mung bean were also flowering while late planted cotton was flowering. Therefore Chick pea, Mung bean and pigeon pea, including that planted as early season refuges, likely acted as genetic diluters for the late planted cotton (Fig. 1.4). The strength of attractive, flowering pigeon pea late in the season also may have diverted *Helicoverpa* pressure from the late Bollgard 3 crop, partly explaining why late planted cotton did not yield any moths in this study.

In the 2016/17 season, early planted Bollgard 3 cotton during most of its flowering period, may not have had a known alternative attractive (flowering) crop to provide genetic dilution (Fig. 1.4). While there may be other crops and unstructured refuges in the Emerald region, these results indicate that refuges may struggle to be genetic diluting RMP tools in early season cotton in Emerald, which could make early season Bollgard 3 crops more susceptible to *Helicoverpa* pressure. These findings suggest that there is a stronger resistance risk in early season Emerald cotton. One possible response would be to undertake more sampling in BG3 cotton in Emerald to clarify the risk. Another would be to closely monitor for Bt resistance in *Helicoverpa* moths emerging early season in Emerald.

## Section 2. C<sub>4</sub> moths in cotton systems.

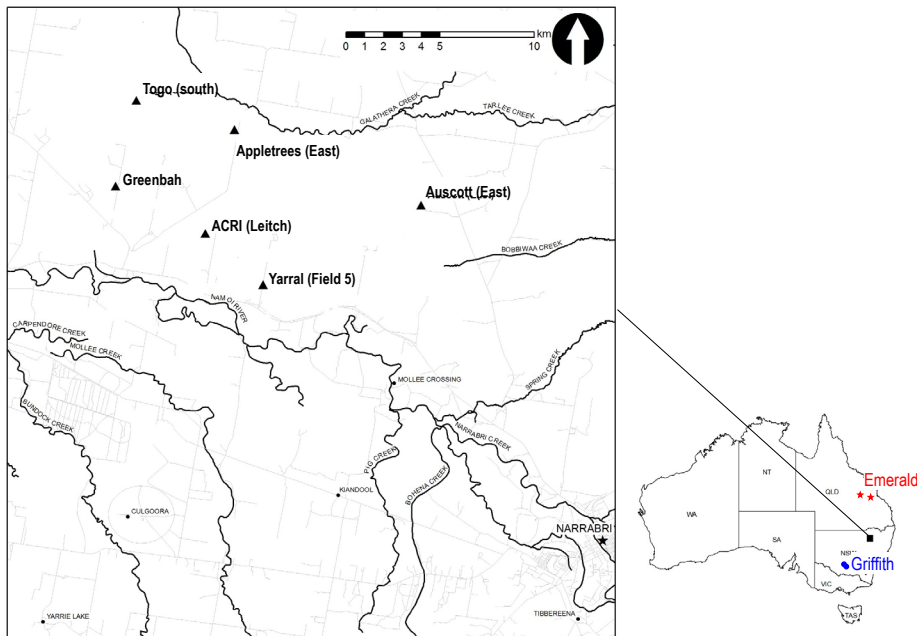
### 2.1 Methods

This section tests whether (1) the presence of C<sub>4</sub> moths in cotton regions in the Namoi is consistent over time (reflecting patterns seen in previous studies); and (2) the presence of C<sub>4</sub> moths in cotton systems, which has been established in the Namoi, Darling Downs, and St George, is also present in Emerald and Griffith. If so, then this would indicate that C<sub>4</sub> moths are widespread and present in cotton throughout the industry.

#### *Carbon Isotopes and moth catches from the Namoi*

From October 2016 until June 2018, six sites, each containing a pair of pheromone canister traps (one targeting *H. armigera* and the other *H. punctigera* males) were monitored weekly. These sites formed part of a previous CSIRO trapping grid which had been recently dismantled. The sites were in an area that covered about 50 km<sup>2</sup>, centring on 30° 12' latitude and 149° 36' longitude (Fig 2.1).

For each sample at each trap all moths were counted but only a maximum of ten were collected. All collected moths were then transferred into 25ml vials filled with absolute ethanol and stored in a minus 80 freezer. Of these about 2 per pheromone site per species were processed in preparation for carbon and nitrogen isotope analysis (explained elsewhere; Baker et al 2019) at the University of New England by Leanne Lisle. Lures in the traps were changed each month, and pest strips in the traps (releasing dichlorvos vapours to kill the moths) were changed every two months.



**Fig. 2.1** Location of 6 pheromone sites in the upper Namoi (inset) and the two farms sampled at Emerald (red) and Griffith (Blue).

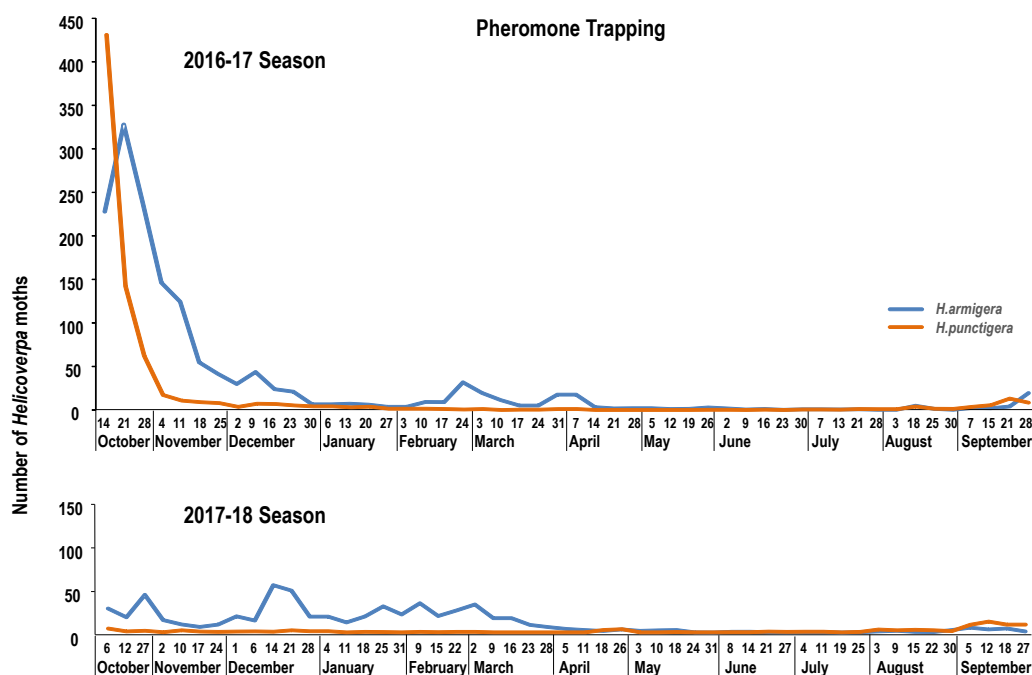
#### *Carbon Isotopes from Emerald and Griffith*

In 2016 and 2017 moths were sampled during early-mid flowering in Griffith and Emerald (January and December respectively). At both sites, pheromone traps supplemented with light traps, were set up on two farms over two nights (Fig 2.1). All moths collected from the pheromone traps were used in the analysis. If the number collected from pheromone traps

were less than 10, the moths were supplemented with those collected from light traps. Between 10 to 20 samples per farm were collected of each species and prepared for isotope analysis as above.

## 2.2 Results

### *Carbon Isotopes and moth catches from the Namoi*

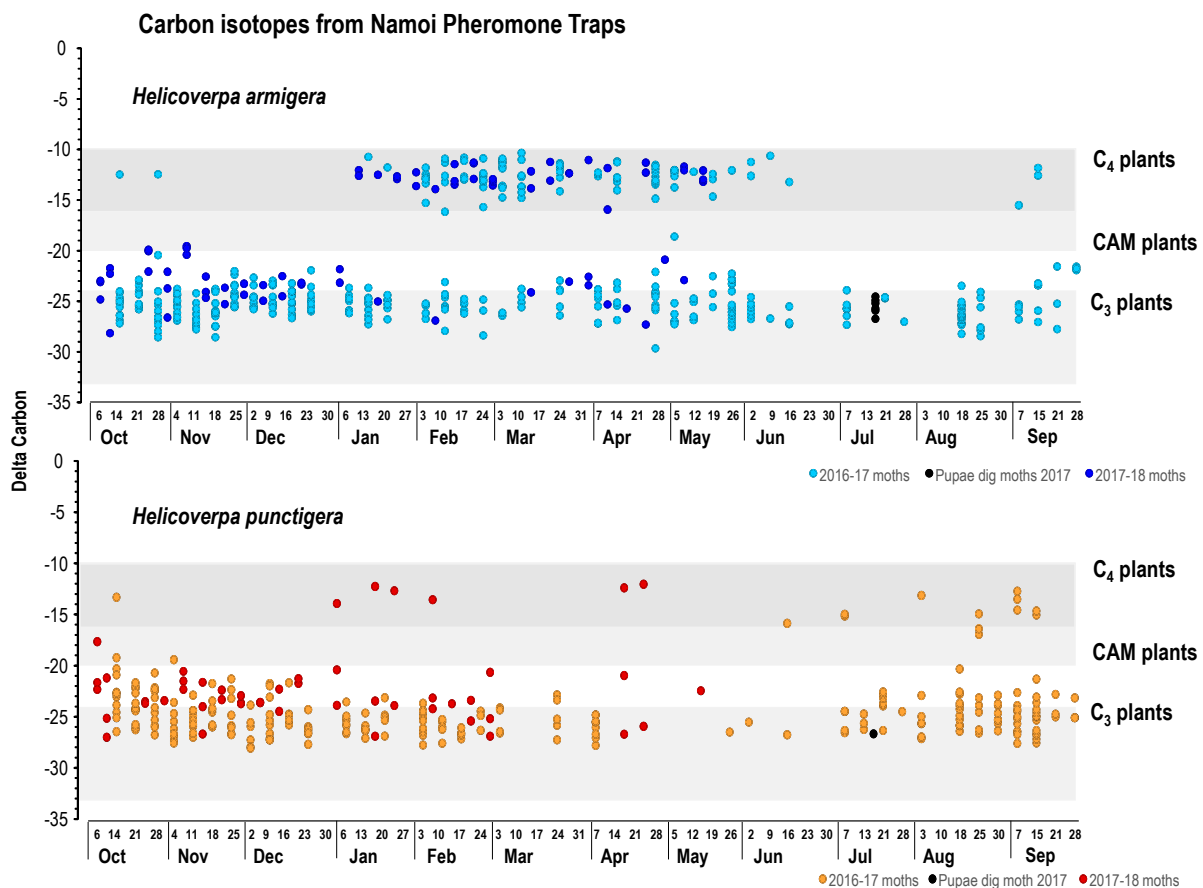


**Fig. 2.2** Number of moths caught in pheromone traps during 2016/17 and 2017/18.

On 7 occasions (29.12.2016, 30.06.2017, 28.07.2017, 28.12.2017, 9.03.2018, 8.06.2018, 4.07.2018) we were unable to check the traps (usually due to bad weather) so the number collected after two weeks was divided between the two dates.

The pheromone traps in the upper Namoi revealed that 2016/17 and 2017/18 were distinctly different seasons (Fig. 2.2). The 2016/17 season caught a large number of both *H. armigera* and *H. punctigera* moths at the beginning of the season, whereas in 2017/18 there was no large spike in the numbers of either species. Of these moths the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  readings of 500 *H. armigera* moths and 303 *H. punctigera* moths were analysed from the upper Namoi.

From October to December, when moths from outside the cotton growing region are thought to move into cotton growing regions (eg Gregg et al 1993, Zalucki and Furlong 2005) the carbon isotopes for *H. armigera* in the upper Namoi for the 2016/17 and 2017/18 seasons differed. During this period there were only two moths that had  $\delta^{13}\text{C}$  values in the C<sub>4</sub> plant range (above  $-15 \delta^{13}\text{C}$ ) and these were collected in the 2016/17 season. Nevertheless overall moths collected in 2016/17 had a  $\delta^{13}\text{C}$  range significantly lower (range:  $-33$  to  $-24$ ; mean =  $-25.03$ ; Stddev =  $2.16$ ,  $n=120$ ) than those collected in 2017/18 (mean =  $-23.21$ ; Stddev =  $2.05$ ,  $n=28$ ;  $t=-4.04$ ;  $df=146$ ;  $P<0.001$ ; Fig. 2.3).



**Fig. 2.3** Carbon isotope signatures of moths collected from the Namoi in pheromone traps and pupae digs.

This pattern was repeated for *H. punctigera*. From October to December, the average  $\delta^{13}\text{C}$  readings for moths collected in the 2016/17 season (mean = -24.59; Stddev=2.28, n=101) were lower than those collected in 2017/18 (mean = -22.94; Stddev= 1.89, n=26; Fig. 2.3). These values were significantly different ( $t=-3.38$ ;  $df=125$ ;  $P<0.001$ ).

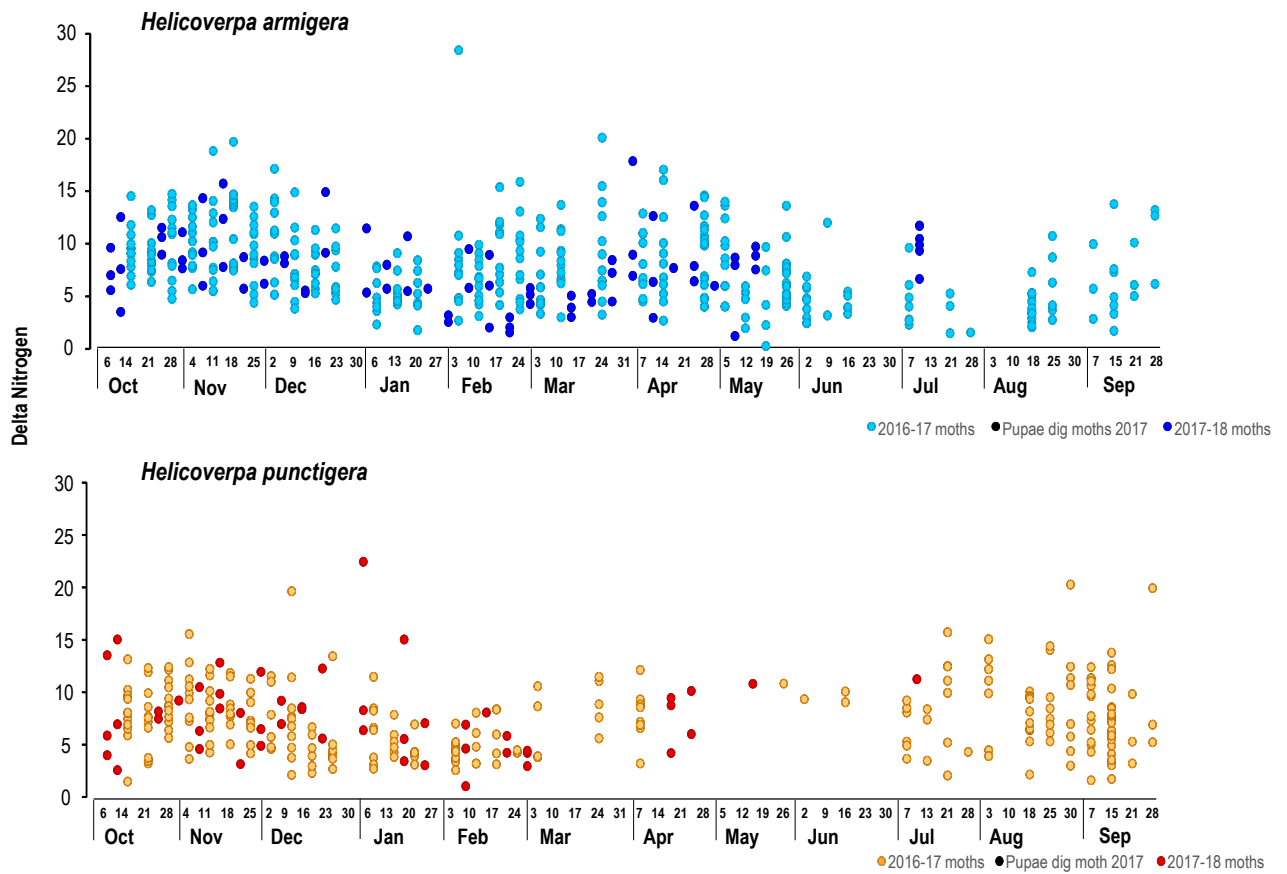
Most of the *H. armigera* moths carrying the  $\text{C}_4$  carbon isotope profile were collected between January and June (Fig. 2.3). The increase in the  $\text{C}_4$  profiled moths was greatest in the 2017/18 season (Chi-sq =13.01;  $df=1$ ;  $P<0.001$ ), where 71.74% of the moths collected between January and June had a  $\delta^{13}\text{C}$  reading greater than -16 (in 2016/17; 42.29% had this  $\delta^{13}\text{C}$  value).

With *H. punctigera*, the seasonal difference in  $\text{C}_4$  profiled moths was not so pronounced, although again none were found in Nov/Dec in both seasons. The presence of  $\text{C}_4$  profiled moths also varied between the two seasons. In 2016/17 only one moth collected between January and June had  $\delta^{13}\text{C}$  readings within the  $\text{C}_4$  range, whereas in 2017/18 there were 6 moths in that range (Fig 2.3). In the 2016/17 season there were 9 moths in the  $\text{C}_4$  range from July to September 2017 (the project finished on July 1 2018, so no moths could be tested after this date).

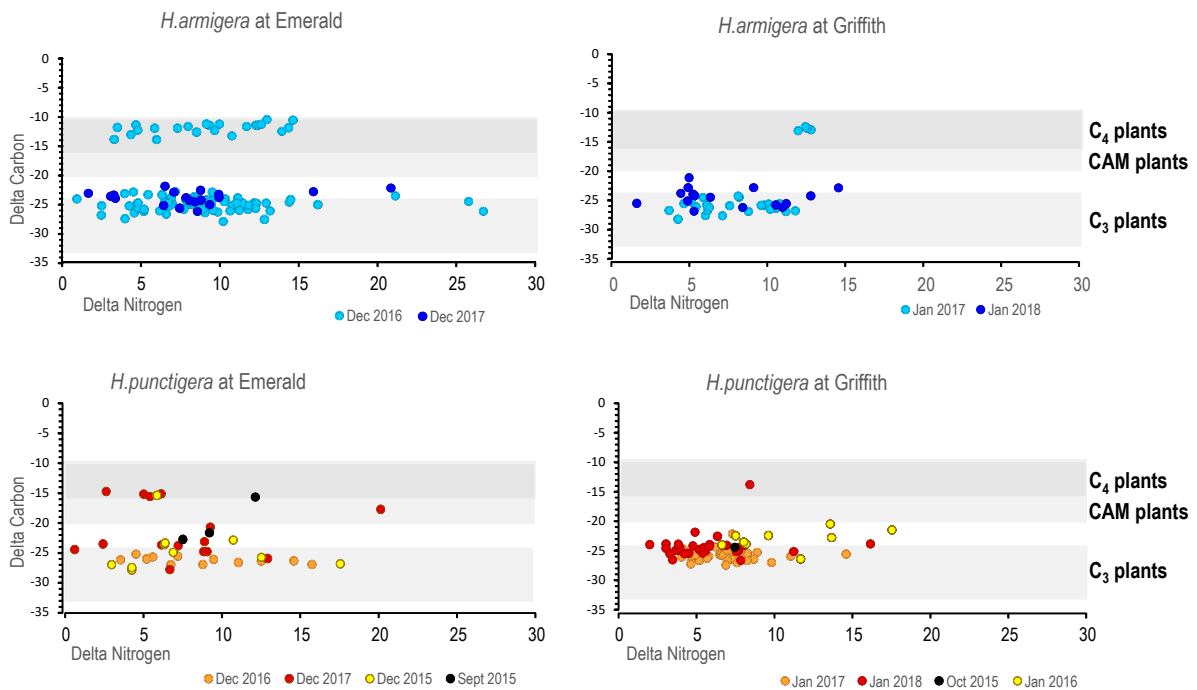
The moths collected from pupae digs under pigeon pea had  $\delta^{13}\text{C}$  readings all within the  $\text{C}_3$  range (Fig 2.3).

Unlike the carbon isotope readings, there was no difference in the October to December *H. armigera*  $\delta^{15}\text{N}$  readings between the two seasons in the Namoi ( $t=0.92$ ,  $df=150$  NS; Fig 2.4). In the 2016/17 season there was a significant difference in the  $\delta^{15}\text{N}$  readings between  $\text{C}_4$  and non- $\text{C}_4$  moths between January and June ( $t=-2.64$ ;  $df=137$ ;  $P=0.009$ ) as  $\text{C}_4$  *H. armigera* moths had higher  $\delta^{15}\text{N}$  values (mean values:  $\text{C}_4=8.2$ ; non- $\text{C}_4=6.7$ ). This pattern was not repeated in the 2017/18 season where there was a trend for  $\text{C}_4$  moths to have lower  $\delta^{15}\text{N}$  values between January and June ( $t= 1.7$ ,  $df=44$ ,  $P=0.096$ ;  $\text{C}_4= 5.86$ , non- $\text{C}_4=7.77$ ). There

were no differences in *H. punctigera* delta values between C<sub>4</sub> and non-C<sub>4</sub> moths, nor between seasons.



**Fig. 2.4** The Nitrogen isotope profiles of moths collected in pheromone traps in the 2016/17 and 2017/18 season in the upper Namoi.



**Fig. 2.5**  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  readings of *H. armigera* and *H. punctigera* collected from Emerald and Griffith over three seasons.

### Carbon Isotopes from Emerald and Griffith

The  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  readings of 43 *H. armigera* moths and 82 *H. punctigera* moths from Griffith; and 120 *H. armigera* moths and 40 *H. punctigera* moths from Emerald were analysed. From these moths there was again no clear pattern in the  $\delta^{15}\text{N}$  readings, although there were clear separations in the  $\delta^{13}\text{C}$  values. In December 2016/January 2017 23 and 4 moths of *H. armigera* had  $\delta^{13}\text{C}$  values within the  $\text{C}_4$  range at both Emerald and Griffith respectively, while the following year there were no  $\text{C}_4$  *H. armigera* moths recorded from either location (Fig.2.5). Conversely, in December 2016/January 2017 there were no recordings of  $\text{C}_4$  *H. punctigera* moths in Emerald and Griffith respectively, while the following year at least one  $\text{C}_4$  *H. punctigera* moth was recorded at both locations (Fig 2.5).

Baker et al (2019) following Baker and Tann (2013) classified  $\text{C}_4$  moths as those with  $\delta^{13}\text{C}$  readings greater than  $-20\text{‰}$ , and  $\text{C}_3$  moths as those with  $\delta^{13}\text{C}$  readings less than  $-20\text{‰}$  (because  $-20\text{‰}$  was the least frequent value recorded between the bimodal peaks). If we follow Baker's et al (2019) classification, and compare moths with  $\delta^{13}\text{C}$  readings greater or less than  $-20\text{‰}$   $\delta^{13}\text{C}$ , then *H. armigera* moths from the three regions with values higher than  $-20\text{‰}$  range from 9.3 to 27.6% of the total moths tested, while those of *H. punctigera* moths range from 1.22 to 17.5% (Table 2.1). Using this classification, in Emerald there is a significant change between seasons in the number of moths emerging with higher carbon readings. With *H. armigera*, there are more moths with higher carbon readings in the 2016/17 season (Fishers exact test,  $P=0.021$ ); while with *H. punctigera* there are more moths with higher carbon readings in the 2017/18 season (Fishers exact test,  $P=0.047$ ). Again, these differences emphasize the variability in the contribution of moths from unstructured refuges.

$\delta^{13}\text{C}$ values	<i>H.armigera</i>			<i>H.punctigera</i>		
	Namoi	Griffith	Emerald	Namoi	Griffith	Emerald
<-20 "C3"	72.4	90.7	80.83	92.74	98.78	82.5
>-20 "C4"	27.6	9.3	19.17	7.26	1.22	17.5
<b>Totals</b>	<b>500</b>	<b>43</b>	<b>120</b>	<b>303</b>	<b>82</b>	<b>40</b>

**Table 2.1** Percentage of moths above and below  $\delta^{13}\text{C}$  values of  $-20$  collected from the Namoi, Griffith and Emerald regions over two seasons.

### 2.3 Discussion

The pheromone trap data indicates that the 2016/17 and 2017/18 seasons were quite distinct. In the 2016/17 season, a large number of moths were collected in October, which is indicative of a large local recruitment or a large migration of spring moths into the Namoi. This used to be an annual event (eg Fitt et al 1989, Zalucki and Furlong 2005). In the 2017/18 season the traps did not capture large numbers of moths in October, suggesting that no migration had occurred. These findings agree with those of Baker and Tann (2017a,b) that a large influx of moths to the Namoi in the spring is not consistent between years. This has ramifications to the RMP of *Helicoverpa* because in years of no migration there will be less genetic dilution in spring of the locally generated Bt exposed moths that emerge from under last season's crops.

The difference in the population structure between the two seasons (indicated by the pheromone trap data) is supported by the carbon and nitrogen isotope readings. The nitrogen readings for both years had distinct peaks and troughs, but particularly for *H.armigera*, their timings were not synchronized. The carbon readings in the 2016/17 season, indicated that  $\text{C}_4$  moths of both species were found in the traps in October, while in the 2017/18 season, none

were found (Fig. 2.3). In addition, the C<sub>3</sub> profiles were significantly different, with higher readings in the 2017/18 season.

These differences may reflect a difference between years in the diet of the moths early season. In the 2017/18 season with no evidence of immigration, the average  $\delta^{13}\text{C}$  was high for a C<sub>3</sub> plant ( $\delta^{13}\text{C}$  value = -23.21‰ and -22.94‰ for *H. armigera* and *H. punctigera* respectively) but similar to that found from pupae collected from cotton by Baker and Tann (2013,  $\delta^{13}\text{C}$  value = -23.2‰). It is unclear if readings from larvae feeding on cotton consistently tend to be high for a C<sub>3</sub> plant. Another explanation for a high  $\delta^{13}\text{C}$  reading is that larvae could be feeding on a combination of C<sub>3</sub> and C<sub>4</sub> plants, thereby raising their  $\delta^{13}\text{C}$  values, or that their  $\delta^{13}\text{C}$  values may increase if they cannibalise each other, as  $\delta^{13}\text{C}$  readings can increase slightly with trophic levels (Ponsard and Arditì 2000). More work testing larvae feeding on cotton is needed to test if larvae feeding on cotton have a clear  $\delta^{13}\text{C}$  signature.

In this report pupae collected under pigeon pea had values ranging from -26.85‰ to -24.65‰, well within the C<sub>3</sub> range, and with values that agree with  $\delta^{13}\text{C}$  values reported for *Helicoverpa armigera* feeding on pigeon pea by Baker and Tann (2013).

Few C<sub>4</sub> moths were caught early in the season (November and December) in the Namoi, and so genetic dilution from C<sub>4</sub> moths is limited at this time. During this period pigeon pea is also unattractive and a poor food source for *Helicoverpa* (see section 4.2.1) reducing its ability to contribute to genetic dilution. Consequently, RMP through genetic dilution seems to be problematic early in the season.

However, from January to June in both seasons a large proportion of the *H. armigera* population originated from C<sub>4</sub> plants. This period covers the critical growing period for cotton when it is actively flowering, setting fruit, and most attractive to *Helicoverpa*. It suggests that the RMP is well supported by genetic dilution at this time, at least for *H. armigera*.

*H. punctigera* is not strongly associated with C<sub>4</sub> plants. Only 3% of eggs laid on sorghum and 0.5% of eggs laid on Maize between 2012-15 were *H. punctigera* (Downes pers comm in Baker et al 2019). Known inland plant hosts of *H. punctigera*, such as *Senecio gregorii*, (Asteraceae), *Cullen cinereum* (annual verbine, Fabaceae) and *Nicotiana velutina* (wild tobacco, Solanaceae) have been tested and are not C<sub>4</sub> plants (P.Gregg pers comm). Therefore *H. punctigera* originating from inland are unlikely to be contributing to the C<sub>4</sub> populations in cotton growing regions. Nevertheless Baker et al (2019) who defined moths emerging from C<sub>4</sub> plants as having a  $\delta^{13}\text{C}$  value of greater than -20‰, reported that between 2012 and 2015, over 30% of *H. punctigera* caught in the Darling Downs were C<sub>4</sub> while in Maranoa and the Namoi for the same period about 10% and 40% respectively were C<sub>4</sub>. In Table 2.1 we used the same criterion to define C<sub>4</sub> moths as that used by Baker et al (2019), and found that the moths we collected were at the lower end of the percentages found by Baker et al (2019), but still indicated that C<sub>4</sub> plants were playing a role as unstructured refuges, even for *H. punctigera*, but that this was variable between seasons.

Nitrogen isotopes, as in other studies, show no clear differentiation between potential host species, despite the large range in  $\delta^{15}\text{N}$  values. While Nitrogen isotopes could be used to identify different food sources in unstructured refuges (different Australian C<sub>4</sub> grasses vary in their  $\delta^{15}\text{N}$  values; Murphy and Bowman 2009) other factors can also affect this ratio, such as water availability, which can reduce  $\delta^{15}\text{N}$  values (Murphy and Bowman 2009).  $\delta^{15}\text{N}$  values also increase 2-3 ‰ each trophic level (Tykot 2004, Ponsard and Arditì 2000) so some of the high values found in this study could indicate cannibalism or predation by some larvae. Different  $\delta^{15}\text{N}$  values have been found in different parts of a plant (Kolb and Evans 2002) so changes in the  $\delta^{15}\text{N}$  values during a season could indicate that the larvae are feeding on different parts of the plant at different times during the season, which could be useful in further studies.

## **Section 3: *Helicoverpa* survival on cotton and pigeon pea refuges.**

### **3.1 Methods**

Critical to the efficacy of refuges/ Bollgard 3 is the ability of *Helicoverpa* to survive to adulthood or be killed on these crops.

#### ***Helicoverpa punctigera* survival on Non-flowering Pigeon pea.**

In January 2018, we tested in the laboratory whether there were differences in survival between *H. punctigera* larvae feeding on non-flowering pigeon pea and flowering cotton. Fifty stems about 10 cm long of non-flowering pigeon pea (variety Sunrise) and flowering non-Bt cotton (variety Sicot 746 RRF) were set up in individual vials (12 cm long, diameter 4 cm). Each vial contained a smaller pot (height 2 cm, lid diameter 4 cm) that fitted snugly at the bottom of the vial and served as the water reservoir for the plant. The stem of the plant to be tested was inserted through a small hole in the lid of the pot. All plant material was collected from the field and replaced every few days. Collected plant material was washed with 10% bleach to destroy bacteria and other harmful substances, then allowed to dry before it was inserted in a vial for the larvae to consume. One larvae per vial was tested.

*Helicoverpa punctigera* larvae (from the laboratory colony) were raised individually on larval diet (Teakle and Jensen 1985) in 45 well trays following standard laboratory protocol (eg Downes et al 2010), until their second instar. At this point fifty larvae were allowed to continue developing on larval diet as controls, while the rest were transferred to a vial containing their test plant. At 5<sup>th</sup> instar the larvae were moved into a larger container with a diameter of around 6.5cm and a gauze lid for ventilation to enable pupation. A water pot (described above) into which the test plant was inserted, was placed in the centre of the container and was surrounded by vermiculite to provide a medium into which the larvae could borrow to pupate.

Control larvae were continued on diet using standard laboratory protocols: young larvae are maintained in 45 trays, and moved to new trays with fresh diet weekly until they reach 3<sup>rd</sup> instar. After third instar larvae are moved to fresh diet in 32 well trays until they are 5<sup>th</sup> instar, at which point they put in small pots (height 2 cm, lid diameter 4 cm) containing a one cm<sup>3</sup> of diet on top of vermiculite, to enable the larvae to burrow down into the vermiculite to pupate.

Ten days after pupation the pupae were removed and placed in fresh small pots until they emerged. We recorded the number of larvae that died, pupated, and emerged as viable moths.

The experiment was repeated in February (when the pigeon pea began flowering) using the same methods but including an additional treatment, flowering pigeon pea. When pupae were removed from the vermiculite ten days after pupation, they were sexed and their width was measured (at the base of the wings). We chose pupae width rather than length because *Helicoverpa* pupae can extend and protract the length of their pupae, resulting in unreliable readings. Pupae were then placed in clean small pots until they emerged.

#### ***Neonate survival and damage on pre-flowering Bollgard 3 cotton.***

We tested whether neonates on Bollgard 3 cotton could survive long enough to cause damage to pin squares (particularly early in the season), and if they did cause damage, recorded whether the cotton could compensate.

In these experiments we developed a standardized protocol where we suspended at least 20 eggs (either *H. armigera* or *H. punctigera*) in about 300µl of water, and used a pipette to eject these onto the growing tip of the cotton. This egg density mimicked field egg pressure of about 200 eggs per metre. All larvae were from Myall Vale laboratory Bt susceptible colonies. The standard control was an ejection of about 300µl of water without eggs onto the growing tip. These growing tips were enclosed in a breathable mesh bag to provide the larvae protection from predation in the field and to capture any dropped pin-squares (Fig 3.1). After one week the bags were removed, and the dropped and retained pin-squares were counted, along with any surviving larvae.

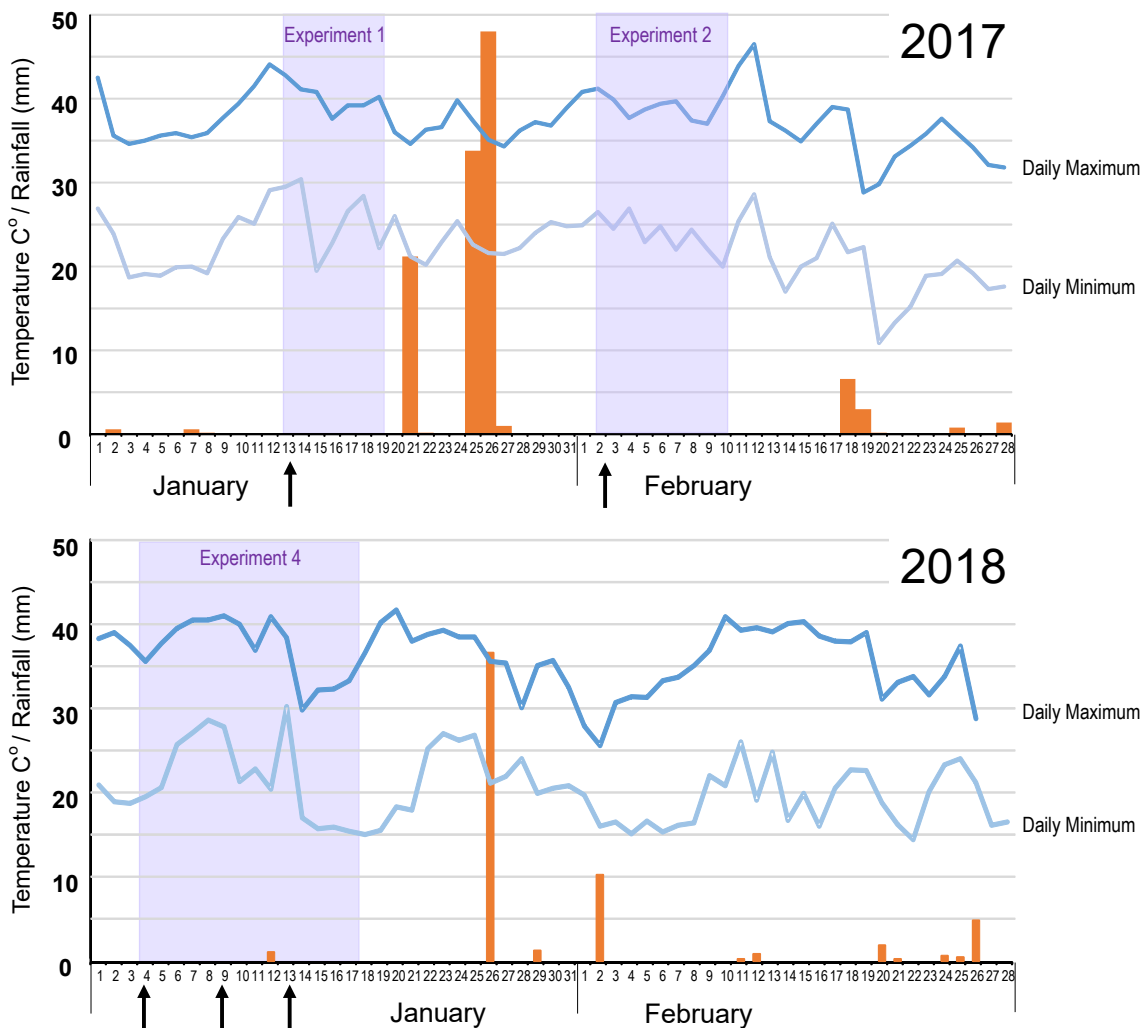


**Fig. 3.1** Ethan Towns (centre) and Abbey Johnson (left with the pipette used to apply the eggs) setting up Experiment 1 on neonate damage at Narrabri.

*Experiment 1*, the pilot experiment, was undertaken from the 13-19<sup>th</sup> of January 2017 at the Australian Cotton Research Institute (ACRI, Lower Namoi) during a heatwave (maximum temperature range: 44 to 37 °C, Fig. 3.2). In this experiment 20 eggs of either *H. armigera* or *H. punctigera* (all from laboratory colonies) were suspended in 200µl of a watery vegetable gum (Xanthan Gum Food grade – E415 Emulsifier) and applied to either the growing tip or first square (10mm wide) of Bollgard 3 (var: Sicot 746B3F, Fig 3.1). They were compared to control tips where only the watery gum was added or no gum was added. To check egg viability and neonate vigour, 300µl of the vegetable gum, with at least 20 *H. armigera* or *H. punctigera* eggs, were applied to the tip and first square of a BG3 plant (one plant for each species that was maintained in water and kept in the shade to check that the eggs hatched, and see if the neonates sampled the cotton). In total there were 8 treatments, 20 reps, 160 samples. After one week the bags were removed, and we recorded any larvae present, and any dropped or retained squares.

*Experiment 2* was undertaken from the 2<sup>nd</sup> to the 10<sup>th</sup> of February 2017 at ACRI, (maximum temperature range: 41 to 36.5 °C; Fig. 3.2) when the cotton was setting fruit. The standard protocol was followed, and the *H. armigera* or *H. punctigera* eggs (all from laboratory colonies) were applied to the growing tips of both Bollgard 3 and non-Bt cotton (var: Sicot 75 RRF) plants. There were controls for both Bollgard 3 and non-Bt cotton plants. In total there were 6 treatments (including the 2 controls) and 10 reps per treatment, equalling 60 samples. To check for egg viability, a 300µl suspension of 20 eggs of *H. armigera* or *H. punctigera* were applied to separate pieces of filter paper and left in a plastic bag in the lab to measure hatching rates.

At the end of the season boll counts and plant mapping was undertaken on the plants used in the experiment.



**Fig. 3.2** The timing of neonate damage experiments (shaded areas), temperature and rainfall (orange histograms) of the lower Namoi (weather station 54038, BOM data) during January and February in 2017 and 2018. The arrows indicate when *Helicoverpa* eggs were inserted into the experiments.

*Experiment 3* was undertaken from the 1<sup>st</sup> to the 8<sup>th</sup> of March 2017 on a private farm at Emerald on late planted (planting date: 17<sup>th</sup> December) cotton that was 5NAWF. The experiment followed the standard protocol, and was undertaken on Bollgard 3 (var: Sicot 746B3F). There were 3 treatments (*H. armigera* or *H. punctigera* eggs and a control) and 12 replicates, producing 36 samples. To check egg viability, 20 eggs of either *H. armigera* or *H. punctigera* (all from laboratory colonies) were suspended in 300µl of water and applied to separate pieces of filter paper (4 replicates each) and kept in the laboratory until emergence. To check for egg viability under field conditions, 20 eggs of either *H. armigera* or *H. punctigera* were suspended in 300µl of water and applied to the growing tip of the adjacent Pigeon pea crop which was not flowering.

*Experiment 4*. In January 2018 we repeated the experiment at ACRI (Fig 3.1), but aimed to test prolonged early season pressure, and the effect of repeated “egglays”. Consequently, using the standard protocol, we applied 20 eggs of either *H. armigera* or *H. punctigera* (all from laboratory colonies) on either Bollgard 3 (var: Sicot 746B3F) or non-Bt cotton (var: Sicot 75RRF) growing tips (6 treatments, 10 reps per treatment, 60 samples). Instead of just containing the larvae at the top of the plant, the whole plant was caged (Fig 3.3) Eggs were reapplied on Day 5 (9<sup>th</sup> Jan) and Day 9 (13<sup>th</sup> Jan). At the beginning of the experiment (January 4<sup>th</sup> 2018) the non-Bt cotton was at first flower (node 15/16), while the BG3 cotton was just before first flower (14 nodes, 10 squares). The experiment ran for two weeks, finishing on the 17<sup>th</sup> of January, by which time the Bollgard 3 crop was on average at 17.9 nodes, and non-Bt cotton at 17.4 nodes. The daily high temperatures ranged from: 29.8 °C to 41 °C (Fig. 3.2).



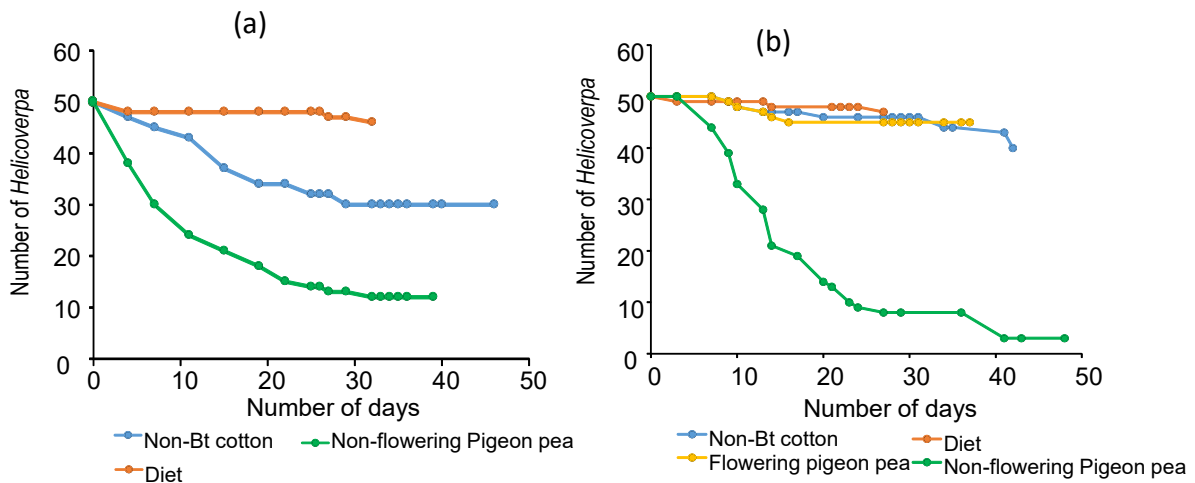
**Fig. 3.3** Field setup for Experiment 4. The plants were completely encaged so that the larvae, if they survived, could move throughout the plant. The zips enabled multiple applications of eggs to mimic sustained heavy egg pressure.

### 3.2 Results

#### *Helicoverpa punctigera* survival on Non-flowering Pigeon pea.

In January there was an overall difference in survival of larvae feeding on the three diets (Chi-square test of independence;  $X=47.73$ ,  $df=2$ ,  $P<0.001$ ) and specifically between those feeding on non-flowering pigeon pea and flowering cotton (Chi-square test of independence;  $X=13.13$ ,  $df=1$ ,  $P<0.001$ ). There was a trend for larvae to die sooner on pigeon pea than non-Bt cotton (Kruskal-Wallis one-way ANOVA,  $H=3.6$ ,  $df=1$ ,  $P=0.053$ ; survival (days): non-Bt cotton- 14.95; non-flowering pigeon pea- 11.1). Out of 50 larvae per treatment, 46 emerged as moths on diet, 30 on cotton plants and 12 on non-flowering pigeon pea (Fig. 3.4).

While larvae emerged as moths faster on the diet (Kruskal-Wallis one-way ANOVA,  $H=62.68$ ,  $df=2$ ,  $P<0.001$ , mean days to emergence: diet: 26.5, non-Bt cotton: 35.1, non-flowering pigeon pea: 36.2), there was no difference in the time it took the moths that developed on non-flowering pigeon pea and cotton to emerge (Kruskal-Wallis one-way ANOVA,  $H=1.27$ ,  $df=1$ ,  $P=0.25$  NS).

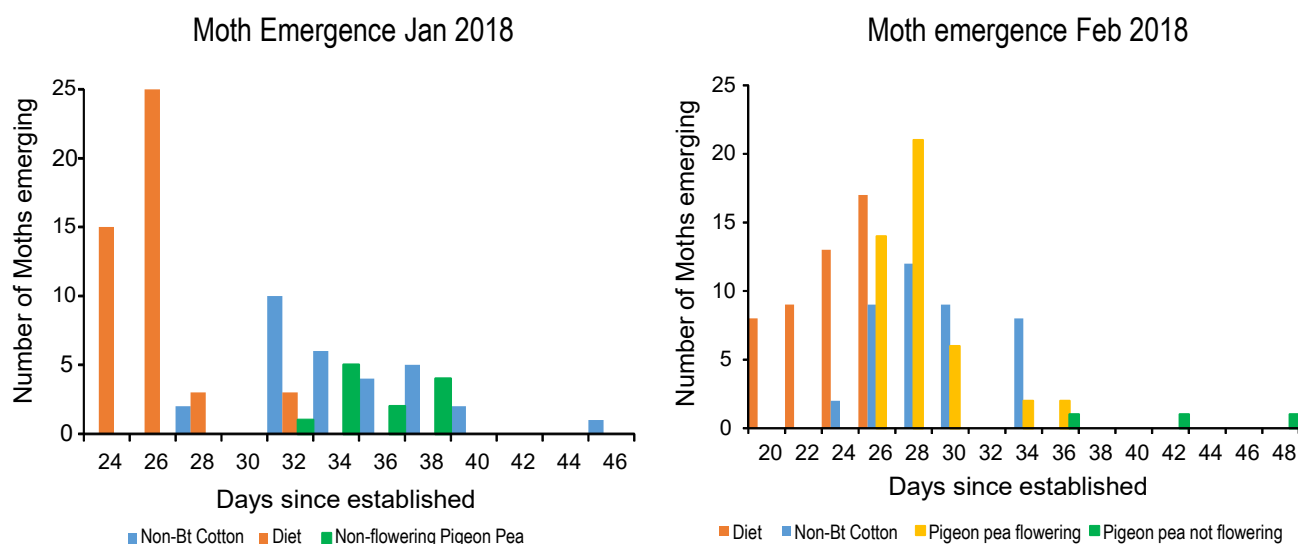


**Fig. 3.4** *Helicoverpa punctigera* survival on non-Bt cotton, non-flowering pigeon pea, larval diet, and (in February) flowering pigeon pea. (a) January experiment; (b) February experiment.

In February, there was an overall difference in larval survival on the four diets, with those on non-flowering pigeon pea showing poor survival (Fig. 3.4 (b), Chi-square test of independence;  $X=117.3$ ,  $df=3$ ,  $P<0.001$ ). Out of 50 larvae per treatment, 46 emerged as moths on diet, 45 on flowering pigeon pea, 40 on non-Bt flowering cotton plants and 3 on non-flowering pigeon pea.

Too few moths emerged from non-flowering pigeon pea to compare emergence times between all four treatments, but all three moths emerging from non-flowering pigeon pea took longer than all of those emerging from cotton and diet (Fig. 3.5). Nevertheless, moths on diet emerged faster than those on either flowering non-Bt cotton (Mann-Whitney U test:  $df=1$ ,  $H=50.09$ ,  $P>0.001$ ) or flowering pigeon pea (Mann-Whitney U test:  $df=1$ ,  $H=56.32$ ,  $P>0.001$ ); but there was no difference in emergence times between flowering pigeon pea and cotton (Mann-Whitney U test:  $df=1$ ,  $H=2.301$ ,  $P=0.129$  NS).

Moths emerging from cotton in February were significantly larger than those emerging from flowering pigeon pea (T-test:  $t=2.33$ ,  $df=62$ ,  $P=0.023$ , mean width: cotton- 5.0mm, pigeon pea- 4.8mm).

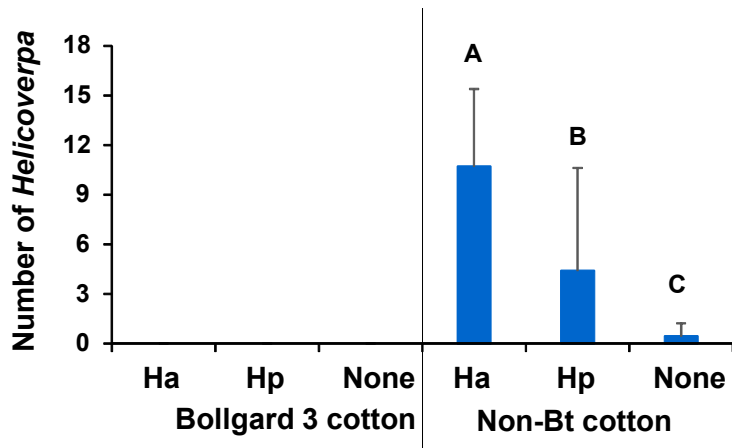


**Fig. 3.5** Time from establishing larvae on non-Bt cotton, non-flowering pigeon pea, larval diet, and (in February) flowering pigeon pea, until their emergence as moths.

### ***Neonate survival and damage on pre-flowering Bollgard 3 cotton.***

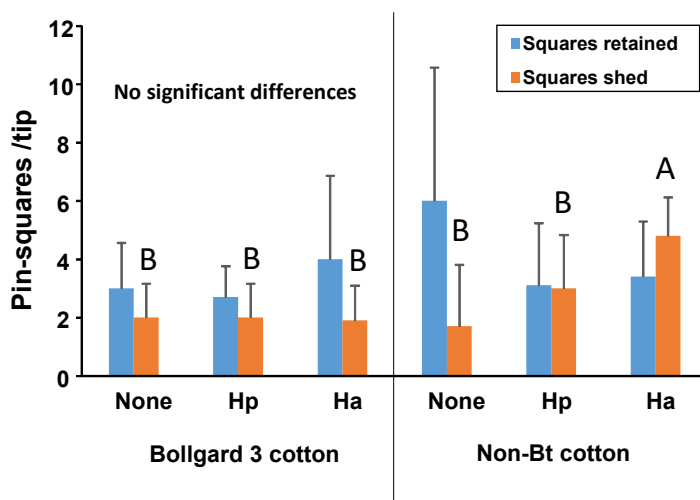
*Experiment 1* (Pilot): After 6 days there was no significant difference between treatments in the number of pin squares retained (ANOVA;  $df=6,152$ ;  $F= 1.4$ ;  $P=0.2$ ; NS), mean pin squares per tip = 6.8) or pin squares dropped (Kruskal Wallis one-way ANOVA combining *H. armigera* and *punctigera* eggs;  $df=3$ ;  $H=0.09$ ;  $P=0.2$ ; NS; mean pin squares per tip dropped = 0.3). However survival of neonates was low, with only 1 to 2 eggs hatching per bag.

*Experiment 2*: In the laboratory viability test, the hatch rate was high (75% for *H. punctigera*, and 85% for *H. armigera*). On the non-Bt cotton treatments, there were 108 larvae in the *H. armigera* treatments, along with 59 larvae in the *H.punctigera* treatments, and 4 larvae in treatments where no eggs had been added (indicating natural eggclays had occurred in those plots). Therefore an average of 11 *H. armigera* (~55% survival, assuming 20 eggs were added) and 5 *H. punctigera* (~25% survival) were found on the growing tips in the *H.armigera* and *H.punctigera* treatments respectively (Fig 3.6).



**Fig.3.6** Neonate survival in Experiment 2 (lower Namoi) after 8 days on Bt and non-Bt cotton showing standard errors. Ha=*H. armigera*; Hp=*H. punctigera*, None= no eggs added (Control).

There was a significant difference in the number of squares dropped overall (ANOVA:  $df=4,54$ ;  $F=5.3$   $P=0.001$ ), with no difference between the Bollgard 3 treatments (ANOVA:  $df=2,27$ ;  $F=0.02$ ;  $P=1$ ; NS) but a significant difference between the non-Bt treatments (ANOVA:  $df=2,27$ ;  $F=7.6$ ;  $P=0.002$ ). In the non-Bt treatments, *H. armigera* treatments dropped significantly more squares than the others (Fig. 3.7). However, there was no difference in the amount of squares retained across all Bollgard 3 and Non-Bt cotton treatments (ANOVA:  $df=4, 54$ ;  $F=2.2$ ;  $P=0.08$ ; NS) although there was a trend for the non-Bt cotton control to retain more fruit (Fig 3.7 ). When total fruit production is compared, more fruit was produced by non-Bt cotton than Bollgard 3, irrespective of treatments (ANOVA:  $df=1,54$ ;  $F=12$ ;  $P<0.001$ ).



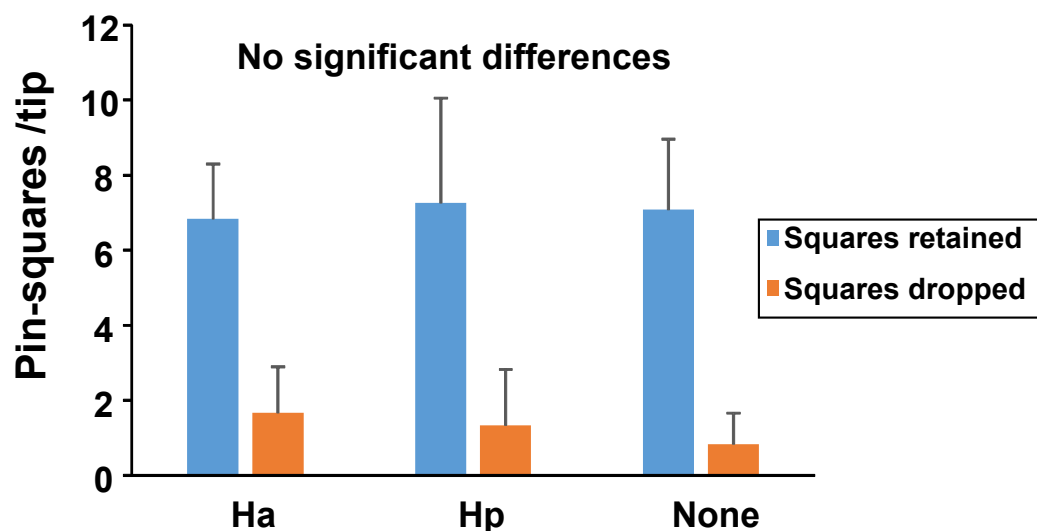
**Fig. 3.7** Pin squares dropped and retained in Experiment 2 on Bt and non-Bt cotton. Ha=*H. armigera*; Hp=*H. punctigera*; None= no eggs added (Control).

At the end of the season we compared between treatments the number of bolls that had developed from fruit that 1) were on the plant when the experiment was set up; and 2) had developed after the experiment had finished. While there was a difference between Bollgard 3 and non-Bt crops on the number of bolls from fruit (1) (ANOVA:  $df=1,54$ ;  $F=4.12$ ,  $P=0.047$ ) with more bolls in the Bollgard 3 crop, there was no effect of adding *H. armigera* or *H. punctigera* eggs on fruit (1) bolls (ANOVA:  $df=4,54$ ;  $F=0.58$ ,  $P=0.68$ ; NS). Likewise there was no difference between these treatments in the number of bolls from fruit (2) (Kruskal-Wallis ANOVA:  $df=5$ ;  $H=1.6$ ;  $P=0.8$ ; NS). The treatments had no effect on total number of bolls (ANOVA  $df=4,54$ ;  $F=0.8$ ;  $P=0.5$ ; NS; mean number of bolls: 8.93).

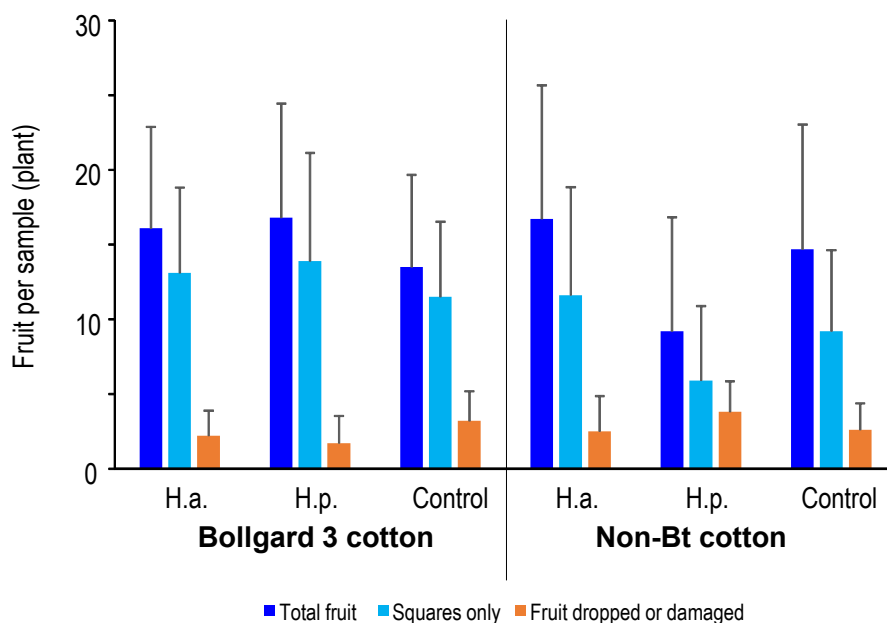
*Experiment 3:* In the laboratory viability test, the hatch rate averaged 43.8% for *H. punctigera*, and 65.8% for *H. armigera*. Survival on the pigeon pea was not high, with an

average of 1.25 *H. punctigera* and 1.75 *H. armigera* larvae (6.25% and 6.74% respectively) found per tip after a week. As the pigeon pea was not flowering, this low survival rate was not surprising (see above).

There was again no significant difference in the number of fruit dropped (ANOVA: df=2,33; F=1.4; P=0.26; NS) or retained (ANOVA: df=2,33; F=0.12; P=0.9; NS; Fig. 3.8) in any of the treatments on Bollgard 3.



**Fig. 3.8** Experiment 3: There was no difference between treatments in the number of pin-squares retained or dropped in the Emerald experiment on Bollgard 3 cotton. Ha=*H. armigera*; Hp=*H. punctigera*; None= no eggs added (Control).



**Fig. 3.9** Experiment 4: The number of fruit per sample after 3 applications of *Helicoverpa* eggs (mimicking 200 eggs/metre each application) over the course of 2 weeks. There was no difference between treatments in the total number of fruit, nor the number of dropped fruit. There were significantly more squares in the Bollgard 3 samples than the non-Bt cotton samples.

*Experiment 4:* The laboratory viability tests gave variable results. For *H. punctigera* and *H. armigera* respectively, there was a 42% and 37% hatching success for Day 1 eggs; 9% and 22% for Day 5; and 26% and 43% for Day 9. After two weeks of adding eggs, no larvae were found on Bollgard 3 samples, and only four non-Bt samples contained larvae: two control cages with 1 larvae; and two *H. punctigera* cages: one with 15 and another with 24 larvae.

After 2 weeks of high egg pressure, neither crop (ANOVA; df=1,54; F=0.9; P=0.33; NS) nor any of the treatments (treatments: adding *H.armigera* eggs, adding *H.punctigera* eggs, not

adding eggs on both Bollgard 3 and non-Bt cotton; ANOVA;  $df=4,54$ ;  $F=1.6$ ;  $P=0.2$ ; NS) affected the total amount of fruit produced. There also was no difference in the amount of fruit dropped between treatments (Kruskal Wallis ANOVA;  $df=5$ ;  $H=7.7$ ;  $P=0.16$ ; NS). The number of squares produced was affected by crop, with Bollgard 3 samples producing more squares (mean = 15.4 squares) than non-Bt samples (mean= 13.5 squares; ANOVA,  $df=1,54$ ;  $F=6.4$ ,  $P=0.014$ ; Fig 3.9).

### 3.3 Discussion

#### ***Helicoverpa punctigera survival on Non-flowering Pigeon pea.***

Our previous work (CSE1304) showed that non-flowering pigeon pea is not attractive to laying moths. These current results indicate that *H. punctigera* larvae that developed on flowering pigeon pea were smaller than those emerging from flowering non-Bt cotton that these larvae took longer to mature than those on flowering non-Bt cotton, and that *H. punctigera* larvae have lower survival on non-flowering pigeon pea. The results suggest that non-flowering pigeon pea is a poor food source for *H. punctigera*, and therefore not an effective refuge for Bollgard 3 cotton with respect to *H. punctigera*.

However some care must be taken with these results because the larvae tested were from a laboratory colony. Nevertheless, the difference in survival in these larvae between non-flowering pigeon pea and flowering cotton is instructive; especially as more recent work in the 2018/19 season indicates that unlike *H. punctigera*, laboratory raised *H. armigera* can grow as well on non-flowering pigeon pea as on non-Bt cotton (Townsend and Whitehouse, unpubl. data). The differences between *H. punctigera* and *H. armigera* in their response to non-flowering pigeon pea again highlight challenges in developing refuge requirements for the industry that is pertinent to both species, and provides a caveat in relying on genetic dilution by non-flowering pigeon pea to support the RMP of *H. punctigera*.

#### ***Neonate survival and damage on pre-flowering Bollgard 3 cotton.***

In the upper Namoi and Emerald we found no effect on fruit load of heavy *Helicoverpa* egg applications either on pre-flowering, flowering, or mid-season cotton crops. Even multiple applications of eggs over a 2 week period, simulating successive heavy eggclays at the onset of flowering, did not affect fruit loss. This indicates that Bollgard 3 is well protected against neonates, reducing the pressure on growers to spray should they encounter heavy egg lays.

However, we also found that hatching rates of *Helicoverpa* eggs was very variable, and could be quite low, contributing to the lack of damage on non-Bt cotton. The use of eggs from laboratory colonies (rather than eggs from wild-caught moths) could have contributed to low survival, although in the second experiment we had high rates of emergences, and good survival on non-Bt cotton after one week (55% and 25% respectively for *H. armigera* and *H. punctigera*) despite the high temperatures. Daytime highs exceeded 40°C during all of the trials undertaken in the Namoi. Constant high temperatures kill *H. armigera* (Zalucki et al 1986, Mironidis et al 2008) so these high temperatures may have contributed to the low hatching rate of *Helicoverpa*, reducing neonate pressure (but see Experiment 2). Additional work looking at survivorship of *Helicoverpa* under high field temperatures would reveal if these temperatures reduce *Helicoverpa* pressure in cotton crops.

Temperature can also affect the plant's response. Reddy et al (1992) found that 93% of squares were aborted when cotton was subjected to a diurnal range of 40°C to 32°C. Thus any square loss when day temperatures are above 40°C are likely to be caused by the temperature

rather than an insect. The interplay between temperature extremes and pest damage needs further investigation.

#### 4. Acknowledgements

I would like to thank our collaborators in Emerald, Sharna Holman, Gail Spargo, and Saba Sinai, for their hard work; Paul Grundy for overseeing their work and providing us with Sunrise seed; and Rob Ingram and Aron Kiely for allowing us to use their farms for our experiments. I would like to thank Leanne Lisle for the carbon isotope analysis, and Colin Tann for undertaking moth collections and playing a central role in the carbon isotope work before retiring; Peter Gregg for in depth discussions on all things about *Helicoverpa* in the field, and Sharon Downes and Tracey Parker for advice and access to ACRI *Helicoverpa* colonies. I also thank my technical staff, Abbey Johnston, Giulio Heimoana, Joel Sampson, Jade Williams and students Ethan Towns, Edwina Murray, Lisa and Zoe Paisley.

#### References

- Ambika, t., W. James, L. Bird, and C. Beard. 2005. Identifying that dietary source of polyphagous *Helicoverpa armigera* (Hubner) using carbon isotope signatures. *Current Science* **89**:1982-1984.
- Baker, G. H., T. Leven, T. May, and C. R. Tann. 2016a. Planting window requirements for Bt cotton in Australia: do they limit the exposure of *Helicoverpa* spp. (Lepidoptera: Noctuidae) to Bt toxins? *Austral Entomology* **55**:32-42.
- Baker, G. H., H. Parry, and C. R. Tann. 2016b. Pigeon pea refuge crops are likely to provide patchy delivery of *Helicoverpa* (Lepidoptera: Noctuidae) within Bt cotton production regions in eastern Australia. *Austral Entomology* **55**:439-448.
- Baker, G. H. and C. R. Tann. 2013. Mating of *Helicoverpa armigera* (Lepidoptera: Noctuidae) moths and their host plant origins as larvae within Australian cotton farming systems. *Bulletin of Entomological Research* **103**:171-181.
- Baker, G. H. and C. R. Tann. 2017a. Broad-scale suppression of cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae), associated with Bt cotton crops in Northern New South Wales, Australia. *Bulletin of Entomological Research* **107**:188-199.
- Baker, G. H. and C. R. Tann. 2017b. Long-term changes in the numbers of *Helicoverpa punctigera* (Lepidoptera: Noctuidae) in a cotton production landscape in northern New South Wales, Australia. *Bulletin of Entomological Research* **107**:174-187.
- Baker, G. H., C. R. Tann, P. Verwey, and L. Lisle. 2019. Do the plant host origins of *Helicoverpa* (Lepidoptera: Noctuidae) moth populations reflect the agricultural landscapes within which they are caught? *Bulletin of Entomological Research*: **109**:1-14.
- Downes, S., T. L. Parker, and R. J. Mahon. 2010. Characteristics of Resistance to *Bacillus thuringiensis* Toxin Cry2Ab in a Strain of *Helicoverpa punctigera* (Lepidoptera: Noctuidae) Isolated from a Field Population. *Journal of Economic Entomology* **103**:2147-2154.
- Downes, S., T. Walsh, and W. T. Tay. 2016. Bt resistance in Australian insect pest species. *Current Opinion in Insect Science* **15**:78-83.
- Fitt, G., J. Daly, C. Mares, and K. Olsen. 1998. Changing efficacy of transgenic Bt cotton-patterns and consequences. .in 5th Australian Applied Entomological Research Conference Sept 29-Oct 2, Brisbane, Australia.
- Fry, B., W. Brand, F J. Mersch, K. Tholke, and R. Garritt. 1992. Automated analysis system for coupled  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  measurements. *Analytical Chemistry* **64**:288.
- Gregg P. C., Fitt G. P., Zalucki M. P., Murray D. A. H. & McDonald G. (1993). Winter breeding and spring migration of *Helicoverpa* spp. in inland Australia, 1989–1991. In: *Pest Control and Sustainable Agriculture* (eds S. A. Corey, D. J. Dall and W. M. Milne). *CSIRO, Canberra*
- Grundy, P. 2018. Sunrise™ – A New Refuge Cultivar of Pigeon Pea for the Bollgard 3 RMP. Bayer.
- Kolb, K. L. and R. D. Evans. 2002. Implications of leaf nitrogen recycling on the nitrogen isotope composition of deciduous plant tissues. *New Phytologist* **156**:57-64.
- Mahon, R. J., S. J. Downes, and B. James. 2012. Vip3A Resistance Alleles Exist at High Levels in Australian Targets before Release of Cotton Expressing This Toxin. *PLOS ONE* **7**:e39192.
- Mironidis, G. K. and M. Savopoulou-Soultani. 2008. Development, Survivorship, and Reproduction of *Helicoverpa armigera* (Lepidoptera: Noctuidae) Under Constant and Alternating Temperatures. *Environmental Entomology* **37**:16-28.
- Murphy, B. P. and D. M. J. S. Bowman. 2009. The carbon and nitrogen isotope composition of Australian grasses in relation to climate. *Functional Ecology* **23**:1040-1049.
- Ponsard, S. and R. Arditì. 2000. What can stable isotopes ( $\delta\text{N-15}$  and  $\delta\text{C-13}$ ) tell about the food web of soil macro-invertebrates? *Ecology* **81**:852-864.

- Reddy, K. R., H. f. Hodges, and V. R. Reddy. 1992. Temperature effects on Cotton Fruit retention.
- Teakle, R. E. and J. M. Jensen. 1985. *Heliothis punctigera*. Pages 313-322 in P. Singh and R. F. Moore, editors. Handbook of Insect Rearing. Eksevier, Amsterdam
- Tykot, R. H. 2004. Stable isotopes and diet: You are what you eat. in M. Martini, M. Milazzo, and M. Piacentini, editors. Proceedings of the International School fo Physics "Enrico Fermi" Course CLIV. IOS Press, Amsterdam.
- Whitehouse, M. E. A., D. Cross, S. Mansfield, S. Harden, A. L. Johnson, D. J. Harris, W. Tan, and S. J. Downes. 2017. Do pigeon pea refuges in Bt cotton pull their weight as “genetic diluters” to counter Bt resistance in *Helicoverpa* moths? *Crop Protection* **100**:96-105.
- Zalucki, M. P., G. Daglish, S. Firempong, and P. Twine. 1986. The biology and ecology of *Heliothis armigera* (Hubner) and *H. punctigera* Wallengren (Lepidoptera: Noctuidae) in Australia: What do we know? *Australian Journal of Zoology* **34**:779-814.
- Zalucki, M. P. and M. J. Furlong. 2005. Forecasting *Helicoverpa* populations in Australia: A comparison of regression based models and a bioclimatic based modelling approach. *Insect Science* **12**:45-56.

**5. Please describe any:-**

- a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.);**
- b) other information developed from research (eg discoveries in methodology, equipment design, etc.); and**
- c) required changes to the Intellectual Property register.**

None applicable

## 6. Conclusion

**Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. What are the take home messages?**

1. Bollgard 3 appeared to be more effective than Bollgard II at managing *Helicoverpa*, but too few moths were attracted to cotton crops to robustly quantify any differences.
2. Very heavy egg lays applied artificially early in the season (pre flower) did not cause fruit loss and yield loss in Bollgard 3 in Emerald nor in the lower Namoi.
3. Four *Helicoverpa* emerged from Bollgard 3 plots at Emerald (over 1680 cage days) while none were caught from Bollgard 3 plots in the lower Namoi (over 11880 cage days). The moths emerged in early season when there were few refuges functioning well. CSE1301 also reported large numbers of moths emerging from earlier Bt crops in Emerald. The risk of resistance evolution may be increased in early season *Helicoverpa* from Emerald
4. The new pigeon pea variety, Sunrise, produced more moths than the old pigeon pea variety Quest, but only once it flowered.
5. *H. punctigera* larvae were less likely to survive on non-flowering Sunrise than on flowering Sunrise or flowering cotton.
6. Summer scholarship work demonstrated that *Helicoverpa punctigera* readily moved between refuges and cotton, so refuges should be close enough for moths to fly between refuges and cotton, but separated so that larvae cannot walk between them (see Appendix 17).
7. In our experiments in all three seasons, Pigeon pea, including Sunrise, flowered a month after cotton. Non-flowering pigeon pea is not attractive to laying moths. Therefore, pigeon pea was only functioning as genetic diluter, particularly for *H. punctigera*, late in the season when it was flowering (and when cotton was becoming less attractive). If this finding is widespread it could be a concern for resistance management.
8. During 201/17 and 2017/18 season, moths emerged from cotton crops and pigeon pea refuges much latter than in previous years, even though cotton was flowering from late December/early January. The cause of the delay is unclear (but may be weather related) and needs further investigation as it assists the RMP.
9. As Sunrise pigeon pea is very productive at the end of the season, it may be an effective trap crop, supporting the RMP by season quarantining. The relative importance of genetic dilution in comparison to seasonal quarantining needs further exploration.
10. In the lower Namoi, unstructured refuges of C<sub>4</sub> plants in both seasons were a consistent source of unexposed *H. armigera* throughout the cotton growing season, but were a less reliable source of *H. punctigera*. In Emerald and Griffith, unstructured refuges of C<sub>4</sub> plants were a source of unexposed *H. armigera* and *H. punctigera*, but not reliably between the two seasons sampled.
11. In summary, our results suggest that Bollgard 3 and the refuge complex are providing good protection to the cotton crop. However, caution is needed around early season genetic dilution, particularly in Emerald and in respect to *H. punctigera*.

## 7. *Extension Opportunities*

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

- (a) to further develop or to exploit the project technology.
- (b) for the future presentation and dissemination of the project outcomes.
- (c) for future research.

### **Bt Stewardship Award:**

The plenary talk that MW gave at the 2017 Australian Cotton Research Conference on IPM increased interest in the Bt stewardship award, which has broadened its scope. After discussions with other researchers including Simone Heimoana, Graham Charles and Karen Kirkby, we will develop its focus to be a good citizenship award for best practise in refuges, insect, weed, and disease management. We are in discussion with a couple of organizations about financing the award (\$2000). We are planning to use the myBMP system to identify potential candidates.

To increase the profile of refuges we are preparing a submission in support of a “Bt stewardship” award for each region. The Bt stewardship award would recognize a grower from each region who had taken care of their refuges and strongly contributed to the Resistance Management Plan.

The finding that *H. punctigera* did poorly on non-flowering pigeon pea lead us to test the ability of *H. armigera* to survive on non-flowering pigeon pea. Analysis is incomplete, but results so far suggest that *H. armigera* does much better on non-flowering pigeon pea than *H. punctigera* (Towns and Whitehouse unpubl. data).

Work from CSE1304 indicated that non-resistant *H. punctigera* larvae may be able to survive on Bollgard II bolls and pass this tolerance onto their offspring. We have been testing if these larvae can do likewise with Bollgard 3. Analysis is incomplete, but to date it appears that they cannot (Towns and Whitehouse unpubl data).

Work with Sharna Holman indicated that *Helicoverpa* egg lays pre-flowering did not affect fruit load. We followed up this work by collaborating with extension officers lead by Amanda Thomas to test if pre-flowering mirid pressure at different sites around Australia reduced fruit counts and yield. We found that overall there was no effect of early season pre-flowering mirid pressure on yield (see Appendix 11) but the results suggested further studies combining early season damage with environmental factors such as cloudiness. This work is ongoing in the CRDC project (1819FRP038).

## 8. A. List the publications arising from the research project and/or a publication plan.

(NB: Where possible, please provide a copy of any publication/s)

### Peer reviewed

- Appendix 1:* Lowe, E.C., Latty, T., Webb, C.E., Whitehouse, M.E.A., Saunders, M.E. 2019. Engaging urban stakeholders in the sustainable management of arthropod pests. **Journal of Pest Science**. <https://doi.org/10.1007/s10340-019-01087-8>
- Appendix 2:* Baker, G. H., C. R. Tann, P. Verwey, and L. Lisle. 2019. Do the plant host origins of *Helicoverpa* (Lepidoptera: Noctuidae) moth populations reflect the agricultural landscapes within which they are caught? **Bulletin of Entomological Research** **109**:1-14.
- Appendix 3:* Walsh, T.K., Joußen, N., Tian, K.T., McGaughran, A., Anderson, C.J., Qiu, X., Ahn, S.J., Bird, L., Pavlidi, N., Vontas, J., Ryu, J., Rasool, A., Barony Macedo, I., Tay, W.T., Zhang, Y.J., Whitehouse, M., Sylvie, P., Downes, S., Nemeč, L. and Heckel, D.H. 2018. Multiple recombination events between two cytochrome P450 loci contribute to global pyrethroid resistance in *Helicoverpa armigera*. **PLoS ONE**.**13 (11) e0197760**.
- Appendix 4:* Lewis J. Wilson, Mary E.A. Whitehouse, Grant A. Herron 2018. The management of Insect Pests in Australian Cotton: An Evolving Story. **Annual review of Entomology** **63**:215-237.
- Appendix 5:* Baker, G. H. and C. R. Tann. 2017a. Broad-scale suppression of cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae), associated with Bt cotton crops in Northern New South Wales, Australia. **Bulletin of Entomological Research** **107**:188-199.
- Appendix 6:* Baker, G. H. and C. R. Tann. 2017b. Long-term changes in the numbers of *Helicoverpa punctigera* (Lepidoptera: Noctuidae) in a cotton production landscape in northern New South Wales, Australia. **Bulletin of Entomological Research** **107**:174-187.
- Appendix 7:* Whitehouse, M.E.A., Cross, D., Mansfield, S., Harden, S., Johnson, A., Harris, D., Tan, W., Downes, S. 2017 Do pigeon pea refuges pull their weight as “genetic diluters” to counter *Bt* resistance in *Helicoverpa* moths? **Crop Protection** **100**:96-105. <http://dx.doi.org/10.1016/j.cropro.2017.05.030>
- Appendix 8:* Baker, G. H., T. Leven, T. May, and C. R. Tann. 2016a. Planting window requirements for Bt cotton in Australia: do they limit the exposure of *Helicoverpa* spp. (Lepidoptera: Noctuidae) to Bt toxins? **Austral Entomology** **55**:32-42.
- Appendix 9:* Baker, G. H., H. Parry, and C. R. Tann. 2016b. Pigeon pea refuge crops are likely to provide patchy delivery of *Helicoverpa* (Lepidoptera: Noctuidae) within Bt cotton production regions in eastern Australia. **Austral Entomology** **55**: 439-448.

### Industry

- Appendix 10:* Downes, S., Whitehouse, M., Heimoana, S., Tay, W., Walsh, T. 2018. Terminating the mega-pest. Biosecurity a key to effective Integrated Pest Management. **The Australian Cottongrower** **39(4)**: 14-17.
- Appendix 11:* Thomas, A., Williams, S., Whitehouse, M., Dickinson, S., Twine, A. 2018. Does high mirid pressure at early squaring have an effect on yield? **The Australian Cottongrower** **39(4)**: 28-31.

*Appendix 12:* Sequeira, R., Whitehouse, M.E.A., Williams, S., Maas, S. 2018. Revised mirid spray thresholds – what they mean and how to use them. **The Australian Cottongrower** **39(3):** 42-45.

*Appendix 13:* Whitehouse, M.E.A., Herron, G.A., Heimoana, S.C., Wilson, L.J. 2017. What is the value of IPM in cotton production systems? **The Australian Cottongrowers Cotton Yearbook 2017** **38:** 154-160

*Appendix 14:* Whitehouse M.E.A., Holman S. 2017. Helicoverpa egg lays: should you spray? **Spotlight** Spring 2017.

*Appendix 15:* Whitehouse M.E.A. 2016. Healthy, Attractive and rogue free. **Spotlight** Summer 2016-2017.

### **Student Theses**

*Appendix 16:* Murray E. 2016. Detecting Emerging Moth Pests in Genetically Modified Cotton Crops (AFNR4102 Research Project B, University of Sydney)

Tompkins C. 2017. Flood Irrigation: how ants react, respond and recover in cotton. (Masters Thesis, University of Sydney)

*Appendix 17:* Paisley L. 2017. Investigating the Movement of *H. punctigera* Larvae from Pigeon Pea to Bollgard III Cotton. (Fourth year Honours Thesis, University of Sydney)

*Appendix 18:* Paisley Z. 2017. The role of inter- and intra-specific competition in larval movement off refuges by *Helicoverpa armigera* and *Helicoverpa punctigera*. (Fourth year Honours Thesis, University of Sydney)

### **Conference presentations**

2017. **3<sup>rd</sup> Australian Cotton Research Conference** (Australia). **Plenary speaker:** What is the value of IPM in cotton production systems?

2015. **The 34<sup>th</sup> International Ethological Conference** (Australia) The ramifications of oviposition and foraging decisions on resistance management and induced tolerance.

2015. **2<sup>nd</sup> Australian Cotton Research Conference** (Australia). The response of *Helicoverpa* to Bt toxins and refuges: the role of tolerance and the loss of efficacy.

### **Additional publications of associated work (Peer reviewed)**

Dreyer, D., Frost, B., Mouritsen, H., Günther, A., Green, K., Whitehouse, M., Johnsen, S., Heinze, S., Warrant, E. 2018. The Earth's magnetic field and visual landmarks steer migratory flight behaviour in the nocturnal Australian Bogong moth. **Current Biology** **28:2160-2166**.

Rendon, D., Hagler, J., Taylor, P.W., Whitehouse, M.E.A. 2018. Integrating immunomarking with ecological and behavioural approaches for assessing consumption of *Helicoverpa* spp. larvae by wolf spiders in cotton. **Biological Control**. **122:51-59**.

Rendon D., Whitehouse M. E. A., Taylor P. W. 2016 Consumptive and nonconsumptive effects of wolf spiders on cotton bollworms. **Entomologia Experimentalis et Applicata**. **158 (2):**170-183. DOI: 10.1111/eea.12390

Rendon D., Whitehouse M. E. A., Hulugalle N.R., Taylor P.W. 2015. Influence of Crop Management and Environmental Factors on Wolf Spider Assemblages (Araneae: Lycosidae) in an Australian Cotton Cropping System. **Environmental Entomology** **44(1):** 174-185. DOI:10.1093/ee/nvu025.

### **B. Have you developed any online resources and what is the website address?**

No online resources have been developed

## ***Part 4 – Final Report Executive Summary***

---

The aim of the Resistance Management Plan (RMP) in Australian cotton is to stop *Helicoverpa* developing resistance to Bt cotton. There are two main strategies to this plan. Season Quarantining, where potentially resistant *Helicoverpa* are stopped from passing their genes between seasons; and Genetic Dilution, where so many moths are produced by other vegetation that any moth emerging out of Bt cotton will mate with these moths, thereby reducing the chance of homozygous resistant offspring (see front cover). The aim of structured refuges, such as pigeon pea, is to produce enough moths to provide genetic dilution. Unstructured refuges, which are other vegetative sources in the environment that produce moths that have not been exposed to Bt cotton, also contribute to genetic dilution.

The aim of this project was to test the efficacy of Bollgard 3 and to test if the RMP was well supported by structured and unstructured refuges. To do this the project compared the ability of these crops to produce moths; and tested the survival of larvae on Bt cotton and pigeon pea.

We found that there was no larval survival or crop damage in the lower Namoi and Emerald on Bollgard 3 resulting from pre-flower *Helicoverpa* egg pressure of 200 eggs per metre. This is good news for resistance management and crop protection.

No moths emerged from Bollgard 3 cotton in the Namoi, but *Helicoverpa* pressure in cotton in this study was lower than in previous studies, as few moths emerged from Bollgard II or non-Bt cotton, but in the case of *H. armigera*, pigeon pea did experience similar pressure to other years. The reduced pressure on cotton in comparison to pigeon pea by *H. armigera* assists the RMP of *H. armigera*.

Four moths emerged from early season Bollgard 3 cotton in Emerald (none from pigeon pea). Cotton can be in the ground all year round in Emerald (therefore there is continual pressure on *Helicoverpa* by Bt toxins). Also, apparently few refuges provide genetic dilution for early season cotton in Emerald. These findings suggest that there is a stronger resistance risk in early season Emerald cotton. One possible response would be to closely monitor for Bt resistance in *Helicoverpa* moths emerging early season in Emerald.

Pigeon pea, including Sunrise, flowered a month after cotton. Non-flowering pigeon pea is not attractive to laying moths. In addition, summer scholarship work showed that *H. punctigera* larvae will walk off non-flowering pigeon pea onto flowering cotton, and we found that *H. punctigera* larvae have difficulty surviving on non-flowering pigeon pea. Therefore, pigeon pea was only functioning as genetic diluter, particularly for *H. punctigera*, late in the season when it is flowering (and when cotton is becoming less attractive). This is a concern for resistance management.

However, in this project moths emerged from both cotton crops and pigeon pea refuges much latter than in previous years, even though cotton was flowering from late December/early January. The cause of the delay is unclear and needs further investigation as it assists the RMP.

In addition, other factors may counter the lack of genetic dilution early in the season. This study found that early season genetic dilution could be supported by unstructured refuges including C<sub>4</sub> plants, other crops and locations such as the stock route (but the support may not be consistent between years). These factors assist the RMP.

In summary, our results suggest that Bollgard 3 and the refuge complex are providing good protection to the cotton crop. However, caution is needed around early season genetic dilution, particularly in Emerald and in respect to *H. punctigera*.