Australian Government

Cotton Research and Development Corporation

SUMMER SCHOLARSHIP REPORT: 2014-15 SEASON

1.	**Project Title** (Maximum 15 words)	: Testing for a 'critical exposure period' for developing tolerance to Bt toxin
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3.	**Summer Scholar and University**	: Sharna Holman, The University of Sydney
4.	**Organisation & Location for the project**	**: Australian Cotton Research Institute, Narrabri**

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SUMMER SCHOLARSHIP REPORT

Testing for a 'critical exposure period' for developing tolerance to Bt toxin

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1. Executive Summary:

Helicoverpa punctigera, along with *Helicoverpa armigera*, are major pests in Australian cotton. They are currently controlled using "Bt cotton" which contain genes derived from the bacteria *Bacillus thuringiensis*, that produce proteins toxic to *Helicoverpa*. While most effort has focused on preventing *Helicoverpa* spp. developing genetic resistance to these toxins, laboratory studies have shown that larvae which are not resistant are able to tolerate low to medium levels of toxin. This "induced tolerance" could lead to larvae surviving on Bt cotton without being resistant, and it could provide a stepping stone to the development of resistance. While *Helicoverpa* are known to develop some tolerance after one generation of exposure to Bt toxins, we did not know whether exposure is required throughout the entire larval period or only during particular instars. The aim of this summer project was to test if exposing the larvae *Helicoverpa punctigera* at different larval stages to 2% or 5% toxin concentrations of the discriminating dose of Cry1Ac toxin (used by CSIRO's Resistance Team) would affect larval development and lead to tolerance in their offspring.

The results confirmed that after exposing only one generation of larvae to low levels of Cry1Ac toxins their offspring were able to tolerate higher levels of Cry1Ac than the controls. In addition we found that larvae exposed to Cry1Ac in early instars overcompensated their growth once they fed on non-toxin diet, and those exposed as late instars actively tried to avoid the toxin and developed into smaller moths. While the offspring of larvae exposed to 5% toxin as late instars showed the most tolerance, those exposed to 2% toxin as late instars also produced significantly more tolerant offspring. These results indicate that the critical period for the development of tolerance is late in larval development.

These results have implications in respect to Bt cotton efficacy, and could have implications in respect to the placement of refuges. They suggest that larvae moving off other crops and completing their development in Bt cotton could produce offspring at least as tolerant as those completing their development within the Bt crop. Therefore ideally refuges need to be far enough away from Bt cotton to avoid older larvae moving into the cotton.

2. Background:

Helicoverpa spp. are economically important pests of agriculture throughout the world, including Australia where *Helicoverpa armigera* and *Helicoverpa punctigera* can dramatically reduce cotton yield. The economic losses to Australian cotton growers that occur from yield reduction and control mechanisms of all *Helicoverpa* spp. (such as spray applications and scouting) has been upwards of $250 million per annum (CSIRO 2010). In addition, *Helicoverpa armigera* in particular has developed resistance to most chemical sprays used in its control, making it very difficult to manage with insecticides alone.

The use of transgenic Bt cotton by the industry has overcome many of these management issues, leading to a reduction in chemical insecticides used in cotton, allowing for better control of insect pests and assisting the establishment of integrated pest management schemes within the Australian cotton industry (Naranjo 2011). Transgenic Bt cotton contains insecticidal proteins derived from the bacteria *Bacillus thuringiensis*. The current Bt cotton, Bollgard II, contains Cry1Ac and Cry2Ab toxins. With the extensive use of Bt toxins in cotton, the development of resistance is a serious threat. Currently Bt toxins are effective in managing *Helicoverpa*, however the cotton industry needs to remain vigilant to ensure the ongoing success of these insecticidal proteins and Bt cotton.

Most previous research on the ability of *Helicoverpa* spp. to overcome Bt toxins focused on their ability to develop resistance. Genetic resistance is based on target site mutations producing individuals resistant to concentrations of very high toxin. However other ways by which Helicoverpa can overcome Bt toxins may exist. Under laboratory conditions larvae which do not have genetic resistance are able to tolerate low to medium levels of toxin. The tolerance slowly increases over many generations of exposure and is referred to as inducible tolerance (Rahman *et al*. 2004).

Inducible tolerance seems to be caused by gene and protein regulatory mechanisms, however the underlying mechanisms are still largely unknown (Rahman *et al*. 2011). Ma observed and suggested that the glycolipids in gut derived lipid particles may be the target for mature toxin allowing for sequestration in the gut lumen (Ma *et al*. 2012). This sequestration reaction removes lipid particles from lipid metabolism with the observed increases in the tolerance to Bt toxin being associated with high fitness costs (Rahman *et al*. 2011). Knowing how *Helicoverpa* spp. develop tolerance is important to profitable risk management in cotton cropping systems. *Helicoverpa* are known to develop some level of tolerance after one generation of exposure, however we do not know whether exposure is required throughout the entire larval period or only during particular instars.

This project focused on exposing *Helicoverpa* to 2% or 5% toxin concentrations of the discriminating dose (used by CSIRO's Resistance Team to detect genetic resistance) to early and late instar larvae. Results from a current project on refuge management and tolerance (CSE1304) indicated that the grandchildren of *Helicoverpa punctigera* moths emerging from Bt cotton expressed higher levels of tolerance to Cry1Ac (M. Whitehouse & M. Rahman, pers. com.). Consequently, for this study we used *Helicoverpa punctigera* and the toxin Cry1Ac.

3. Aims and Objectives:

Due to the nature of the experiment there were several distinct yet related aims and objectives. In particular, we set out to answer: i) Does exposure to 2% or 5% of the discriminating dose of toxin enable larvae to develop some tolerance after only one generation? ii) Does exposure to toxin at different larval stages affect the development of tolerance? iii) How does exposure to different levels of toxin alter life history characteristics such as lifespan and size?

The aims of the project align with CRDC's strategic outcome of ensuring that the Australian Cotton Industry is the global leader in sustainable agriculture.

4. Materials and Methods:

Part I. Raising colonies under different toxin exposure treatments

Insect strains. An established Bt toxin-susceptible *H. punctigera* laboratory strain was provided by CSIRO, Narrabri.

Larval rearing. *H. punctigera* were maintained on seven artificial diets incorporating two different levels of sub-lethal toxin concentrations throughout their lifecycle in multi-well plastic trays heat-sealed with polyester film in the Australian Cotton Research Insectary, Narrabri. The seven treatments included i) control of 0% toxin, ii) 2% toxin in early larval stages until 3rd instar then being placed onto non-toxin diet until pupation, iii) 2% toxin in later larval stages, being on a non-toxin diet until 3rd instar, iv) 2% toxin diet throughout larval stages, v) 5% toxin in early larval stages until 3rd instar then being placed onto non-toxin diet, vi) 5% toxin exposure in later larval staged, being on a non-toxin diet until 3rd instar and vii) 5% toxin exposure throughout their larval stages.

Cry1Ac toxin. Cry1Ac toxin derived from *B. thuringiensis* strain GHD 73, was incorporated into the artificial diets. Two levels of concentration were used throughout the experiment, 2% and 5%. Preliminary assays were conducted using a broad range of Cry1Ac concentrations to determine the amount to be formally used and the 2 and 5% values were chosen because larval survival rates were 60-80% on these toxin concentrations (data not shown). As the aim was to ensure about 80 larvae in each treatment completed development and we expected different survival rates with different treatments, we varied the number of larvae initially set up in each treatment: 180 control larvae; 360 2% early exposure, 360 2% late exposure, 720 2% continual exposure, 675 5% early exposure, 675 5% late exposure, 1260 5% continual exposure. In total 4230 larvae were used in these experiments.

Moths and egg collection. Forty male and female moths from the seven treatments were used for the next stage of the experiment. The 80 moths from each treatment were placed in their respective buckets with honey solution for food. Moths were chosen on the basis of emerging early, being healthy and having no defects in their wings. The open end of the buckets were covered with nappy liners as an egg laying substrate. After three days the liners containing *H. punctigera* eggs were collected and replaced by another liner for further egg laying and collection. The first eggs collected were kept as a back-up, while the eggs from days four and five were used as the main collection of viable eggs for tolerance testing. The collected liners were placed inside resealable plastic bags and kept in a cool room before being transported to The University of Adelaide, Waite Campus. Eggs were collected for a further three days after the main collection as further back up.

Developmental Penalties and Delays. To determine the effects of varying exposure periods to sub-lethal concentrations of Bt toxins, development and growth rates from the seven treatments were determined and compared. The duration of the larval period was recorded by noting larval stage and survivorship every 4th and 3rd day periodically from placement of neonates until pupation. The number of larvae that escaped the trays was also recorded and used as a measure of avoidance behaviour. Larvae were given up to 50 days to reach pupation before being removed from the experiment. The pupal weights were recorded from varying days

throughout pupation. The moths' body size were recorded by measuring the shoulder width of the moth, from the joint of the wings.

Part II. Tolerance Bioassays

Tolerance Bioassays. Tolerance bioassays were performed by using artificial diet overlaid with a crude bacterial suspension containing Cry1Ac. In each bioassay fresh artificial diet was poured into 45-well trays and left to solidify in a fume hood. Bioassays for each of the seven toxin exposure treatments were then conducted with 8 concentrations, including a Milli-Q water control, with an aim of 45 larvae for each concentration. The toxin containing stock solutions had been diluted in Milli-Q water to specific concentrations, and 75µl aliquots were pipetted evenly over the artificial diet. After being left to dry, one neonate was placed in each well and sealed as described previously. The efficiency of the concentrations on the varying treatments were assessed on day 10.

Statistical analysis. Analysis is only in the preliminary stage at this point in time. Nevertheless, Mortality data for the tolerance bioassays were analysed through POLO-PC Software (LeOra Software, Berkeley, CA) to estimate the Lethal Concentrations (LC). Differences in susceptibility were considered significant when the 95% confidence intervals did not overlap, with attention being paid to low toxin levels around LC50.

Results:

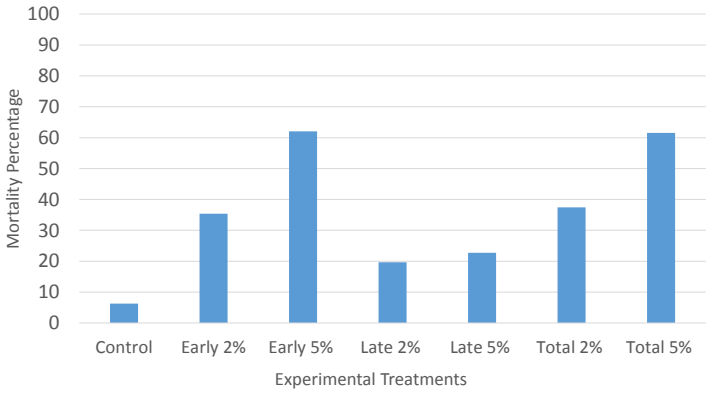

Figure 1. The averaged mortality rates of the colonies associated with the various toxin treatments. Total exposure and early exposure to toxin had similar mortality rates, however larvae exposed to 5% toxin had a higher mortality rate than those exposed to 2% toxin.

Mortality and Survivorship Rates. Toxin exposure period had a significant impact on mortality rate of larvae (Figure 1). The control cohorts had an average mortality rate of 6.28%. For all exposure periods, larvae exposed at a 5% toxin concentration had a higher mortality rate than those at a 2% toxin concentration. Larvae exposed to 2% toxin concentration in late larval stages had a mortality rate of 19.68% while larvae exposed to 5% toxin concentration had a mortality rate of 22.73%. The treatments exposed to toxin in early larval stages had similar mortality rates to the larvae exposed to toxin consistently. Early exposure to 2% toxin concentration had a mortality rate of 35.4% while exposure at 5% toxin concentration resulted in an average mortality rate of 62.03%. The average mortality rate of cohorts exposed to 2% toxin concentration continuously was 37.45% while continuous exposure at a 5% toxin concentration level was 61.55%.

Effect of toxin and exposure period on larval development. The toxin concentration and exposure period both had an impact on larval development and the time required to reach pupation (Figure 2). The control took an average of 14 – 18 days to reach pupation. The late exposure treatments both initially experienced the same growth in the first 14 days. Toxin concentration impacted the later development with the 2% late exposure taking approximately 28 days to reach pupation and the 5% late exposure treatment approximately 32 days to reach pupation. The early and total exposure treatments both had similar growth rates for the initial 6 days. The 2% early exposure took approximately 32 days to reach pupation while the 5% early exposure treatment took approximately 35 days to reach pupation. The total exposure treatments were most affected and had the largest impact on larval development. The 2% total exposure took 42 days to reach pupation while it took the 5% total exposure treatment approximately 46 days to reach pupation.

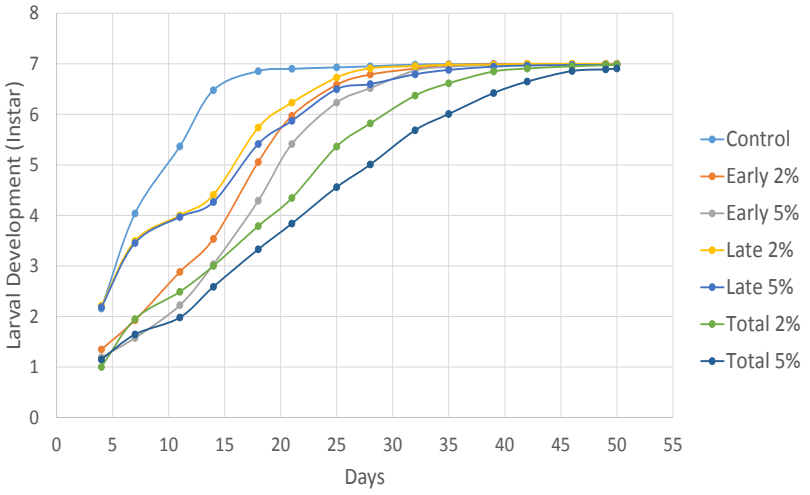

Figure 2. The effect of toxin concentration and exposure period on larval development and growth curves.

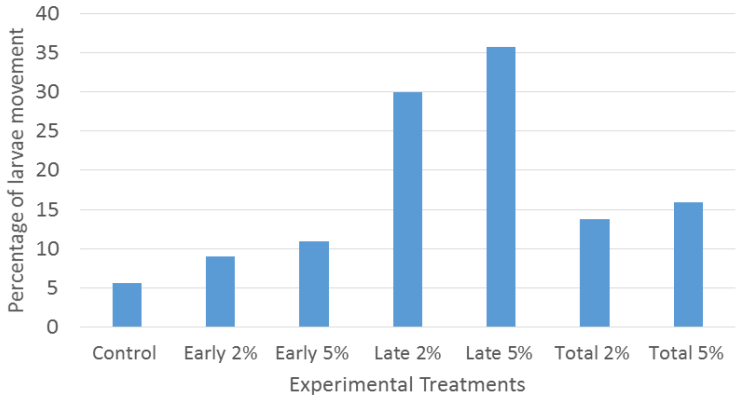

Figure 3. Percentage of larvae attempting to escape calculated by averaging the larvae that escaped in the cohort from the initial number of larvae.

Larval Behaviour and Movement. There was greater larval movement (escape attempts) in the larvae exposed to toxins in late instars, than those exposed in early instars or throughout their larval stage (Figure 3). Those exposed to toxins throughout their larval life stage were slightly more likely to escape than those only exposed in early instars. Larvae exposed to higher levels of toxin concentration (5%) were more likely to escape than those exposed to lower concentrations within each exposure period.

Correlation between pupae mass and moth body span. Similar to larval development and growth rates, toxin concentration and exposure period also had an impact on the pupae mass and later moth size of the individual. The correlation between pupae mass and moth size for all treatments were positive (Figure 4). Compared to the control group, the larvae exposed to Bt toxin in early instars 'over' compensated once put on non-toxic diet, as per shoulder span they had heavier pupae. Alternatively, the larvae that had been reared on toxin throughout their whole larval life have the same ratio of pupae mass to moth size as the controls; while the larvae exposed to toxin in the latter instars were lighter than the controls of the same size. The larvae that were reared on a late 5% toxin diet suffered the greatest penalties as they were smaller and lighter than the controls.

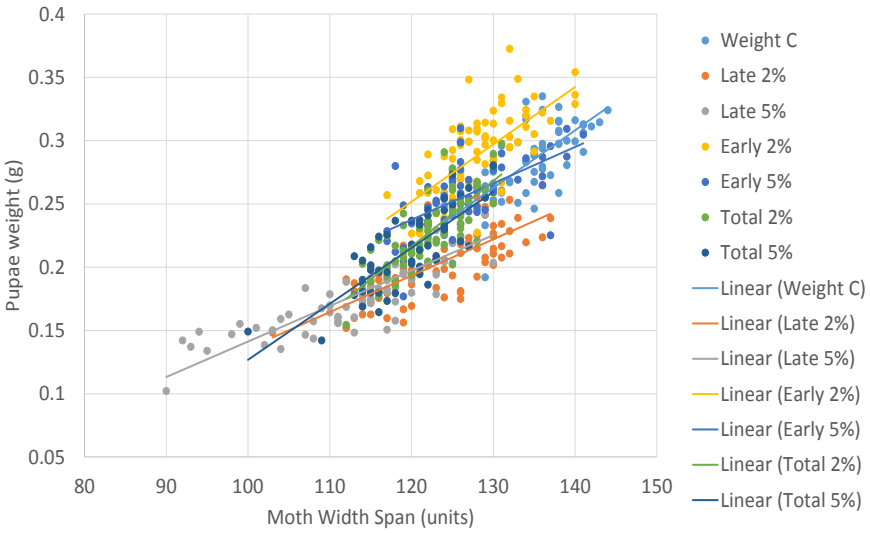

Figure 4. The correlation between pupal mass (g) and moth shoulder width span for the healthy moths of the different treatments.

Inducible Tolerance Bioassays. The inducible tolerance bioassays confirmed that only exposing larvae for one generation to low levels of toxin can increase tolerance to their offspring (Figure 5). Larvae exposed to 5% toxin, late in their larval development, produced offspring with the highest levels of tolerance.

The data also suggests that exposure to higher levels of sub-lethal Bt toxins will have an effect on the rate of developing tolerance. Overall there were statistically significant differences between the control and the late 2%, late 5% and total 5%.

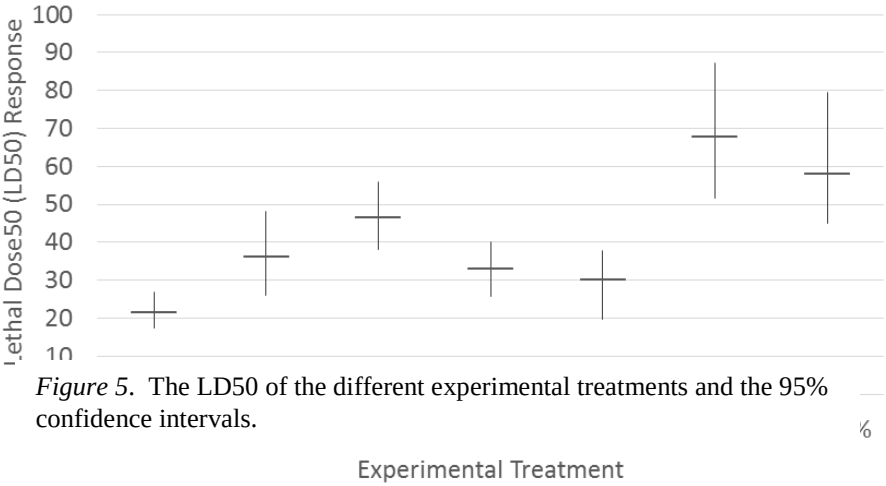

Figure 5. The LD50 of the different experimental treatments and the 95% confidence intervals.

5. Discussion and Conclusions:

We examined various developmental penalties associated with exposure to low levels of Cry1Ac toxin at different concentrations and exposure time periods in a *H. punctigera* population under laboratory conditions. We were focused on whether this level of exposure would manifest in the next generation as low level tolerance to Cry1Ac toxins in the offspring. As the population used

was a pre-existing laboratory susceptible strain of *H. punctigera* provided by CSIRO, any increased larvae survival could not be due to pre-existing recessive resistance alleles.

Our data suggest developmental penalties were not only influenced and associated with toxin concentrations but also with when the larvae were exposed to the toxin. Larvae exposed as early instars (up to 3rd instar) and throughout their larval life had similar mortality and survivorship rates, indicating the larvae were most susceptible to toxin in early instars. The larvae that were exposed to toxin only as latter larval stages suffered significantly less mortality (although it was still higher than that of the control).

The growth curves and larval developmental time varied under different experimental treatments, indicating possible penalties to larvae caused by heightened immune activities. Exposure to toxin at an early stage resulted in slow growth rates, with those exposed to 2% toxin growing slightly quicker (approximately 3 days) than those on 5% toxin. The initial delayed growth rates disappeared once the larvae were placed on the normal non-toxic diet, where growth increased significantly. While these larvae still required a longer period of time to reach pupation, their pupae mass was on average heavier than that of the other experimental treatments. When larvae were exposed to toxin at a late stage, the initial growth rates for both 2 and 5% exposure were similar to no exposure (control). However, when these same larvae were moved from non-toxin to toxin diets, their growth rate plateaued before increasing at a rate that exceeded their growth rate on a normal non-toxin diet. Larvae in the late toxin exposure treatments were able to recognise and reject the toxins in their diets with these larvae actively attempting to escape out of their wells. The total toxin exposure treatments were delayed throughout larval life stage however their growth rate increased slightly once they reached third instar. Previous studies also noted that sub-lethal exposure of Btk to *H. armigera* neonates is detrimental to development and fitness. Studies speculate that the increase in the length of time larvae take to reach pupation is caused by physiological alterations resulting from interactions between Bt toxins and the gut epithelium (Sedaratian, A et al., 2013). Sedaratian also suggests that the larvae exposed to toxin in early instars may have a longer growth period in order to compensate for the costs associated with recovering from the damage of the Bt toxins, including increased feeding activity. Both the mortality and growth rate data indicate that once reaching third instar the likelihood of death decreases and growth rates increase for all treatments.

These data from a laboratory experiment with *H. punctigera* have practical implications for the Australian Cotton Industry. The results align with the findings from previous studies which show that *H. punctigera* can detect Bt toxins in their diet and will actively avoid feeding on these toxins. The avoidance behaviour is more significant when larvae suddenly encounter toxin in latter instars. This finding has implications to larvae in cotton fields. As different parts of Bt cotton plants have different toxin levels, larvae initially feeding on areas with lower levels of toxin are probably more likely to seek out other parts of the plant that have the least amount of toxin.

The inducible tolerance bioassays indicated that the critical exposure period required for *H. punctigera* to develop tolerance to Cry1Ac toxin is in the late instars (from third instar to pupation). The offspring from these treatments were significantly more tolerant of Cry1Ac toxins that those from the control and the other treatments. The results also suggest that the toxin concentration level exposed to the larvae does affect the rate at which the offspring will develop tolerance. Interestingly, the late and total 2% treatments had higher increased tolerance levels than the early 5% treatments suggesting that the time period of toxin exposure may be more important than toxin level.

The tolerance data as well as escape behaviour could impact on the spatial arrangement of refuges and cotton crops. There is a greater chance that if larvae are forced off refuges into Bt cotton crops they will be able to tolerate the Bt toxin in the crop as well as more able to effectively search for parts of the cotton plant that express lower amounts of toxin.

Overall, in this summer project we were able to conclude that the exposure period in which larvae consume toxin influences the developmental penalties caused by toxins, such as changes in pupal mass, moth shoulder width span and larval life span. *H. punctigera* can develop tolerance (in laboratory conditions) after one generation of exposure to 2% and 5% toxin concentrations, with the late larval stages as the 'critical exposure period' for the development tolerance to Cry1Ac toxin.

6. Highlights:

- *H punctigera* larvae exposed to low levels of Cry1Ac toxin develop more slowly than those not exposed to toxin.
- Exposure to Cry1Ac toxin in later instars has a greater detrimental effect on the body mass of adults than exposure at earlier instars.
- After reaching third instar the likelihood of larval death decreases and the rate of growth increases.
- Larvae exposed (as late instars) to 2% and 5% of the discriminating dose of Cry1Ac toxin for only one generation produced offspring that had higher levels of tolerance to Cry1Ac.
- The offspring of larvae exposed to 2% or 5% Cry1Ac toxin as late instars showed higher tolerance than those exposed as early instars respectively. The offspring of larvae exposed as late instars to 5% toxin developed the highest levels of induced tolerance.
- These results are relevant to the placement of refuges, as they indicate that refuges need to be far enough away from Bt cotton to avoid older larvae moving into the cotton.
- I have thoroughly enjoyed my time at the Australian Cotton Research Institute and would love to pursue different opportunities that will allow me to continue to complete and undertake research in Australian cotton.

7. Future Research:

This research project has been extremely interesting in potentially leading to other areas of research. These future areas of research include larvae movement within the cotton crop and refuges, as well as a 'longer' version of the experiment where future generations of offspring are subjected to the same toxin and exposure treatments as their parents to see how many generations it requires for the developmental delays to decrease to achieve similar growth rates, body size and mass to that of the controls. From our research we observed there was a difference in the ratio between pupae mass and moth body size in the different treatments. It would be interesting if we could see how these differences then effect reproductive potential e.g. effects on reproductive system and/or low egg production.

8. Presentations and Public Relations:

Jan 2015: Talk at the Australian Cotton Research Institute titled: Testing for a critical exposure period for developing tolerance to Bt toxin.

I hope to be able to present my research at the Behaviour 2015 Conference in Cairns, 9th – 14th August 2015

9. Reference List:

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