**Australian Government**
**Cotton Research and**
**Development Corporation**

# FINAL REPORT 2015

## For Public Release

*Part 1 - Summary Details*

**CRDC Project Number:** DAN 1201

## Project Title: Characterisation of neonicotinoid resistance in the cotton aphid, *Aphis gossypii* Glover

**Project Commencement Date:** 01/01/12  **Project Completion Date:** 30/06/2015

**CRDC Research Program:** 2 Industry

*Part 2 – Contact Details*

| | | |
|---|---|---|
| **Administrator:** | Cara Brooks, External Project Systems Administration | |
| **Organisation:** | NSW DPI | |
| **Postal Address:** | Locked Bag 21, Orange, NSW, 2800 | |
| **Ph:** 02 6391 3651 | **Fax:** 02 6391 3336 | **E-mail:** cara.brooks@dpi.nsw.gov.au |
| **Principal Researcher:** | Kate Marshall, Research Entomologist | |
| **Organisation:** | NSW DPI | |
| **Postal Address:** | Private Bag 4008, Narellan, NSW, 2567 | |
| **Ph:** 02 4640 6389 | **Fax:** 02 4640 6300 | **E-mail:** kate.marshall@dpi.nsw.gov.au |
| **Primary Supervisor:** | Dr Grant Herron, Principal Research Scientist | |
| **Organisation:** | NSW DPI | |
| **Postal Address:** | Private Bag 4008, Narellan, NSW, 2567 | |
| **Ph:** 02 4640 6471 | **Fax:** 02 4640 6300 | **E-mail:** grant.herron@dpi.nsw.gov.au |
| **Supervisor 2:** | Prof. Steven Djordjevic, Professor of Infectious Disease | |
| **Organisation:** | University of Technology, Sydney | |
| **Postal Address:** | PO Box 123 Broadway 2007 | |
| **Ph:** 9514 4127 | **Fax:** 9514 4143 | **E-mail:** steven.djordjevic@uts.edu.au |

**Signature of Research Provider Representative:** _____

**Date Submitted:** _____

## Part 3 – Final Report

*Background*

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

Worldwide the cotton aphid, *Aphis gossypii* (Hemiptera:Aphididae), is a highly polyphagous species that inflicts serious damage to a broad range of agricultural, horticultural and greenhouse crops (Blackman and Eastop 2000). Damage may be directly via feeding on the phloem sap of young plants leading to significant yield reductions (Blackman and Eastop 2000); indirectly via the transmission of plant virus diseases (Blackman and Eastop 2000, CABI 2005); and lastly through honeydew contamination of the open boll lint (Schepers 1989) which can severely downgrade cotton fibre quality (Miller et al. 1994).

The pest status of *A. gossypii* in Australian cotton has steadily increased since the 1990's when it was considered a late season secondary pest suppressed by insecticides used against other insect species (Wilson 1996). The introduction of Bt-transgenic cotton into Australia in the 1990's, which contains a toxin deadly to the primary cotton insect pest *Helicoverpa* spp., significantly reduced the number of insecticide sprays required for their control (Fitt 2003). These sprays were inadvertently controlling secondary pest populations including *A. gossypii*, which consequently increased (Wilson 1996). In the late 1990s significant damage from *A. gossypii* via the transmission of the poleovirus Cotton Bunchy Top (CBT) disease (Reddall et al. 2004) led to reduced aphid tolerance by growers, and an increase in the number of targeted sprays against them. These sprays led to resistance in pest populations of *A. gossypii* that have caused the chemical control to fail. Spray failures against aphids can permanently tarnish Australia's reputation for producing high quality lint if failures lead to 'sticky cotton'. Failures also increase grower costs and the likely hood of unforeseen environmental consequences.

Recently in Australian cotton there have been failures against aphids belonging to the neonicotinoid insecticide group (Herron and Wilson 2011). This group is among the most effective chemical class for control of sucking insect pests (Jeschke and Nauen 2008). Like nicotine, they act on the nicotinic acetylcholine receptors (nAChRs) of insects where they mimic the agonistic action of acetylcholine (Ach), an abundant neurotransmitter, to cause irreversible hyper-excitation and death (Matsuda, Buckingham et al. 2001). In Australian cotton two neonicotinoid compounds, (thiamethoxam and imidacloprid) provide up to 92% of all insecticide seed treatment and are used by industry to provide seedling cotton protection against a range of sucking and chewing pests (Mass 2012, AVPMA 2013a). Thiamethoxam is by far the most popular with an estimated share of 80% of all seed treatment use (Herron and Wilson 2011). The neonicotinoid insecticide clothianidin

(Shield®) was registered for use in Australian cotton in the 2008-09 cotton season (AVPMA 2013b). It has established itself as the most effective foliar neonicotinoid insecticide for control of the green mirid *Creonaties dilutus* and *A. gossypii* due to fast knockdown and competitive pricing (Sumitomo Chemical Australia 2010). It became clear with the neonicotinoid failures that the sustainable management of *A. gossypii* in Australian cotton was at risk. As a result, research to restore neonicotinoid efficacy was seen as an industry priority as part of an integrated program to better manage mites and mirids in Australian cotton.

To restore efficacy of neonicotinoid insecticides, an understanding of their resistance status, along with the underlying resistance mechanisms was considered necessary. Outside Australia, target site insensitivity was found to confer high level resistance to neonicotinoids in the closely related green peach aphid *Myzus persicae* (Bass et al. 2011). A point mutation of the nAChR β1 subunit (Loop D) (termed R81T) resulted in an arginine to threonine substitution at amino acid position 81 (Bass et al. 2011). As *M. persicae* and *A. gossypii* share close homology in their genetic make-up, it is reasonable to assume that mutations in R81T may also be associated with neonicotinoid resistance in *A. gossypii*. If an R81T link is not found then the role of metabolic detoxification in conferring resistance would need to be explored.

In summary, this study will produce improved resistance management of neonicotinoid insecticides by increasing the repertoire of knowledge we currently have regarding insecticide resistance mechanisms in Australian *A. gossypii*. By firstly understanding the status of neonicotinoid resistance, we may then elucidate its underlying genetics so that development of a molecular diagnostic for rapid and precise monitoring of resistant genotypes within a population is achievable. Importantly, this information can significantly contribute to the design and application of sustainable pest management strategies; the ultimate aim of which are to restore efficacy of chemical compounds so that their value is not lost to agriculture.

*Objectives*

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

*2.1 Objective 1: PhD study on neonicotinoid resistance*

*2.1 Milestone 1: PhD student enrolled*

*2.1.1 Performance indicator 1.1: Enrolled student on site at EMAI*

Objective met: Ms Kate Marshall enrolled at the University of Technology with PhD candidature confirmed in January 2012. Ms Marshall was previously employed by NSW DPI so was immediately on site at the Elizabeth MacArthur Agricultural Institute (EMAI).

**2.2 Objective 2:** *Determine the practical consequence of neonicotinoid resistance in the field*

**2.2 Milestone 2:** *Determine the practical consequence of resistance of neonicotinoid resistance in the field.*

**2.2.1 Performance indicator 2.1:** *Relate the phenotypic expression of resistance detected via bioassay to potential field control failures via a glasshouse based trial.*

Objective met: This objective was met via two randomized complete block design (RCBD) efficacy glasshouse trials completed at EMAI. The first trial required potted cotton plants grown from thiamethoxam treated seed (Cruiser® and Cruiser Extreme®) to be challenged with thiamethoxam-susceptible and -resistant *A. gossypii* to see if aphids could complete their development. Against susceptible *A. gossypii* each treatment was highly effective providing control of >90% for 42 days. For resistant *A. gossypii* the study found continued use of either thiamethoxam treatment would select for resistant phenotypes and probably restrict the useful life of neonicotinoid insecticides against this pest. As all of the seed treatments currently registered for control of *A. gossypii* on cotton belong to the neonicotinoid mode of action (MoA) group 4A, management options including alternative chemical rotations are very limited. At-planting or in-furrow granular insecticides are one possible alternative to seed coated treatments but their use must be carefully considered requiring a second RCBD trial. This was achieved with an organophosphate at-planting side dressing utilizing phorate (Thimet®), effective at controlling a range of sucking insect and mite species present in seedling cotton. Thus, in the second trial, side-dressing of cotton seed with phorate 200 g/kg (Thimet®) was investigated as a replacement for established neonicotinoid seed treatments. The trial found phorate to effectively provide plants with protection from pirimicarb-susceptible *A. gossypii* but against pirimicarb-resistant *A. gossypii*, control was not statistically different to that of untreated cotton plants (P>0.05). This was critical information as it demonstrated cross resistance between phorate and pirimicarb. To maintain the effectiveness of pirimicarb and phorate in Australian cotton their use must be carefully managed. As a result of this second trial, it was recommended that the first foliar spray applied to cotton following a phorate side dressing should not be pirimicarb or any other insecticide affected by insensitive cholinesterase (*Ace*1) type resistance.

**2.3 Objective 3:** *Molecular genetics*

**2.3 Milestone 3:** *Molecular genetics used to characterise neonicotinoid resistance*

**2.3.1 Performance Indicators 3.1:** *Molecular genetics identifies a single point mutation*

Objective met: Molecular genetic techniques were employed to elucidate a region of DNA known to contain a mutation conferring resistance to neonicotinoid insecticides. The point mutation termed

R81T, is located in the loop D region of the nAChR β1 subunit and has been found previously in *M. persicae* from Europe and *A. gossypii* from China and Korea to cause neonicotinoid resistance. Thus, in three thiamethoxam-resistant *A. gossypii* strains (F 101, Glen twn S and Carr) studied here from Australia the R81T mutation was proposed as the likely causal mechanism of resistance. Unexpectedly however, PCR amplification of that mutation site and comparative sequence analysis between susceptible (Sus F 96) and resistant strains revealed that the R81T mutation was not correlated with resistance in Australian *A. gossypii*. Therefore, metabolic detoxification was investigated as an alternate resistance causing mechanism using the synergist piperonyl butoxide (PBO). This new avenue of research was completely successful and the use of PBO in tandem with thiamethoxam in bioassays either completely or partially suppressed resistance, suggesting that thiamethoxam resistance in Australian *A. gossypii* from cotton is at least in part, mediated by overexpression of metabolic detoxification enzymes.

Detoxification as the cause of neonicotinoid resistance in *A. gossypii* was further studied via Illumina next generation sequencing (NGS) technology. The aim was to identify differentially expressed genes (DEGs) in response to thiamethoxam stress, by comparing the transcriptomes of thiamethoxam-resistant strains (F 101, Glen twn S and Carr) to the reference susceptible strain (Sus F 96). Bioinformatics analysis revealed a number of significantly differentially expressed genes in resistant strains as candidates for a role in thiamethoxam resistance ($P \leq 0.001$). Transcript expressions (CL1190 and CL1418) were confirmed by quantitative real-time PCR (qRT-PCR) and the trends in gene expression observed by qRT-PCR matched those of the Illumina expression profiles. Unfortunately initial sequencing did not detect any allelic variants in the gene sequence (of transcripts CL1190 and CL1418) which may have corresponded with the increased level of gene expression observed in RNA-Seq and qRT-PCR analysis. However, there is great potential for future research to build on this study to develop a molecular diagnostic for thiamethoxam resistance. This would provide a rapid and cost effective assay for monitoring of resistant genotypes in the field.

*2.4 **Objective 4**: PhD degree*

*2.4 **Milestone 4:** PhD Degree finalised*

*2.4.1 **Performance Indicator 4.1** PhD written, examined and passed*

<u>Objective met:</u> A thesis has been written and submitted to the University of Technology for examination. Supervisors Dr. Grant Herron and Prof. Steven Djordjevic both consider the thesis well written and good quality with Prof. Djordjevic noting 'well done'.

*Methods* [**Note:** The following methods section has been condensed from the attached thesis (Appendix E)].

3. **Detail the methodology and justify the methodology used. Include any discoveries in methods that may benefit other related research.**

### 3.1 General methods

### 3.1.1 Aphid collection and culturing

Aphid strains were collected by researchers, CRC Regional Extension Officers, consultants and growers from commercial cotton fields or cotton plants in the vicinity of commercial crops. All were sent to the bioassay laboratory at Menangle (EMAI) and each field strain was cultured separately on pesticide-free cotton at $25 \pm 4°C$ under natural light. Strain integrity was assured by maintaining populations in purpose built insect proof cages. A reference laboratory susceptible strain (Sus SB) collected from an unsprayed source was maintained under insecticide-free conditions and its susceptibility to several chemicals has been documented (Herron et al. 2001). A second susceptible strain Sus F 96 was collected off commercial cotton in the Queensland (QLD) region of St. George during 2011 and has previously been shown susceptible to a range of chemicals used for *A. gossypii* control (Herron et al. 2013). Field strains F 101 and Glen twn S were collected during the 2010-11 growing season off cotton from St. George (QLD) and Toobeah (QLD), respectively. In 2012, a third field strain termed Carr was collected off cotton in Moree New South Wales (NSW). Lastly, strain Mon P was collected off commercial cotton and had previously been determined to be pirimicarb resistant (Herron et al. 2013).

### 3.1.2 Pressuring

Each resistant strain was routinely pressured (every 8-12 weeks) whilst they were maintained in culture to prevent reversion from resistant to susceptible. Pressuring required a potted insecticide free cotton plant to be placed into a fume cupboard where it was insecticide sprayed to run off. Once the sprayed plant had dried it was transferred into a cage of the correct chemical / strain combination so that *A. gossypii* could infest it (as was done with routine culturing above). This was achieved by picking at random 30-40 leaves from the old plant and placing them onto the newly sprayed plant. Importantly, when a newly sprayed plant was placed into a cage the old plant was immediately removed so there was no unsprayed harbourage for susceptible aphids.

### 3.2 Bioassay

### 3.2.1 Chemicals tested

Aphids were treated with commercial proprietary formulations of clothianidin (Shield[®]), thiamethoxam (Actara[®]) or PBO (Endura PB 80 EC-NF).

*3.2.2 Methodology*

Adult apterous aphids were tested by placing them in a 35 mm Petri dish on an excised cotton plant leaf disc fixed in agar (Herron et al. 2001). Briefly, batches of thirty aphids per leaf disc were then sprayed with a single discriminating dose (to separate susceptible from resistant) of insecticide with the aid of a Potter spray tower (to yield percent insecticide susceptible). All tests were replicated and included a water-only sprayed control. After spraying, clear plastic film was used to cover the Petri dishes, which were then maintained at 25 ± 0.1°C in 16:8 L:D for 24 h. Mortality (unable to walk when prodded) was evaluated with the aid of a stereo microscope by counting the number of live aphids on the leaf disc and subtracting the survivors from the pre-treatment count. To yield full log-dose probit regressions from which resistance factors (RFs) could be calculated, serial concentrations of formulated thiamethoxam selected to achieve $0 < x < 100\%$ were sprayed using the methods outlined above. Each full log-dose probit regression was replicated three to four times (on different days) and included a water only sprayed control that produced <10% mortality.

For synergist bioassays, methodology was the same as insecticide only tests except PBO was prepared in reverse osmosis (RO) water at a rate of 0.2 mL PBO / 100 mL RO water and that was used in all synergist study insecticide dilutions (in place of water alone).

*3.3.3 Analysis*

For discriminating dose tests percent mortality was calculated to yield percent susceptible. Log-dose probit analysis was done without replicate pooling using a stand-alone probit program developed by Barchia (2001) that ensured variability between replicates is taken into account during the analysis. The program applies the method of Finney (1971) including data adjustment for natural mortality (Abbott 1925). Significant heterogeneity is identified using a $\chi^2$ test. If significant at the 5% level the variance of the estimated parameter is scaled by the corresponding heterogeneity factor equal to the residual mean deviance (Finney 1971). RFs were calculated by dividing the $LC_{50}$ of the resistant strain by the $LC_{50}$ of a reference susceptible strain. The corresponding 95% confidence interval (CI) of the calculated $LC_{50}$ ratio was used to determine significance (Robertson et al. 2007).

## 3.4 *Glasshouse trial*

*3.4.1 Chemicals tested*

Cotton seed treatments included: Untreated Control (cotton seed variety Sicot 71); two thiamethoxam containing seed treatments: 2.76 g a.i./kg seed (Cruiser[®]); 5.52 g a.i./kg seed (Cruiser Extreme[®]) and the organophosphate at-planting side treatment; phorate 200 g/kg (Thimet[®]). For the thiamethoxam trial approximately 60 seeds of the following treatment groups: untreated control;

Cruiser®; and Cruiser Extreme® were individually planted into plastic pots (11.5cm diameter) filled with NativeMix™ premium potting mix (180 treated pots in total) and held in a room maintained at 28 ± 2˚C (Figure 1). In the phorate trial, approximately 60 seeds of treatment groups: untreated control and Thimet® (side dressing of phorate equivalent to 3 kg/ha) were planted (120 treated pots in total) and maintained as above.



**Figure 1** Approximately sixty seeds of each treatment group were individually into plastic pots and held in a growth room under grow light (that appears purple).

*3.4.2 Methodology*

At planting and on another three occasions over the following six days 150 mL of water was poured over the soil surface of each pot. During the trial, pots were watered by filling their saucers when necessary. When dicotyledons emerged [week after planting (termed Day 0)], six pots from each treatment group were transferred onto individual saucers in insect proof cages maintained at 25 ± 4˚C and subject to natural light.

Strains were randomized to cages ("whole-plots") and treatments were randomized to two and three pot positions within cages ("sub-plots") for phorate and thiamethoxam trials, respectively, forming a RCBD. Two apterous adult aphids (susceptible or resistant) were placed onto each of the plants within each cage and each cage contained only susceptible or resistant aphids. On Day 7 all leaves were removed from each plant and final aphid numbers were counted with the aid of a stereo microscope. This process was repeated with new plants at weekly intervals until Day 49 by which time susceptible aphids could survive on both thiamethoxam treatments.

### 3.4.3 Analysis

Trial analysis was done via generalised linear mixed models in ASReml (Gilmour et al. 2009). The response (number of aphids) was analysed as quasi-poisson (over-dispersed Poisson with log link) for each trial using a mixed model comprising fixed strain, treatment (within strain) and linear day effects and all associated interactions. Also analysed were random factor day effects and interactions with treatment: strain, strain by treatment, as well as cage, cage by day and position. Wald type F-tests for fixed terms in the model are reported, as well as contrasts to test for treatment efficacy and interactions between treatment efficacy and (linear) day.

The Henderson-Tilton formula (Henderson and Tilton 1955), was used to calculate the corrected percentage efficacy of each insecticide treatment against susceptible and resistant *A. gossypii*.

### 3.5 **PCR amplification of the R81T mutation site**

### 3.5.1 Methodology

Briefly, RNA was extracted from individually pooled samples of 200 adult apterous female aphids for each of the different field strains and transcribed to cDNA. Following cDNA synthesis, each extraction was subject to PCR amplification using primers designed to amplify a 350 bp fragment covering the R81T mutation site. Resultant PCR products for each strain were sequenced by the Australian Genomic Research Facility (AGRF).

### 3.5.2 Analysis

Sequencing data of each strain were aligned to a reference imidacloprid resistant *A. gossypii* strain (IMI-R) (GenBank accession number: JQ627836) containing the R81T mutation using the sequencing software program Sequencher®.

### 3.6 **Transcriptome analysis**

### 3.6.1 Assembly and functional annotation

Approximately 10-20 μg total RNA per strain (Sus F 96, F 101, Glen twn S and Carr) were sent to the Beijing Genomics Institute (BGI), Shenzhen, China for cDNA library construction and Illumina sequencing using the HiSeq™ 2000 platform.

### 3.6.2 Gene ontology (GO) and Clusters of orthologous groups (COGs) classification

Transcriptome de novo assembly was carried out using the Trinity short reads assembling program (Grabherr et al. 2011). Unigene sequences were annotated using the following protein databases: non-redundant (NR) protein database in NCBI, Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG) and COGs using BLASTX searches (e-value<0.00001). The BLAST results were used to perform a tentatively functional annotation of the unigenes. ESTScan software was

also used to determine the annotation of sequences that were not aligned to any of the databases mentioned above (Iseli et al. 1999). Functional annotation by GO terms (GO; http://www.geneontology.org) was analysed with the program Blast2GO program (Conesa et al. 2005).

### 3.6.3 Quantitative RT-PCR

Four differentially expressed transcripts between thiamethoxam resistant and susceptible strains were selected for independent validation of their gene expression via qRT-PCR. Total RNA was isolated from susceptible (Sus F 96) and resistant (F 101, Glen twn S and Carr) strains and then used in subsequent cDNA synthesis reactions. Synthesized cDNAs were used as templates for qRT-PCR in a 7500 Real-Time PCR Detection System. Gene specific primers were designed using Primer3Plus and synthesized by Sigma Aldrich®, Australia.

### 3.6.4 Analysis

#### 3.6.4.1 Transcript expression differences between resistant and susceptible transcriptomes

To compare the transcriptomes from resistant and susceptible *A. gossypii*, gene expression levels were calculated by mapping clean reads to the reference transcriptome using SOAPaligner / SOAP2 (http://soap.genomics.org.cn/soapaligner.html) (Li et al. 2009). The expression level for each gene was calculated by using the RPKM method (Reads Per kb per Million reads) (Mortazavi et al. 2008). The transcript fold change was then calculated by the formula of log2(Res_ RPKM/Sus_RPKM). Genes were classified as differentially expressed using an algorithm derived from "The significance of digital gene expression profiles" (Audic and Claverie 1997). False discovery rate (FDR) $\leq$ 0.001 and the absolute value of log2Ratio $\geq$ 1" were thresholds used to judge the significance of gene expression difference.

#### 3.6.4.2 Quantitative RT-PCR

Fold changes in gene expression between resistant and susceptible strains were derived by the comparative cycle threshold (CT) method using the endogenous control β-actin to standardize expression (Livak and Schmittgen 2001).

**Results** [Note: The following results have been condensed from the original thesis (Appendix E)].

## 4. Detail and discuss the results for each objective including the statistical analysis of results.

### 4.1 Objective: Relate the phenotypic expression of resistance detected via bioassay to potential field control failures via a glasshouse based trial

### 4.1.1 Bioassay

*4.1.1.1 Discriminating dose tests*

Strain F 101 contained the lowest frequency of thiamethoxam susceptible individuals (47%) whilst strain Carr contained the highest frequency of susceptible individuals (82%) (Table 1). Against clothianidin, strain F 101 also contained the lowest proportion of susceptible individuals (67%), whilst strains Carr and Glen twn S contained 92 and 96%, respectively. Discriminating dose tests with thiamethoxam at pressuring rates of 0.05 g a.i./L for strains F 101 and Glen twn S and 0.1 g a.i./L for strains Carr, confirmed that resistant phenotypes in each strain were maintained for the duration of this study.

**Table 1** Resistance detection (percent susceptible) in *A. gossypii* strains Sus SB, F 101, Glen twn S and Carr using bioassay [Thia (thiamethoxam) and Clo (clothianidin)] methodology.

| Strain | Thia 0.02* | Clo 0.05* |
|---|---|---|
| Sus SB | 100% | 100% |
| F 101 | 47% | 67% |
| Glen twn S | 67% | 96% |
| Carr | 82 | 92% |

* Dose sprayed in g a.i./L; results control corrected according to Abbott (1925)

*4.1.1.2 Full log-dose probit tests*

For strains F 101, Glen twn S and Carr full log-dose probit analysis yielded RFs of 49.20- (95% CI 35.43-68.33), 51.31- (30.55-86.19) and 85.00- (65.29-110.66) fold against thiamethoxam, respectively, when initially field collected (Figure 2). As indicated by overlapping 95% CIs at the $LC_{50}$ level no significant difference between strain responses was observed. Strains F 101, Glen twn S and Carr all showed significant heterogeneity (P<0.05) (as indicated by $\chi^2$ values of 33.01, 91.63 and 49.59, respectively) and so were not a good fit to the probit model with excessive heterogeneity accounted for by a scaled fiducial limit calculation. Regression slope values for strains F 101 (1.59 ± 0.16), Glen twn S (1.18 ± 0.20) and Carr (2.18 ± 0.19) were less than that of Sus SB which had the highest slope value recorded at 2.40.

*4.1.2 Glasshouse efficacy trial* (related output may be found in Appendix D)

*4.1.2.1 Thiamethoxam*

Both Cruiser[®] and Cruiser Extreme[®] provided 100% protection of strain Sus F 96 for 14 days (Figure 3). On Cruiser[®] treated seed control of strain Sus F 96 remained very high (>90%) until day 49 where residual efficacy was reduced to 87%. In contrast, residual efficacy of Cruiser Extreme[®] provided greater control at 49 days of 93%. Cruiser Extreme[®] also provided higher initial protection
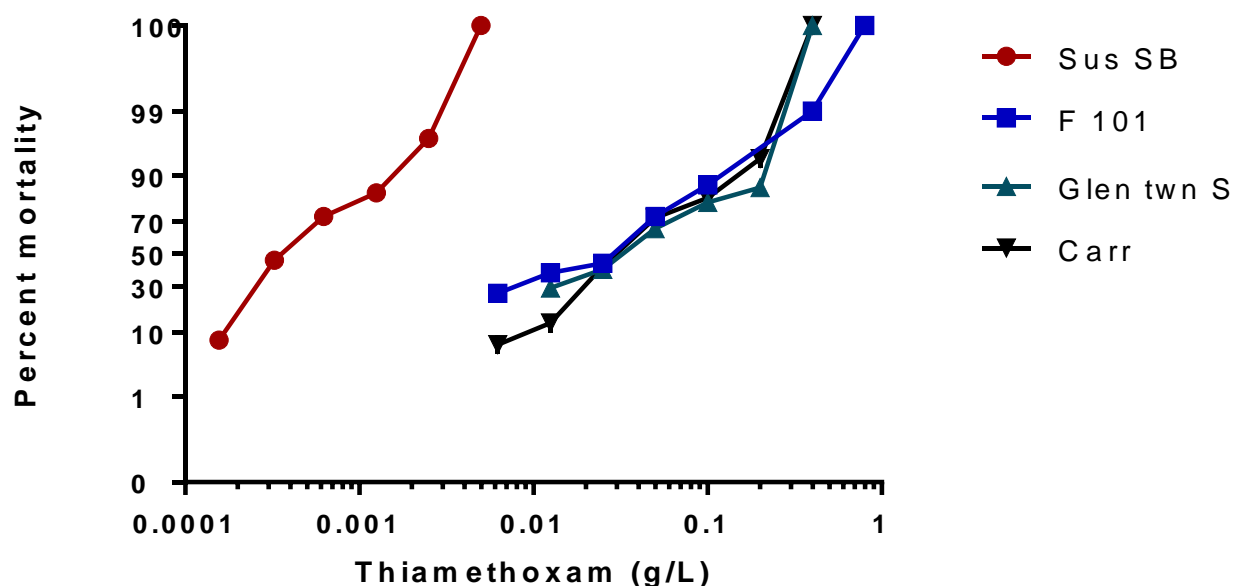
**Figure 2** Dose response for thiamethoxam-resistant strains (F 101, Glen twn S and Carr) compared to a reference susceptible strain (Sus SB) against thiamethoxam (Actara 250 g/kg)

compared to Cruiser[®] (Figure 3). Against resistant *A. gossypii*, neither treatment provided adequate control. Indeed, from day 28 the effectiveness of Cruiser[®] against resistant strain Glen twn S was similar to untreated cotton (Figure 3).

*4.1.2.2 Phorate*

Phorate provided robust protection of strain Sus F 96 for the duration of the trial, with control only decreasing below 90% at day 35 (Figure 4). From day 42, phorate provided residual control of 81%, decreasing to 67% control at day 49. Pirimicarb resistant strain Mon P survived well on phorate treated cotton from day 0 (Figure 4). Population size of strain Mon P when challenged with phorate showed no statistical difference compared with untreated cotton suggesting cross resistance (Figure 4).

*4.1.3 Discussion*

*4.1.3.1 Bioassay*

Results confirm neonicotinoid resistance in three field strains of *A. gossypii* used so demonstrating their suitability for use in the following experimental work. Each strain contained thiamethoxam resistant individuals and demonstrated $LC_{50}$ resistance levels greater than those previously linked to field control failure (Herron and Wilson 2011). Herron and Wilson (2011) documented the highest $LC_{50}$ level RF against thiamethoxam at 22-fold in their field strain (Elra) collected from the Darling Downs (QLD) in the 2007-08 cotton season. Here, strain Carr collected off cotton from Moree (NSW), yielded the highest $LC_{50}$ level RF of 85-fold some three seasons later.
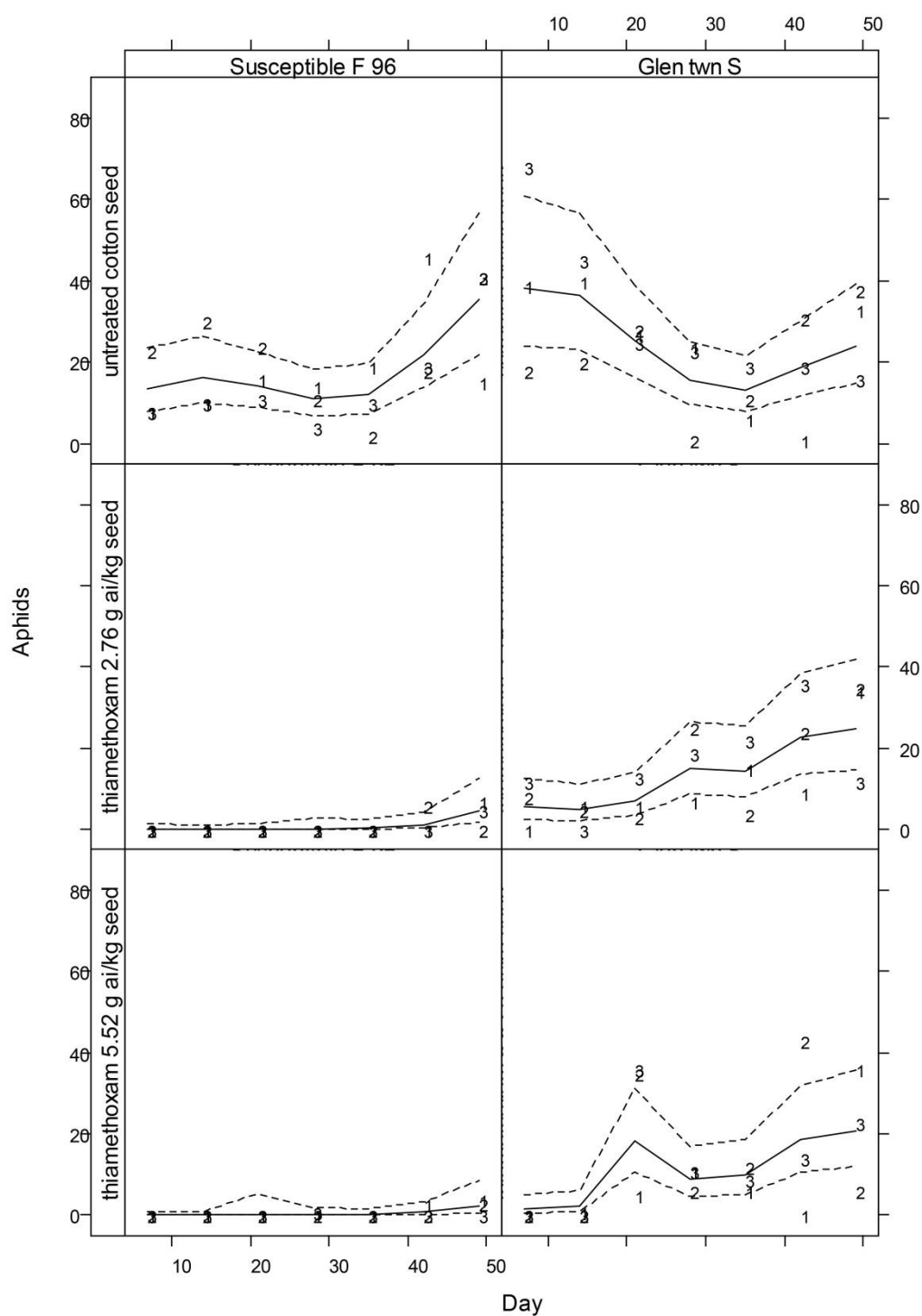
**Figure 3** Fitted trend for the thiamethoxam analysis, for each strain by treatment combination (thiamethoxam at 5.52 g a.i./kg seed, Cruiser Extreme® Insecticide Seed Treatment; thiamethoxam at 2.76 g a.i./kg seed, Cruiser® Insecticide Seed Treatment; untreated cotton seed, variety Sicot 71). The solid line represents the fitted trend, with dotted lines representing the 95% confidence interval. The raw data for each replicate is numbered 1 to 3 in each panel (with replicates 1 and 3 shifted slightly left and right, respectively, to avoid overlap).
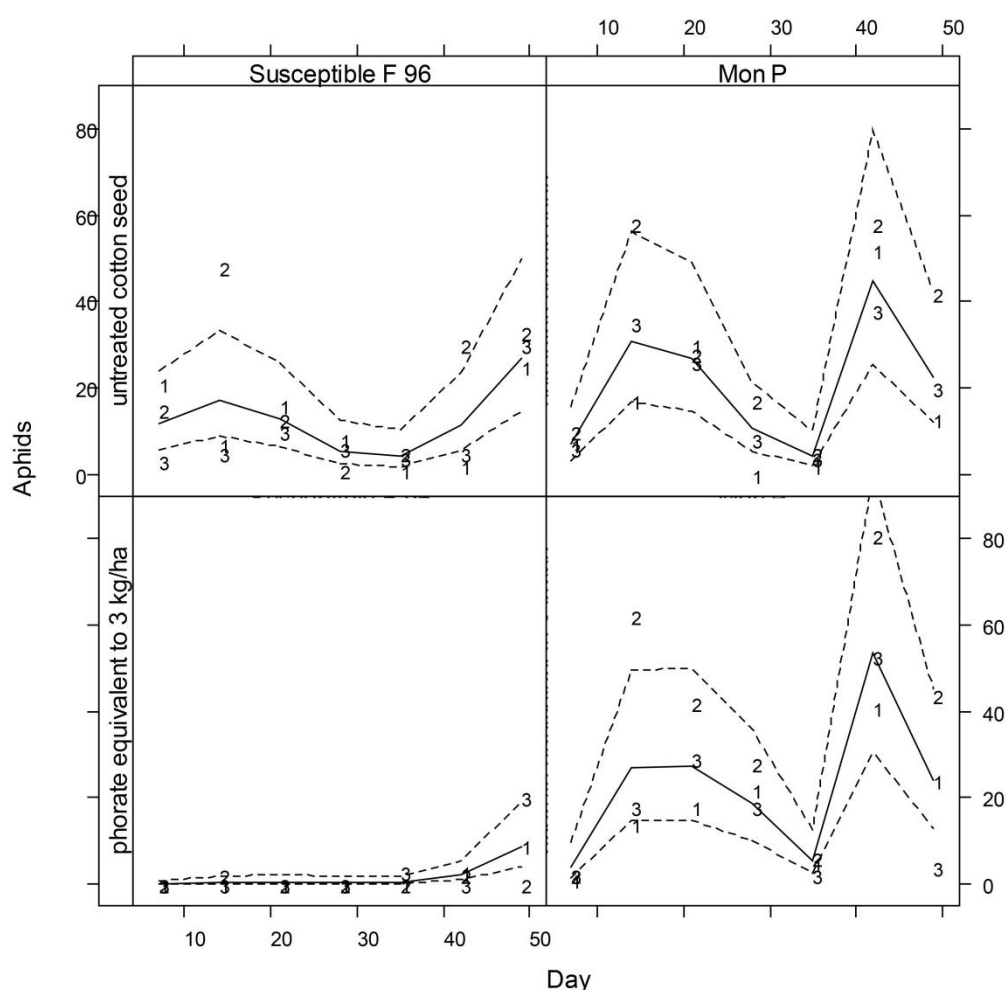
**Figure 4** Fitted trends for the phorate analysis, for each strain by treatment combination (phorate equivalent to 3 kg/ha, Thimet® 200 G Systemic Granular Insecticide; untreated cotton seed, variety Sicot 71). The solid line represents the fitted trend, with dotted lines representing the 95% confidence interval. The raw data for each replicate is numbered 1 to 3 in each panel (with replicates 1 and 3 shifted slightly left and right respectively, to avoid overlap).

Each of the strains used were collected from sites where neonicotinoid insecticides had been used either directly for *A. gossypii* control or used against other insect pests such as *C. dilutus* whereby *A. gossypii* was indirectly selected. This study indicates that between the 2007-08 and 2011-12 cotton seasons, continued use of neonicotinoid insecticides caused resistance to increase in *A. gossypii*. In the 2010-11 cotton season, foliar applications of neonicotinoids, including thiamethoxam and clothianidin rose to 7.5% of the total foliar application in Bollgard II planted cotton (APVMA 2013). This increase was largely attributed to the registration of clothianidin (Shield®) in 2008-09 for control of *C. dilutus* and *A. gossypii* (Sumitomo Chemical Australia Pty

Ltd 2010). Furthermore, the percentage of cotton seed planted that was coated with a neonicotinoid insecticide rose from 80 to 92% between seasons 2008-09 and 2011-12 (APVMA 2013).

*4.1.3.2 Glasshouse trial*

Results clearly show that formulated thiamethoxam at either rate (2.76 g a.i./kg seed and 5.52 g a.i./kg seed) is highly effective against neonicotinoid susceptible *A. gossypii* and continues to be a viable option for aphid control. Results support previous studies investigating the efficacy of thiamethoxam against susceptible *A. gossypii* with Maienfisch et al. (2001) finding rates between 105-350 g a.i./100 kg seed gave excellent control for 21-45 days. Further, Prasanna et al. (2004) found thiamethoxam 70WS at a rate of 2.85 g a.i./kg seed effective until 40 days post seedling emergence. In contrast to neonicotinoid susceptible *A. gossypii*, neither rate of thiamethoxam gave adequate control against neonicotinoid resistant *A. gossypii*. It is likely then that the ongoing and widespread reliance on neonicotinoid seed treatments will continue to select for resistant genotypes. Cross resistance between members of the neonicotinoid MoA group 4A in *A. gossypii* has been reported elsewhere (Wang et al. 2007, Shi et al. 2011) and suggests that control of resistant populations is likely to be lost if neonicotinoid use is not better managed. The Insecticide Resistance Management Strategy (IRMS) for control of sucking insect pests of cotton recommends chemical rotation as the primary strategy for control of resistant *A. gossypii* (Mass 2012). Other well defined strategies in the IRMS such as use of refugia for control of *Helicoverpa* spp. are limited in their practicality for *A. gossypii* due to its short life cycle and there being no sexual phase of reproduction in Australia (Smith et al. 2006) for outcrossing. If chemical rotation is maintained over successive generations, then in the absence of selection the resistant population should return to susceptibility. It should be mentioned that this strategy relies on a fitness cost to be associated with resistance causing resistance frequencies to decrease with time. Fortunately, reversion to susceptibility in the absence of insecticide pressure has been noted in laboratory strains of neonicotinoid resistant *A. gossypii* [see attached thesis (Appendix E)]. This would suggest that at least in some populations of *A. gossypii*, genes conferring neonicotinoid resistance do not become fixed.

Neonicotinoid seed dressings are primarily targeted against other pests where they continue to provide cost-effective control (Mass 2012), so restricting their use without a viable alternative is impractical. Phorate is registered for the control of *A. gossypii* at planting and has previously been shown to control neonicotinoid resistant *A. gossypii* as it possesses a distinct MoA to neonicotinoid insecticides (Herron et al. 2013). However, established cross resistance between the organophosphate and carbamate chemical classes via *Ace*1 type resistance will select for high level resistance in *A. gossypii* pest populations if used sequentially and will lead to control failures

(Herron et al. 2001, Andrews et al. 2004, Benting and Nauen 2004). The IRMS lists the carbamate, pirimicarb as a favourable first foliar spray for use against *A. gossypii* due to its softness on beneficials (Mass 2012). However, Herron et al. (2013) suggested that pirimicarb-resistant *A. gossypii* would not be controlled by phorate. The results of the glasshouse trial confirm those laboratory findings. If phorate is to successfully substitute for a neonicotinoid seed dressing its interaction with pirimicarb must be carefully considered. If phorate is used to control neonicotinoid resistant *A. gossypii* then pirimicarb, or any other chemical associated with *Ace*1 type resistance, should not immediately follow as the first foliar spray.

### 4.2 Objective: *Molecular genetics*

*4.2.1 PCR amplification of the R81T mutation site* (for related publications please see Appendix C)

Amplification of the loop D region of the nAChR β1 subunit gene produced 350 bp of quality cDNA sequence in strains Sus F 96, F 101, Glen twn S and Carr (Figure 5). Comparative sequence analysis between strains Sus F 96, F 101, Glen twn S, Carr and the reference *A. gossypii* strain IMI-R (Imidacloprid resistant, GenBank accession number: AFH00994.1) identified that the region amplified was the loop D region of the nAChR β1 subunit gene. Sequences belonging to two additional aphid species: the Soybean aphid *Aphis glycines* (GenBank accession number: JN681174.1) and *M. persicae* (GenBank accession number: AJ251838.1) (Figure 5), were also included for further validation. Unexpectedly, the amino acid substitution at position 81, resulting in an arginine (R) to threonine (T) substitution was present in strain IMI-R (China) but absent in Australian strains Sus F 96, F 101, Glen twn S and Carr (Figure 5).

*4.2.2 Dose responses with and without synergist*

For strains F $101_P$, Glen twn $S_P$ and $Carr_P$ [where $_P$ denotes that strains were routinely pressured while maintained in laboratory culture over a three year period) (see Appendix E: Chapter 2)] full log-dose probit analysis yielded RFs of 4.00- (2.53-6.32), 7.37- (4.44-12.23) and 7.53- (5.52-10.27) fold against thiamethoxam, respectively (Table 2). As indicated by overlapping 95% CIs at the $LC_{50}$ level no significant differences between strain responses were observed. Calculated $LC_{50}$ values ranged from a low of 0.00030 g/L to a high of 0.00056 g/L in strains F $101_P$ and $Carr_P$ respectively (Table 2). Significant synergism was observed for the P450 inhibitor, PBO, in strains F $101_{P,}$ and $Carr_P$ as indicated by non-overlapping 95% CIs at the $LC_{50}$ level (Table 2). The $LC_{50}$ values of strains F $101_P$ and $Carr_P$ were lower in the presence of PBO when compared to Sus F 96 suggesting complete synergism of resistance. In strain Glen twn $S_P$ the $LC_{50}$ of thiamethoxam in the presence of PBO was slightly higher than in strain Sus F 96. In contrast, in strain Sus F 96, the effect of PBO was negligible.

**Figure 5** Amino acid alignment of a partial sequence of the nAChR β1 subunit containing the R81T mutation site. Strains include: Sus F 96, F 101, Glen twn S, Carr, IMI-R (Imidacloprid resistant, GenBank accession number: AFH00994.1), and *A. glycines* (GenBank accession number: JN681174.1) and *M. persicae* (GenBank accession number: AJ251838.1). A conserved loop (Loop D) within the ligand binding domain is marked by a red box. The R81T mutation is marked in bold.

**Table 2** Probit mortality data for thiamethoxam + PBO against *A. gossypii* strains thiamethoxam-susceptible F 96 and thiamethoxam-resistant pressured F 101$_P$, Glen twn S$_P$ and Carr$_P$.

| | Treatment | | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | Thiamethoxam | | | Thiamethoxam + PBO | | |
| **Strain** | No. of aphids treated | LC$_{50}$ (95% FL) | RR[a] (95% CI) | No. of aphids treated | LC$_{50}$ (95% FL) | RR[a] (95%CI) |
| **Sus F 96** | 544 | 0.000074 (0.000063-0.000086) | - | 568 | 0.000061 (0.000050-0.000072) | - |
| **F 101$_P$** | 607 | 0.00030 (0.00017-0.00044) | 4.00 (2.53-6.32) | 542 | 0.000052 (0.000014-0.00011) | 0.85 (0.33-2.19) |
| **Glen twn S$_P$** | 598 | 0.00055 (0.00029-0.00084) | 7.37 (4.44-12.23) | 523 | 0.00017 (0.000096-0.00024) | 2.74 (1.68-4.47) |
| **Carr$_P$** | 585 | 0.00056- (0.00042 0.00072) | 7.53 (5.52-10.27) | 601 | 0.000015 (0.000001-0.000053) | 0.24 (0.039-1.51) |

FL, fiducial limits; CI, confidence intervals; [a]RR = LC$_{50}$ resistant strain/ LC$_{50}$ susceptible strain

*4.2.3 Discussion*

Target site insensitivity via mutations in nAChR subunits have repeatedly been implicated as causal mechanism(s) of neonicotinoid resistance in many insect species (Liu et al. 2005, Bass et al. 2011, Shi et al. 2012, Puinean et al. 2013, Kim et al. 2015). Those reports demonstrated a reduced binding affinity of neonicotinoid compounds at their target site as one of the main reasons for resistance. Surprisingly then, sequencing data presented here shows that mutation R81T (in loop D of the nAChR β1 subunit) responsible for resistance in aphid species overseas is not present in Australian strains. Encouragingly, it is consistent with the recent finding of Pan et al. (2015) who demonstrated a thiamethoxam resistant strain of *A. gossypii*, was also not linked to the R81T mutation (Pan et al. 2015).

It is interesting then that strains of *A. gossypii* where the R81T mutation has been demonstrated seem to anecdotally show resistance strongly correlated to imidacloprid (Shi et al. 2012, Koo et al. 2014). Although there is confirmed cross resistance between members of the neonicotinoid MoA group 4A (Wang et al. 2007), the spectrum of resistance displayed between members is confounding; some reports show evidence of cross resistance between all group 4A members (Koo et al. 2014) and others demonstrate resistance to one and susceptibility to another (Shi et al. 2011). For example, Shi et al. (2011) demonstrated no cross resistance in an imidacloprid resistant strain of *A. gossypii* to dinotefuran, clothianidin or thiamethoxam. Similarly, preliminary discriminating dose data here [obtained via treating whole cotton plants with 0.004 g a.i./L imidacloprid (Confidor®) 200 g/L and transferring resistant aphids to the treated plant once dried] also demonstrated that strains F 101, Glen twn S and Carr were susceptible to imidacloprid.

In Australia, both imidacloprid and thiamethoxam are available as pre germination seed treatments; however, 80% of cotton seed planted is coated with thiamethoxam (Herron and Wilson 2011). For that reason, it may be considered that the limited use of imidacloprid in Australian cotton may be responsible for the metabolic resistance detected rather than target site resistance associated with imidacloprid. Clearly, in this study there is reasonable evidence to suggest that neonicotinoid resistance is at least in part, mediated by metabolic detoxification enzymes. Such a conclusion is consistent with the findings of Khan et al. (2015) who demonstrated that PBO increased toxicity of thiamethoxam against a laboratory selected resistant strain of house fly *Musca domestica*, (Khan et al. 2015). In their study Khan et al. (2015) demonstrated via biochemical analyses that mixed function oxidase activity in their thiamethoxam resistant strain was significantly higher than their susceptible strain, suggesting that P450-mediated resistance was involved. Monoxygenase based resistance has also been detected in thiamethoxam resistant western flower thrips *Frankliniella occidentalis* again based on significant PBO synergism (Gao et al. 2014). Furthermore, in the

closely related aphid species *M. persicae*, pre-treatment with PBO via topical bioassays substantially synergised the effect of four neonicotinoid insecticides in a neonicotinoid-resistance clone of *M. persicae* (5191A clone) (Puinean et al. 2010). In their resistant 5191A clone, over-expression of a single P450 gene was revealed and attributed at least in part, to gene amplification (Puinean et al. 2010). In complete agreement then to the above studies, survival times of thiamethoxam resistant aphids were observed to be at least decreased in the presence of PBO and in two strains complete susceptibility was restored.

Overall, this study demonstrated the potential of synergists to reverse resistance in some instances. However, when using synergists alone, the inclusion of positive data only is often not enough to attribute resistance to a specific detoxifying enzyme (Raffa and Priester 1985). This is because synergists are often capable of detoxifying more than one resistance associated enzyme. For instance, past studies have shown that PBO does not exclusively synergise P450s and instead has been shown to effectively synergise resistance-associated esterases linked to pirimicarb in *A. gossypii* (Bingham et al. 2008) and spinosad resistance in *F. occidentalis* (Herron et al. 2014). Thus, extending this study to the transcriptome level to identify changes in gene expression of transcripts relating to metabolic detoxification as outlined in the following section would be extremely beneficial.

### *4.3 Objectiv*e**: *Molecular genetics*

### *4.3.1 Illumina sequencing and sequence assembly*

To obtain a comprehensive transcriptome of *A. gossypii* when under thiamethoxam stress, a total of 39, 33, 31, and 29 million raw reads were obtained from strains Carr, Sus F 96, F 101, and Glen twn S, respectively after filtering out dirty raw reads. When pooled, the total number of raw reads obtained from the four individual transcriptomes totalled 143,723,328 and 132,159,760 clean reads *de novo* assembled. A total of 37,167 contigs were assembled with an $N_{50}$ length of 906 bp. The contigs were further assembled into 31,042 unigenes with an $N_{50}$ of 1337 bp.

### *4.3.2 GO and COGs classification*

Of these unigenes a total of 23,372 (89.75% of all distinct sequence), 16506 (63.38%) and 15460 (59.37%) transcripts were annotated to NR, Swiss-prot, and KEGG, respectively. The identified *A. gossypii* unigenes were most similar to the pea aphid *Acyrthosiphon pisum* and a high degree of sequence homology (91.6%) between these species was revealed.

GO analysis identified 10,488 transcripts (40.27%) which were categorized into 48 GO terms consisting of three domains: "biological process", "cellular component" and "molecular function". Of the 48 terms, "cellular process", "metabolic process", "cell", "binding" and "catalytic activity"

were over-represented, whilst "extracellular matrix part", "antioxidant activity" and electron carrier activity" were under-represented. The terms "cell killing", "virion", "virion part" and "channel regulator activity" were absent.

All assembled unigenes were aligned to the COG database for functional prediction and classification. COG analysis identified a total of 7,633 transcripts (29.31%) classed into 25 functional categories, the largest five being "general function prediction only" (2572 genes), "transcription" (1249 genes), "replication, recombination and repair" (1247 genes), "translation, ribosomal structure and biogenesis" (1014 genes) and "carbohydrate transport and metabolism" (987 genes). "RNA Processing and Modification" (83 genes), "Extracellular structures" (6 genes) and "Nuclear transport" (4 genes) represented the smallest categories.

*4.3.3 Network of unigene*

31042 unigenes were mapped to the reference canonical pathways in the KEGG database and 15460 of them obtained KEGG annotation and assigned to 255 pathways. Among them, the "metabolic pathway" was the largest group (2109 unigenes, 13.64%), followed by "RNA transport" (549, 3.55%), "focal adhesion" (516, 3.34%) and "regulation of actin cytoskeleton" (491, 3.18%). In contrast, the following pathways contained <10 unigenes: "Phenylalanine, tyrosine and tryptophan biosynthesis" (9, 0.06%), "Vitamin B6 metabolism" (8, 0.05%), "D-Arginine and D-ornithine metabolism" (6, 0.04%), "Lipoic acid metabolism" (5, 0.03%), "Thiamine metabolism" (5, 0.03%), "Lysine biosynthesis" (4, 0.03%), "D-Glutamine and D-glutamate metabolism" (2, 0.01%) and finally "Caffeine metabolism" with only one unigene (1, 0.01%).

*4.3.4 Differential expression and pathway analyses in resistant vs susceptible strain combinations*

*4.3.4.1 Sus F 96 vs F 101*

The results revealed 24,299 genes with significantly differential expression levels between Sus F 96 and F 101 (Figure 6). Among them, 24,265 (99.86%) and 34 (0.0014%) genes were down-regulated and up-regulated, respectively, in strain F 101 compared to Sus F 96.

Providing further insights into twenty of the most differentially up-regulated genes between strain Sus F 96 and F 101 (Table 3), significant matches included the gene homologous to one that encodes a hypothetical protein in the red imported fire ant *Solenopsis invicta*; hypothetical protein in the Florida carpenter ant *Camponotus floridanus*; replicase polyprotein of *Ascaris suum* (large roundworm of pigs); polyprotein-like protein of a Tsetse fly sp., *Glossina morsitans morsitans*; and ten predicted functional genes, including five heat shock protein 68-like (*A. pisum*), partial; two heat shock protein 70 B2-like (*A. pisum*) and three similar to gag-pol polyprotein (rust red flour beetle, *Tribolium castaneum*). The six remaining genes had no functional annotation. The top ten
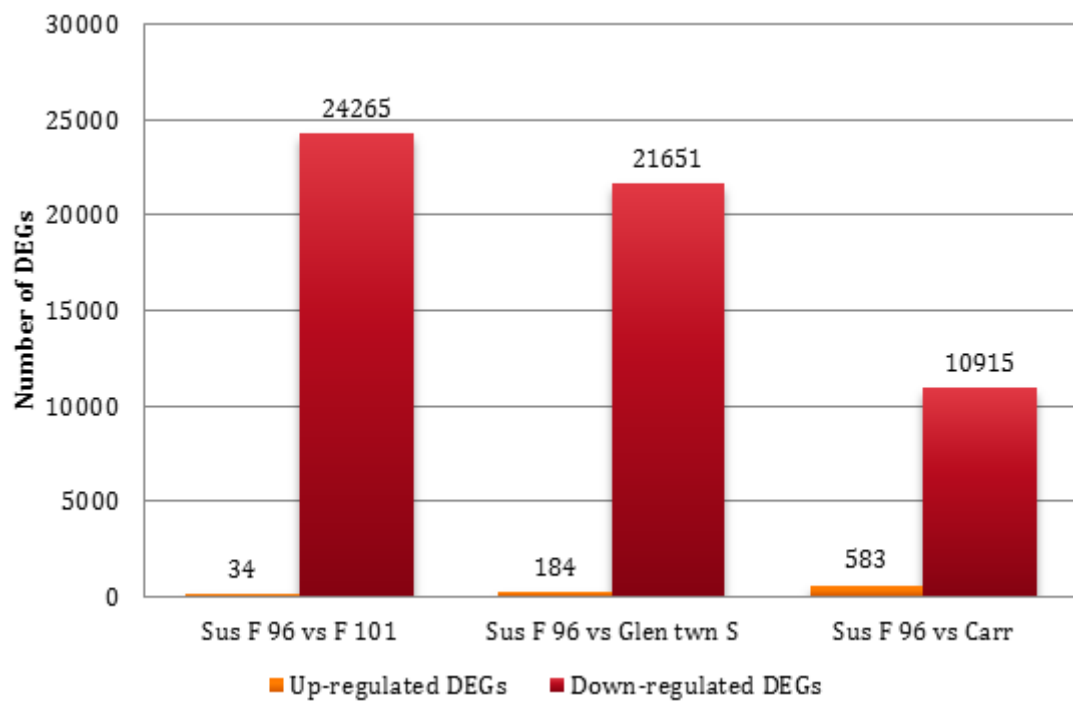
**Figure 6** Number of DEGs expressed between susceptible (Sus F 96) and thiamethoxam-resistant (F 101, Glen twn S and Carr) *A. gossypii* strains.

down-regulated genes in strain F 101 compared to Sus F 96 (Table 3) included one gene homologous to exoribonuclease 1 (*A. pisum*) and eight predicted functional genes: major facilitator superfamily domain-containing protein 6-like isoform 1 (*A. pisum*); oligopeptidase A-like (*A. pisum*); protein msta, isoform A-like (*A. pisum*); two genes encoding probable multidrug resistance-associated protein lethal(2)03659-like (*A. pisum*); hypothetical protein LOC100159424 (*A. pisum*); ATP synthase subunit alpha-like (Common eastern bumblebee, *Bombus impatiens*); and protein toll-like (*A. pisum*). In total, twelve down-regulated genes had no functional annotation.

DEGs between Sus F 96 and F 101 were characterized into three groups from the GO classification: cellular component; molecular function; and biological process. The results showed that 3843, 6112 and 5472 DEGs were annotated to 299, 570 and 2054 GO terms of cellular component, molecular function, and biological process, respectively (corrected P-value ≤1).

To further categorize, the DEGs were significantly enriched to fourteen cellular components, in which "ribonucleoprotein complex" was most strongly presented and the category, "cell" was the largest represented with 3010 DEGs (78.3%)**.** The DEGs were significantly enriched to ten molecular functions, two which contained the majority of DEGs: "catalytic activity" (3775, 61.8%) and "binding" (3598, 58.9%). Under the umbrella of "biological process", twenty three significantly enriched GO terms were reported between Sus F 96 and F 101. Those associated with "metabolic process" (3438, 62.8%) and "cellular process" (3979, 72.7%) accounted for the two largest

**Table 3** Top DEGs between thiamethoxam resistant (F 101) and susceptible (Sus F 96) *A. gossypii* strains. Transcript ID, $\log_2$ Ratio, FDR and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr).

| GeneID | $\log_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Sus F 96 vs F 101 | | | |
| Unigene6303[a] | 8.66 | 1.54E-08 | - |
| Unigene19540[a] | 8.23 | 2.15E-05 | - |
| Unigene19346[a] | 8.10 | 1.93E-06 | - |
| Unigene16958[a] | 7.96 | 0.000237 | - |
| Unigene8236[a] | 2.72 | 2.48E-24 | EFZ13460.1\|hypothetical protein SINV_12007 [Solenopsis invicta] |
| Unigene21349[a] | 2.54 | 1.77E-05 | EFN72115.1\|hypothetical protein EAG_00326 [Camponotus floridanus] |
| Unigene10453[a] | 2.52 | 0 | ADY39838.1\|Replicase polyprotein [Ascaris suum] |
| Unigene10452[a] | 2.46 | 0 | ACY69873.1\|polyprotein-like protein [Glossina morsitans morsitans] |
| Unigene10451[a] | 2.39 | 0 | - |
| CL2116.Contig3[a] | 2.37 | 2.93E-131 | XP_001951915.1\|/PREDICTED: heat shock protein 70 B2-like [Acyrthosiphon pisum] |
| Unigene17291[a] | 2.25 | 4.37E-28 | XP_001807263.1\|PREDICTED: similar to gag-pol polyprotein [Tribolium castaneum] |
| Unigene16335[a] | 2.15 | 1.14E-07 | - |
| Unigene22183[a] | 2.15 | 0.000216 | XP_001807263.1\|PREDICTED: similar to gag-pol polyprotein [Tribolium castaneum] |
| Unigene21691[a] | 2.12 | 1.77E-07 | XP_001807662.1\|PREDICTED: similar to putative gag-pol protein [Tribolium castaneum] |
| Unigene5014[a] | 2.12 | 9.45E-14 | XP_001951915.1\|PREDICTED: heat shock protein 70 B2-like [Acyrthosiphon pisum] |
| CL2116.Contig5[a] | 2.06 | 1.56E-22 | XP_003248918.1\|PREDICTED: heat shock protein 68-like, partial [Acyrthosiphon pisum] |
| Unigene7780[a] | 2.04 | 3.91E-25 | XP_003248918.1\|PREDICTED: heat shock protein 68-like, partial [Acyrthosiphon pisum] |
| Unigene11682[a] | 1.97 | 1.60E-11 | XP_003248918.1\|PREDICTED: heat shock protein 68-like, partial [Acyrthosiphon pisum] |
| Unigene10435[a] | 1.90 | 3.05E-13 | XP_003248918.1\|PREDICTED: heat shock protein 68-like, partial [Acyrthosiphon pisum] |
| CL2116.Contig6[a] | 1.89 | 7.44E-26 | XP_003248918.1\|PREDICTED: heat shock protein 68-like, partial [Acyrthosiphon pisum] |

| GeneID | log$_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| CL857.Contig2[b] | -10.75 | 1.39E-65 | XP_001948792.2\|PREDICTED: major facilitator superfamily domain-containing protein 6-like isoform 1 [Acyrthosiphon pisum] |
| Unigene15451[b] | -10.60 | 1.63E-31 | XP_001945759.1\|PREDICTED: oligopeptidase A-like [Acyrthosiphon pisum] |
| Unigene17197[b] | -10.33 | 7.25E-19 | - |
| Unigene5422[b] | -10.32 | 7.36E-21 | XP_003245146.1\|PREDICTED: protein msta, isoform A-like [Acyrthosiphon pisum] |
| Unigene19845[b] | -10.28 | 3.34E-18 | - |
| Unigene18246[b] | -10.21 | 3.38E-19 | - |
| Unigene1782[b] | -10.12 | 3.46E-23 | - |
| Unigene1872[b] | -10.07 | 1.54E-17 | - |
| Unigene17759[b] | -10.05 | 1.50E-15 | - |
| Unigene18414[b] | -10.03 | 7.58E-29 | - |
| Unigene15063[b] | -10.03 | 3.12E-13 | - |
| Unigene19824[b] | -10.00 | 1.52E-16 | NP_001155946.1\|exoribonuclease 1 [Acyrthosiphon pisum] |
| Unigene7980[b] | -9.95 | 1.62E-25 | XP_001948961.2\|PREDICTED: probable multidrug resistance-associated protein lethal(2)03659-like [Acyrthosiphon pisum] |
| Unigene18326[b] | -9.94 | 7.48E-24 | XP_001948736.2\|PREDICTED: probable multidrug resistance-associated protein lethal(2)03659-like [Acyrthosiphon pisum] |
| Unigene884[b] | -9.93 | 6.89E-15 | - |
| Unigene19195[b] | -9.92 | 7.00E-16 | XP_001943554.1\|PREDICTED: hypothetical protein LOC100159424 [Acyrthosiphon pisum] |
| Unigene17685[b] | -9.92 | 1.60E-22 | - |
| Unigene18285[b] | -9.85 | 1.48E-14 | XP_003492803.1\|PREDICTED: ATP synthase subunit alpha-like [Bombus impatiens] |
| Unigene18341[b] | -9.84 | 1.50E-15 | XP_003243866.1\|PREDICTED: protein toll-like [Acyrthosiphon pisum] |
| Unigene18832[b] | -9.79 | 3.26E-16 | - |
| Unigene19121[b] | -9.76 | 3.22E-15 | - |

[a]Up-regulated transcripts; [b]Down-regulated transcripts; [1]Ratio, RPKM of resistant/RPKM of susceptible samples. RPKM, reads per kilo bases per million reads.

represented terms, although the term "carboxylic acid metabolic process" (p-value 0.00570) was strongly presented. KEGG enrichment analysis revealed 11716 DEGs between Sus F 96 an F 101 which were assigned to 254 pathways. DEG enrichment analyses showed that the first four pathways that involved up- or down-regulated genes in response to thiamethoxam stress were "Ribosome" (104 unigenes), "Protein processing in endoplasmic reticulum" (257 unigenes), "Metabolic pathways" (1766 unigenes) and "RNA transport" (366 unigenes).

### 4.3.4.2 Sus F 96 vs Glen twn S

The comparison between Sus F 96 and Glen twn S also revealed significant variations in expression. A total of 21,835 genes, including 184 (0.0084%) up-regulated genes and 21,651 (99.16%) down-regulated genes were identified (Figure 6). Among the twenty most up-regulated genes (Table 4), matches included the gene homologous to that which encodes GL24774 of ferment fly *Drosophila persimilis* (N-glycan biosynthesis), glutaredoxin-like (glutathione dependent reductase of *A. pisum*), and also eight predicted functional genes (maltase 2-like (*A. pisum*); *A. pisum* hypothetical proteins LOC100575926, LOC10056912, LOC100574103; centrosomal protein of 78 kDa-like (*A. pisum*); similar to SET domain and mariner transposase fusion of Hydra spp., *Hydra magnipapillata*; and deoxynucleotidyltransferase terminal-interacting protein 1-like (*A. pisum*)). Ten highly up-regulated genes had no functional annotation. Of the top twenty down-regulated genes (Table 4), nine had no functional annotation and eleven were predicted functional genes: *A. pisum* hypothetical proteins LOC100571774 partial, LOC100162722, LOC100574363, LOC100163439 and LOC100164810; nose resistant to fluoxetine protein 6-like (*A. pisum*); bifunctional purine biosynthesis protein purH-like (*B. impatiens*) (protein coding gene of IMP cyclohydrolase activity); x-ray radiation resistance- associated protein 1-like (*A. pisum*); probable multidrug resistance-associated protein lethal-like (*A. pisum*) [ATP-binding cassette, subfamily C (CFTR/MRP), member 4] and phosphatidylinositol 3-kinase catalytic subunit type 3-like (*A. pisum*) (enzymes involved in cellular functions).

GO analysis between Sus F 96 and Glen twn S revealed 3543, 5652 and 5064 DEGs annotated to 294, 556 and 1975 terms of cellular component, molecular function and biological process, respectively (corrected P-value ≤1). DEGs were significantly enriched to fourteen cellular components, in which "cell" represented the largest with 2792 genes (78.8%). The terms "intracellular" with 2479 (70%) and "ribonucleoprotein complex" with 267 genes (7.5%) were strongly presented with p-values of 2.85e-08 and 6.41e-08, respectively. Of those DEGs enriched to ten molecular functions, "binding" and "catalytic activity" were again the largest represented terms with 3323 (58.8%) and 3488 (61.7%) genes, respectively. Twenty three significantly enriched terms

**Table 4** Top DEGs between thiamethoxam resistant (Glen twn S) and susceptible (Sus F 96) *A. gossypii* strains. Transcript ID, $\log_2$ Ratio, FDR and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr).

| GeneID | $\log_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Sus F 96 vs Glen twn S | | | |
| Unigene6303[a] | 9.45 | 5.14E-14 | - |
| Unigene5141[a] | 8.90 | 1.54E-09 | - |
| Unigene17389[a] | 8.80 | 2.93E-09 | - |
| Unigene15492[a] | 8.61 | 1.75E-06 | XP_001948285.2\|PREDICTED: maltase 2-like [Acyrthosiphon pisum] |
| CL1027.Contig2[a] | 8.58 | 7.98E-05 | - |
| Unigene14552[a] | 8.48 | 3.80E-24 | XP_002027279.1\|GL24774 [Drosophila persimilis] |
| Unigene15706[a] | 8.44 | 7.98E-05 | - |
| Unigene1875[a] | 8.16 | 6.29E-06 | XP_003241394.1\|PREDICTED: hypothetical protein LOC100575926 [Acyrthosiphon pisum] |
| Unigene19078[a] | 8.12 | 7.98E-05 | XP_003245035.1\|PREDICTED: centrosomal protein of 78 kDa-like [Acyrthosiphon pisum] |
| Unigene19346[a] | 8.00 | 6.29E-06 | - |
| Unigene22343[a] | 7.99 | 4.23E-05 | - |
| Unigene19540[a] | 7.98 | 0.000151 | - |
| Unigene21014[a] | 7.97 | 0.000151 | XP_003247256.1\|PREDICTED: hypothetical protein LOC100569128 [Acyrthosiphon pisum] |
| Unigene22887[a] | 7.78 | 2.24E-05 | XP_002161449.1\|PREDICTED: similar to SET domain and mariner transposase fusion [Hydra magnipapillata] |
| Unigene22994[a] | 7.77 | 7.98E-05 | XP_003247311.1\|PREDICTED: hypothetical protein LOC100574103 [Acyrthosiphon pisum] |
| CL1361.Contig3[a] | 7.65 | 4.27E-10 | XP_003242396.1\|PREDICTED: deoxynucleotidyltransferase terminal-interacting protein 1-like [Acyrthosiphon pisum] |
| Unigene19111[a] | 7.62 | 0.000284 | - |
| Unigene22875[a] | 7.57 | 0.000535 | - |
| Unigene20767[a] | 7.04 | 0.000535 | XP_003241394.1\|PREDICTED: hypothetical protein LOC100575926 [Acyrthosiphon pisum] |
| CL273.Contig1[a] | 7.00 | 3.32E-06 | NP_001155375.1\|glutaredoxin-like [Acyrthosiphon pisum] |

| GeneID | log$_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Unigene13938[b] | -11.58 | 1.36E-36 | - |
| Unigene13783[b] | -11.06 | 2.39E-52 | - |
| Unigene11861[b] | -10.95 | 4.36E-51 | - |
| Unigene15683[b] | -10.76 | 1.10E-40 | XP_003244579.1|PREDICTED: hypothetical protein LOC100571774, partial [Acyrthosiphon pisum] |
| Unigene5239[b] | -10.71 | 1.30E-30 | XP_001946997.2|PREDICTED: hypothetical protein LOC100162722 [Acyrthosiphon pisum] |
| Unigene16205[b] | -10.54 | 2.27E-40 | XP_003244577.1|PREDICTED: nose resistant to fluoxetine protein 6-like [Acyrthosiphon pisum] |
| CL218.Contig3[b] | -10.39 | 4.48E-23 | CBY13234.1|unnamed protein product [Oikopleura dioica] |
| Unigene19845[b] | -10.28 | 4.01E-17 | - |
| Unigene932[b] | -10.17 | 4.48E-23 | - |
| Unigene16990[b] | -10.11 | 1.95E-17 | - |
| Unigene16507[b] | -10.10 | 3.40E-21 | XP_003492800.1|PREDICTED: bifunctional purine biosynthesis protein purH-like [Bombus impatiens] |
| Unigene1872[b] | -10.07 | 1.69E-16 | - |
| Unigene18414[b] | -10.03 | 3.70E-27 | - |
| Unigene18254[b] | -9.99 | 3.95E-33 | XP_003243328.1|PREDICTED: hypothetical protein LOC100574363 [Acyrthosiphon pisum] |
| Unigene18905[b] | -9.94 | 1.09E-13 | XP_003243746.1|PREDICTED: x-ray radiation resistance-associated protein 1-like [Acyrthosiphon pisum] |
| Unigene18326[b] | -9.94 | 1.89E-22 | XP_001948736.2|PREDICTED: probable multidrug resistance-associated protein lethal(2)03659-like [Acyrthosiphon pisum] |
| Unigene17020[b] | -9.83 | 5.32E-19 | - |
| Unigene18322[b] | -9.76 | 1.47E-15 | XP_001943231.1|PREDICTED: phosphatidylinositol 3-kinase catalytic subunit type 3-like [Acyrthosiphon pisum] |
| Unigene19719[b] | -9.69 | 8.10E-12 | - |
| Unigene19097[b] | -9.67 | 4.62E-18 | XP_001950522.1|PREDICTED: hypothetical protein LOC100163439 [Acyrthosiphon pisum] |
| Unigene17628[b] | -9.65 | 3.39E-11 | XP_001943487.1|PREDICTED: hypothetical protein LOC100164810 [Acyrthosiphon pisum] |

[a]Up-regulated transcripts; [b]Down-regulated transcripts; [1]Ratio, RPKM of resistant/RPKM of susceptible samples. RPKM, reads per kilo bases per million reads.

of the category biological process were reported between Sus F 96 and Glen twn S. Similarly, the terms "cellular process" (3677, 72.6%) and "metabolic process" (3203, 63.3%) were the largest represented. "Metabolic process" was also very strongly presented (p-value 0.00273), along with "translation" (176, 3.5%) which was slightly stronger (p-value 0.00031) than "metabolic process". Lastly, 10764 DEGs were assigned to 254 KEGG pathways. DEG enrichment analyses showed that the first four pathways that involved up- or down-regulated genes in response to thiamethoxam stress were "Ribosome" (104 unigenes), "Metabolic pathways" (1637 unigenes), "Herpes simplex infection" (159 unigenes) and "Shigellosis" (88 unigenes).

*4.3.4.3 Sus F 96 vs Carr*

Finally, comparative analysis between Sus F 96 and Carr revealed 11,498 genes with significant expression profile changes, including 583 (5.1%) up-regulated genes and 10,915 (94.9%) down-regulated genes (Figure 6). Of the twenty most up-regulated genes (Table 5), eight were predicted function genes: *A. pisum* hypothetical protein LOC100573940, LOC100574035, LOC100574264 isoform 1, LOC100573859, LOC100574035 and LOC100570532; and *A. pisum* maltase 2-like (alpha glucosidase). Two genes have defined functions: *A. pisum* ACYPI000014 (cathepsin B) and GL24774 (*D. persimilis* N-glycan biosynthesis) and the remaining ten had no functional annotation. Among the ten most down-regulated genes (Table 5), eighteen had no functional annotation, one was homologous to hypothetical protein of the Gulf Coast tick, *Amblyomma maculatum* and one gene had predicted function to *A. pisum* hypothetical protein LOC100571804.

GO analysis revealed that 1503, 2371 and 2092 DEGs were annotated to 217, 392 and 1436 GO terms of cellular component, molecular function and biological process, respectively (corrected P-value ≤1). DEGs were significantly enriched to seventeen cellular components, in which "intrinsic to membrane" was most strongly presented and "cell" the largest category represented with 1113 DEGs (74.1%). Further, DEGs were significantly enriched to ten molecular functions, two of which contained the majority of DEGs: "catalytic activity" (1393 genes, 58.8%) and "binding" (1332, 56.2%). For the GO category, biological process, twenty three significantly enriched terms were reported between Sus F 96 and Carr. "Cellular process" (1498, 71.6%) and "metabolic process" (1242, 59.4%) contained the largest number of represented terms. Interestingly, the terms "cellular response to hormone stimulus" (p-value 0.87749) and "cellular response to endogenous stimulus" (p-value 0.87749) were the most strongly presented. Between strains Sus F 96 vs Carr, 4728 DEGs were assigned to 252 KEGG pathways. DEG enrichment analyses showed that the first four pathways that involved up- or down-regulated genes in response to thiamethoxam stress were "Cardiac muscle contraction" (61 unigenes), "Fatty acid elongation" (29 unigenes), "Neuroactive ligand-receptor interaction" (96 unigenes) and "Glycerophospholipid metabolism" (69 unigenes).

**Table 5** Top DEGs between thiamethoxam resistant (Carr) and susceptible (Sus F 96) *A. gossypii* strains. Transcript ID, $\log_2$ Ratio, FDR and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr).

| GeneID | $\log_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Sus F 96 vs Carr | | | |
| Unigene13810[a] | 10.83 | 7.49E-174 | XP_003242198.1\|PREDICTED: hypothetical protein LOC100573940 [Acyrthosiphon pisum] |
| Unigene5980[a] | 10.76 | 2.60E-21 | - |
| Unigene6303[a] | 10.67 | 2.73E-33 | - |
| Unigene14552[a] | 10.49 | 8.45E-101 | XP_002027279.1\|GL24774 [Drosophila persimilis] |
| Unigene15492[a] | 10.41 | 4.98E-22 | XP_001948285.2\|PREDICTED: maltase 2-like [Acyrthosiphon pisum] |
| Unigene1320[a] | 10.04 | 6.67E-25 | XP_003242199.1\|PREDICTED: hypothetical protein LOC100574035 [Acyrthosiphon pisum] |
| Unigene1246[a] | 10.01 | 6.32E-19 | XP_001948285.2\|PREDICTED: maltase 2-like [Acyrthosiphon pisum] |
| Unigene5979[a] | 9.86 | 1.46E-11 | - |
| Unigene16958[a] | 9.84 | 2.09E-14 | - |
| Unigene18503[a] | 9.82 | 5.65E-18 | XP_003242202.1\|PREDICTED: hypothetical protein LOC100574264 isoform 1 [Acyrthosiphon pisum] |
| Unigene19840[a] | 9.62 | 1.31E-31 | XP_003242197.1\|PREDICTED: hypothetical protein LOC100573859 [Acyrthosiphon pisum] |
| Unigene20836[a] | 9.59 | 3.20E-13 | XP_003242199.1\|PREDICTED: hypothetical protein LOC100574035 [Acyrthosiphon pisum] |
| Unigene17389[a] | 9.58 | 4.53E-16 | - |
| Unigene4404[a] | 9.54 | 3.82E-10 | - |
| CL1560.Contig2[a] | 9.48 | 5.34E-60 | XP_003244804.1\|PREDICTED: hypothetical protein LOC100570532 [Acyrthosiphon pisum] |
| CL1027.Contig2[a] | 9.41 | 2.91E-08 | - |
| CL1708.Contig1[a] | 9.29 | 4.91E-12 | BAH70886.1\|ACYPI000014 [Acyrthosiphon pisum] |
| Unigene19111[a] | 9.28 | 1.66E-12 | - |
| Unigene22140[a] | 9.28 | 2.21E-10 | - |
| Unigene5141[a] | 9.26 | 1.66E-12 | - |

| GeneID | log$_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Unigene13938[b] | -11.58 | 3.31E-42 | - |
| Unigene16132[b] | -10.92 | 7.07E-50 | XP_003241320.1\|PREDICTED: hypothetical protein LOC100571804 [Acyrthosiphon pisum] |
| CL893.Contig3[b] | -10.87 | 2.34E-32 | - |
| Unigene4253[b] | -10.69 | 2.89E-31 | - |
| Unigene17599[b] | -10.66 | 1.02E-28 | - |
| Unigene16085[b] | -10.62 | 4.39E-25 | - |
| Unigene17405[b] | -10.44 | 2.89E-23 | - |
| Unigene185[b] | -10.42 | 1.25E-31 | - |
| Unigene17753[b] | -10.34 | 2.35E-24 | - |
| Unigene19845[b] | -10.28 | 1.23E-19 | - |
| Unigene18008[b] | -10.18 | 1.89E-21 | - |
| Unigene16661[b] | -10.18 | 6.51E-35 | AEO35729.1\|hypothetical protein [Amblyomma maculatum] |
| Unigene17266[b] | -10.08 | 6.50E-19 | - |
| Unigene1872[b] | -10.07 | 6.50E-19 | - |
| Unigene18414[b] | -10.03 | 2.89E-31 | - |
| Unigene18907[b] | -9.97 | 4.17E-17 | - |
| Unigene951[b] | -9.92 | 6.09E-15 | - |
| Unigene19766[b] | -9.90 | 2.66E-15 | - |
| Unigene660[b] | -9.77 | 4.36E-21 | - |
| Unigene8883[b] | -9.71 | 2.43E-11 | - |
| Unigene19129[b] | -9.67 | 3.21E-14 | - |

[a]Up-regulated transcripts; [b]Down-regulated transcripts; [1]Ratio, RPKM of resistant/RPKM of susceptible samples. RPKM, reads per kilo bases per million reads.

### 4.3.5 Candidate resistance (detoxification) genes

The expression of transcripts encoding potential resistance genes is shown in Figure 7.
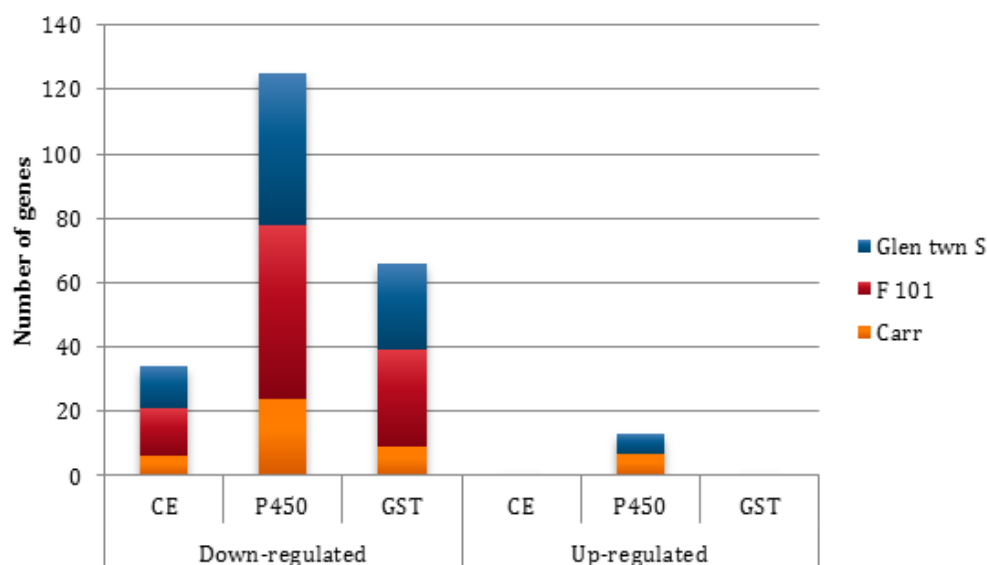


**Figure 7** Expression profiles of detoxification-related proteins in RNA-seq analysis of *A. gossypii* strains: thiamethoxam resistant strain (F 101, Glen twn S and Carr) compared to a reference susceptible strain (Sus F 96). CE, carboxylesterase; GST, glutathione-S-transferase; P450, cytochrome P450-dependent monoxygenase.

Only strains Glen twn S and Carr contained up-regulated genes relating to known insecticide detoxification mechanisms, when compared to Sus F 96. Of these, all were contained to the P450 family, with 6 (Unigene15803, CL627, Unigene4712, CL1190, CL1418 and Unigene12819) and 7 (Unigene15803, CL627, CL1190, Unigene4712, Unigene12819, CL1418 and Unigene12511) genes up-regulated in strains Glen twn S and Carr, respectively (Figure 7). Of these, three had predicted similarity to *CYP305A1* (Unigene15803, CL627 and Unigene4712), two to *CYP6K1* (CL1190 and CL1418) and two to *CYP6A13* (Unigene12511 and Unigene12819). Based on predicted matches, all were contained within the CYP2 and CYP3 clans. In contrast, strain F 101 contained no up-regulated genes in the carboxylesterase (CE), P450 or glutathione-S-transferase (GST) gene families and instead contained the highest number of down-regulated genes with 15, 54 and 30 down-regulated CEs, P450s and GSTs, respectively. It should be noted that contigs CL1190 and CL1418, although not significantly differentially expressed in strain F 101, were up-regulated in strain F 101, and significantly in strains Glen twn S and Carr, when compared to Sus F 96. No GSTs were found up-regulated in any of the resistant strains compared to Sus F 96. Down regulated GSTs showing similarity to the delta, omega, sigma and theta classes were found in some resistant strains. Finally, no CEs were up-regulated in resistant strains compared to Sus F 96.

Seven nAChR subunit gene sequences, including α1, α2, α3, α4, α7, β1, and β2 were matched against known genes. All nAChR subunit genes were downregulated in resistant strains, compared to Sus F 96 (Table 6).

**Table 6** List of differentially expressed nAChR subunit genes among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *A. gossypii* strains. Transcript ID, orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr) and $\log_2$ Ratio are indicated.

| GeneID | Blast nr | $\log_2$ Ratio[1] | | | |
|---|---|---|---|---|---|
| | | Sus F 96 | F101 | Glen twn S | Carr |
| Unigene11848 | CAA57476.1\|nicotinic acetylcholine receptor alpha subunit [Myzus persicae] | 23.72323 | 4.896363 | 6.761522 | 9.049615 |
| Unigene10018 | AFP55242.1\|nicotinic acetylcholine receptor alpha 4 subunit [Aphis glycines] | 15.53073 | 3.691985 | 3.774305 | 6.830007 |
| Unigene12372 | AEV54111.1\|nicotinic acetylcholine receptor alpha 2 subunit [Aphis gossypii] | 21.63213 | 2.951797 | 3.539873 | 7.879289 |
| CL1326.Contig2 | AFP55243.1\|nicotinic acetylcholine receptor beta 1 subunit [Aphis glycines] | 3.810137 | 1.117733 | 1.642734 | 1.910338 |
| Unigene6313 | AEV54111.1\|nicotinic acetylcholine receptor alpha 2 subunit [Aphis gossypii] | 16.03923 | 3.036168 | 2.950106 | 5.5476 |
| Unigene10203 | AFH00994.1\|nicotinic acetylcholine receptor beta 1 subunit [Aphis gossypii] | 9.206973 | 2.114636 | 3.02445 | 3.922346 |
| Unigene12768 | ABR21379.1\|nicotinic acetylcholine receptor alpha 3 subunit [Aphis gossypii] | 20.29044 | 5.893415 | 5.272609 | 8.498577 |
| CL834.Contig2 | AEO91541.1\|nicotinic acetylcholine receptor alpha 2 subunit [Aphis glycines] | 1.891482 | 0.294408 | 0.752958 | 0.845874 |
| Unigene20148 | AEV54113.1\|nicotinic acetylcholine receptor alpha 4-2 [Aphis gossypii] | 3.469474 | 1.103186 | 2.209797 | 2.448757 |
| CL1326.Contig1 | AFP55243.1\|nicotinic acetylcholine receptor beta 1 subunit [Aphis glycines] | 0.362329 | 0.05965 | 0.194163 | 0.260799 |
| Unigene1409 | AEV54113.1\|nicotinic acetylcholine receptor alpha 4-2 [Aphis gossypii] | 4.203021 | 2.126141 | 1.06472 | 2.002179 |
| Unigene20625 | AEV54113.1\|nicotinic acetylcholine receptor alpha 4-2 [Aphis gossypii] | 4.651564 | 1.422168 | 1.234458 | 1.616667 |
| CL834.Contig1 | CAA57477.1\|nicotinic acetyl choline receptor alpha-subunit [Myzus persicae] | - | - | - | 0.024128 |

[1]Ratio, RPKM of resistant/RPKM of susceptible. RPKM, reads per kilo bases per million reads.

*4.3.6 Quantitative RT-PCR*

Four unigenes, of which two had identified functions relating to detoxification (Contig ID 1190 and 1418) and two matched an RNA virus (RhPV6) of the bird cherry-oat aphid *Rhopalosiphum padi* (Contig ID 10451 and 10452) were selected for further validation. The over-transcription of genes,

CL1190 and CL1418, identified from RNA-Seq analysis experiments were confirmed by qRT-PCR in all strain comparisons, although expression ratios obtained from qRT-PCR were frequently higher than those obtained from RNA-Seq analysis (Figure 8). In contrast, expression ratios obtained from qRT-PCR for Unigenes 10451 and 10452, although showing similar trends in up-regulation of expression to RNA-Seq analysis were much smaller in value (Figure 8).
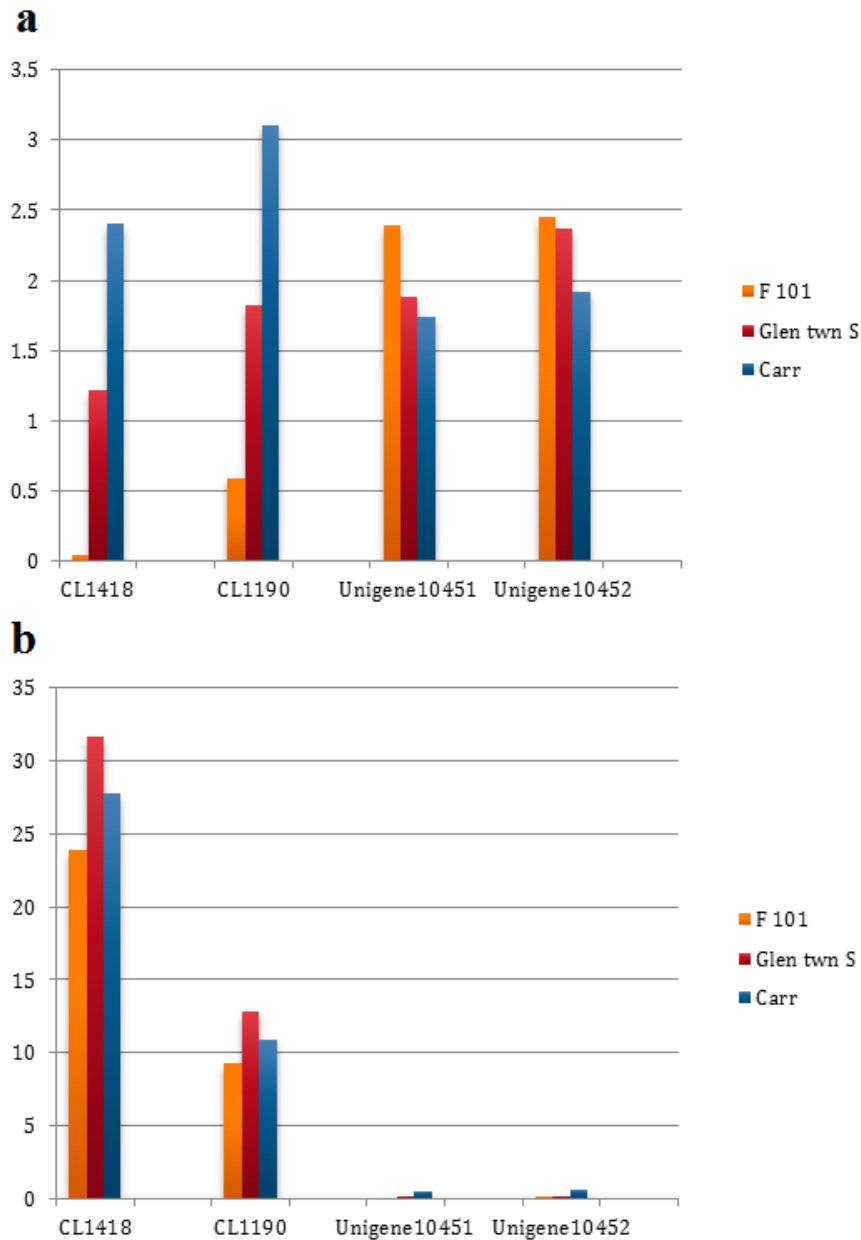


**Figure 8** Validation of gene expression of four transcripts selected from RNA-Seq analysis. (a) The fold change ($log_2$Ratio) for genes from RNA-Seq analysis between strain comparisons: F 101/Sus F 96; Glen twn S/Sus F 96; and Carr/Sus F 96 (b) The fold change of each gene was calculated by qRT-PCR using the comparative CT method.

*4.4 Discussion*

The aim of this study was to investigate insecticide resistance mechanisms associated with neonicotinoid resistant *A. gossypii* from Australian cotton. In the present de novo assembly, a total of 132,159,760 clean reads from the pooled transcriptomes of thiamethoxam resistant and susceptible *A. gossypii* strains were generated resulting in a dramatically increased repertoire of resistance-related genes in *A. gossypii* under thiamethoxam stress. Clean reads were assembled into 37,167 contigs and from this 31,042 unigenes were assembled of which 23,372 matched known genes. Therefore, this study has generated a comprehensive transcriptome resource for *A. gossypii* that has characterized the expression of numerous important transcripts encoding proteins involved in insecticide resistance. Consequently, this study will contribute to future research relating to molecular characterization of insecticide resistance mechanisms of *A. gossypii* and other insect pests.

P450s function in insects as enzymatic proteins involved in a vast number of metabolic processes including insecticide detoxification (Li et al. 2006, Schuler 2011). Although metabolic resistance mediated by overexpression of P450s may be triggered by modifications in *cis/trans* regulatory elements or post translational events (Bass and Field 2011), correlations between gene amplification and overexpression of P450s have been implicated (Puinean et al. 2010, Faucon et al. 2015). According to previous research, the P450 gene families involved in up-regulation and amplification are CYP4, CYP6 and CYP9 (belonging to the CYP3 and CYP4 clans).

In the present study, seven differentially expressed P450 unigenes (Contig ID 627, 1190, 1418, 4712, 12511, 12819 and 15803) were observed belonging to the CYP2 and CYP3 clans that were up-regulated in strain Carr, and six of these up-regulated in Glen twn S (not contig 12511). In strain F 101, only two transcripts (Contig ID 1190 and 1418) were up-regulated when compared to Sus F 96 (although not significantly differentially expressed). Therefore, the transcriptome analysis presented here centred on the role of contigs 1190 and 1418, despite one of the three strains not being significantly differentially expressed, although still up-regulated. These transcripts were predicted as the cytochrome P450 gene 6K1 (*CYP6K1*), and when blast searched against the NCBI database showed 68% and 82% similarity in amino acid sequence to *CYP6K1* of *A. pisum* (XP001948421.1). Contig 1190 matched the amino acid sequence of XP001948421.1 from 1-271 and of the same sequence contig 1418 matched from position 272-514. In the hemipteran insects, the silverleaf whitefly, *Bemisia tabaci* and *M. persicae,* over-expression of two CYP6 P450 genes (*CYP6CM1* and *CYP6CY3* respectively) has previously been linked to resistance of neonicotinoid insecticides (Karunker et al. 2008, Puinean et al. 2010, Yang et al. 2013). Transcriptional profiles of contigs 1190 and 1418 were validated by qRT-PCR and were found to be highly overexpressed in

resistant strains despite significantly lower expression levels obtained from RNA-Seq analysis, especially for strain F 101. This may be explained by the well-known underestimation of expression ratios by RNA-Seq analysis compared with qRT-PCR (Roberts et al. 2011). Discrepancies in the data obtained from RNA-Seq analysis using the Illumina Hi-Seq™ platform and qRT-PCR highlight the importance of qRT-PCR validation of RNA-Seq results.

The expressions of several genes with catalytic/oxidoreductase activity (such as proteins with choline or glucose dehydrogenase activity) were differentially transcribed among resistant and susceptible strains, suggesting a possible relationship between the insecticide resistance phenotype and these physiological processes (Contig ID 273, 324 and 13767). Interestingly, two of these genes, contig 324, annotated as choline dehydrogenase and contig 13767, homologous to *A. pisum* gene ACYPI007791 which encodes dehydrogenase/reductase SDR family member 4, are constitutively overexpressed in all three thiamethoxam resistant strains. These genes encode subunits which function in the mitochondria and belong to complexes of the electron transport and respiratory chain. These observations strongly support the hypothesis that mitochondrial energy/redox metabolism are among the mechanisms partially responsible for detoxification of thiamethoxam. Similar trends in up-regulation of mitochondrial genes were recently reported in the African malaria mosquito, *Anopheles gambiae* after *Plasmodium* sp. infection (Kumar et al. 2003).

Among the DEGs, expression levels of Hsp70 family members were dramatically elevated in resistant strain F 101 (Contig ID 2116, 5014, 7780, 10435 and 11682). In insects, Hsps are modulated in response to a variety of chemical and physical stresses such as heat shock, ultraviolet radiation, chemical pesticides, as well as biotic stresses such as viruses, bacteria and fungi (Parsell and Lindquist 1993, Feder and Hofmann 1999, Sørensen et al. 2003). Their up-regulation is an important part of the cellular stress response induced to maintain stress tolerance and promote cell survival through refolding proteins and preventing their denaturation (Parsell and Lindquist 1993, Feder and Hofmann 1999). Up-regulation of Hsps have been shown to contribute to pesticide tolerance and resistance (Nazir et al. 2001, Feng et al. 2010, Škerl and Gregorc 2010, Chen et al. 2014). For instance, in the ferment fly, *Drosophila melanogaster* and silk worm, *Bombyx mori*, overexpression of Hsp70 was induced by application of the insecticides, chlorpyrifos and pyridalyl, respectively (Nazir et al. 2001, Powell et al. 2011). Similarly, in a spirotetramat resistant strain of *A. gossypii*, up-regulation of five putatively designated Hsp70 unigenes was linked to the resistant phenotype. Therefore, in *A. gossypii*, up-regulation of Hsps may be indicative of an adaptive ability to protect tissues against oxidate stress induced by insecticides (Pournourmahammadi and Abdollahi 2011). Alternatively, as no differentially expressed Hsps were up-regulated in strains Carr and Glen twn S, the dramatically increased expression in strain F 101 may be related to other

abiotic stressors, such as temperature (Jones and Zhao 2012). Indeed, the Hsp70 family appears to be the most prominent contributor to temperature tolerance in insects by enabling increased heat tolerance of organisms to protect them from thermal injury and death (Sørensen et al. 2003). This has been evidenced in numerous insect species, e.g. the oriental fruit moth, *Grapholita molesta* (Chen et al. 2014); ferment flies: *Drosophila buzzatii* (Sorensen et al. 1999) and *D. melanogaster* (Nazir et al. 2001); brown planthopper, *Nilaparvata lugens* (Kim et al. 2008); and the corn earworm, *Helicoverpa zea* (Zhang and Denlinger 2010).

The ABC transporter superfamily is the largest gene family involved in the transport of various substrates across biological membranes, including amino-acids, sugars, lipids, inorganic ions, polysaccharides, metals, peptides, toxic metabolites and drugs (Higgins 1992). Differential expression of certain transporters of B, C and G subfamilies in humans contributes to multidrug resistance of cancer cells against chemotherapeutics (Gottesman et al. 2002): the multidrug resistance proteins (MDR and ABCB subfamily) or P-glycoproteins (Gerlach et al. 1986, Dean et al. 2001); the multidrug resistance associated proteins (MRPs and ABCC subfamily) (Dean et al. 2001); and the breast cancer resistance protein (BCRP and ABCG2 subfamily) (Doyle and Ross 2003). In insects, physiological functions of ABC transporters include roles in molecule transport, and functions that affect metabolism, development and also insecticide resistance (Dermauw and Van Leeuwen 2014). Some ABC transporters of subfamilies B, C and G have been shown to confer resistance to xenobiotics, including insecticides. For example, in the tobacco hornworm, *Manducta sexta*, which feeds on nicotine containing tobacco leaves, nicotine is efficiently excreted by P-glycoprotein-like multidrug transporters in the Malpighian tubules (Murray et al. 1994). Also, in the diamondback moth, *Plutella xylostella*, down-regulation of a novel ABC transporter gene from ABCG subfamily (Pxwhite) is associated with resistance to a Cry toxin, Cry1Ac (Guo et al. 2015). In the present study, down regulation of several transcripts (Contig ID 7980 and 18326) that code for MRPs and include the ABC transporter cassette motif in their structures, are likely to contribute to thiamethoxam resistance in *A. gossypii.*

Genetic changes in genes encoding nAChR subunits are regarded as primary determinants of neonicotinoid resistance in insects (Liu et al. 2006, Bass et al. 2011, Shi et al. 2012, Puinean et al. 2013, Koo et al. 2014). In this study, all nAChR subunits identified through transcriptome analysis were downregulated in each resistant strain compared to Sus F 96. This is consistent with two other studies which have found reduced nAChR subunit expression in neonicotinoid resistant insects, including *A. gossypii*. A transcriptomic survey of thiamethoxam resistant *A. gossypii* by Pan et al. (2015) found downregulation of a nAChR α subunit may contribute to resistance. Elsewhere, in *M. domestica* and *N. lugens*, imidacloprid resistance is correlated with a reduction in expression levels

of the nAChR subunits, α2 and Nlα8, respectively (Markussen and Kristensen 2010, Zhang et al. 2015). These results suggest that depletion of the nAChR subunits may relate to thiamethoxam resistance in Australian *A. gossypii*.

In conclusion, this study has contributed a substantial sequence resource for aphids and is likely to accelerate insecticide resistance mechanism research in *A. gossypii* when under thiamethoxam stress. Comparative transcriptome analysis identified a catalogue of candidate genes that might be involved in conferring neonicotinoid resistance in *A. gossypii*. In particular, some genes encoding Hsps, catalytic/oxidoreductase activity (such as proteins with choline or glucose dehydrogenase activity), ABC transporters, cytochrome P450s and nAChR subunits might play crucial roles in conferring resistance to neonicotinoid compounds. Among the DEGs, up-regulation of cytochrome P450 *CYP6K1* and the role it plays in detoxifying thiamethoxam should be further investigated.

## *References*

**5. Include a list of any material which has been cited throughout this report.**

Abbott, W. S. (1925). "A method for computing the effectiveness of an insecticide." Journal of Economic Entomology **18**: 265-267.

Andrews, M. C., A. Callaghan, L. M. Field, M. S. Williamson and G. D. Moores (2004). "Identification of mutations conferring insecticide-insensitive AChE in the cotton-melon aphid, *Aphis gossypii* Glover." Insect Molecular Biology **13**(5): 555-561.

APVMA. (2013a). "Overview Report: Neonicotinoids and the health of honey bees in Australia." February 2014. Retrieved March 9th, 2014, from https://archive.apvma.gov.au/news_media/chemicals/bee_and_neonicotinoids.php.

APVMA. (2013b). "Australian Pesticides and Veterinary Medicines Authority." Retrieved January 11th, 2015, from https://portal.apvma.gov.au/pubcris.

APVMA. (2014). "Agricultural and Veterinary Permits search." Retrieved 11th May, 2015, from https://portal.apvma.gov.au/permits.

Audic, S. and J. M. Claverie (1997). "The significance of digital gene expression profiles." Genome Research **7**(10): 986-995.

Barchia, I. (2001). Probit analysis and fiducial limits in Genstat. Genstat 2001 Program and Abstracts. V. Doogan, D. Mayer and T. Swain. Mercure Resort, Surfers Paradise, Gold Coast, 31 January–2 February 2001, Australia**:** p. 3.

Bass, C. and L. M. Field (2011). "Gene amplification and insecticide resistance." Pest Management Science **67**(8): 886-890.

Bass, C., A. Puinean, M. Andrews, P. Cutler, M. Daniels, J. Elias, V. Paul, A. Crossthwaite, I. Denholm, L. Field, S. Foster, R. Lind, M. Williamson and R. Slater (2011). "Mutation of a nicotinic acetylcholine receptor beta subunit is associated with resistance to neonicotinoid insecticides in the aphid *Myzus persicae*." BMC Neuroscience **12**(51): 1-11.

Benjamini, Y. and D. Yekutieli (2001). "The control of the false discovery rate in multiple testing under dependency." The Annals of Statistics **29**(4): 1165-1188.

Benting, J. and R. Nauen (2004). "Biochemical evidence that an S431F mutation in acetylcholinesterase-1 of *Aphis gossypii* mediates resistance to pirimicarb and omethoate." Pest Management Science **60**(11): 1051-1055.

Bingham, G., R. V. Gunning, G. Delogu, V. Borzatta, L. M. Field and G. D. Moores (2008). "Temporal synergism can enhance carbamate and neonicotinoid insecticidal activity against resistant crop pests." Pest Management Science **64**(1): 81-85.

Blackman, R. L. and V. F. Eastop (2000). Aphids on the World's Crops: An Identification and Information Guide (2nd Edition). Chichester, UK, John Wiley and Sons.

CABI. (2005). "Invasive Species Compendium."  Retrieved July 12th, 2014, from www.cabi.org.

Chen, E. H., D. D. Wei, G. M. Shen, G. R. Yuan, P. P. Bai and J. J. Wang (2014). "De novo characterization of the *Dialeurodes citri* transcriptome: mining genes involved in stress resistance and simple sequence repeats (SSRs) discovery." Insect Molecular Biology **23**(1): 52-66.

Chen, H., X.-L. Xu, Y.-P. Li and J.-X. Wu (2014). "Characterization of heat shock protein 90, 70 and their transcriptional expression patterns on high temperature in adult of *Grapholita molesta* (Busck)." Insect Science **21**(4): 439-448.

Conesa, A., S. Gotz, J. M. Garcia-Gomez, J. Terol, M. Talon and M. Robles (2005). "Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research." Bioinformatics **21**(18): 3674-3676.

CottASSIST. (2008). "Aphid Yield Loss Estimator."  Retrieved November 17th, 2014, from https://www.cottassist.com.au.

CottonInfo (2015). Cotton Pest Management Guide 2015-16. Toowoomba, QLD, Greenmount Press.

Dean, M., A. Rzhetsky and R. Allikmets (2001). "The human ATP-binding cassette (ABC) transporter superfamily." Genome Res **11**(7): 1156-1166.

Dermauw, W. and T. Van Leeuwen (2014). "The ABC gene family in arthropods: Comparative genomics and role in insecticide transport and resistance." Insect Biochemistry and Molecular Biology **45**: 89-110.

Doyle, L. and D. D. Ross (2003). "Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2)." Oncogene **22**(47): 7340-7358.

Faucon, F., I. Dusfour, T. Gaude, V. Navratil, F. Boyer, F. Chandre, P. Sirisopa, K. Thanispong, W. Juntarajumnong, R. Poupardin, T. Chareonviriyaphap, R. Girod, V. Corbel, S. Reynaud and J. P. David (2015). "Identifying genomic changes associated with insecticide resistance in the dengue mosquito *Aedes aegypti* by deep targeted sequencing." Genome Res **25**(9): 1347-1359.

Feder, M. E. and G. E. Hofmann (1999). "Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology." Annual Review of Physiology **61**: 243-282.

Feng, H., L. Wang, Y. Liu, L. He, M. Li, W. Lu and C. Xue (2010). "Molecular characterization and expression of a heat shock protein gene (HSP90) from the carmine spider mite, *Tetranychus cinnabarinus* (Boisduval)." Journal of Insect Science **10**(112): 1-14.

Finney, D. J. (1971). Probit Analysis. Cambridge, UK., Cambridge University Press.

Fitt, G. (2003). Deployment and Impact of Transgenic Bt Cotton in Australia. The Economic and Environmental Impacts of Agbiotech. N. Kalaitzandonakes, Springer US**:** 141-164.

Gao, C. F., S. Z. Ma, C. H. Shan and S. F. Wu (2014). "Thiamethoxam resistance selected in the western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae): cross-resistance patterns, possible biochemical mechanisms and fitness costs analysis." Pesticide Biochemistry and Physiology **114**: 90-96.

Gerlach, J. H., N. Kartner, D. R. Bell and V. Ling (1986). "Multidrug resistance." Journal of Cancer Survivorship **5**(1): 25-46.

Gilmour, A. R., B. J. Gogel, B. R. Cullis and R. Thompson (2009). ASReml User Guide Release 3.0. Hemel Hempstead, HP1 1ES, UK, VSN International Ltd.

Gottesman, M. M., T. Fojo and S. E. Bates (2002). "Multidrug resistance in cancer: role of ATP-dependent transporters." Nature Reviews Cancer **2**(1): 48-58.

Grabherr, M. G., B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F. di Palma, B. W. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman and A. Regev (2011). "Full-length transcriptome assembly from RNA-Seq data without a reference genome." Nature Biotechnology **29**(7): 644-652.

Guo, Z., S. Kang, X. Zhu, J. Xia, Q. Wu, S. Wang, W. Xie and Y. Zhang (2015). "Down-regulation of a novel ABC transporter gene (Pxwhite) is associated with Cry1Ac resistance in the diamondback moth, *Plutella xylostella* (L.)." Insect Biochemistry and Molecular Biology **59**: 30-40.

Henderson, C. F. and E. W. Tilton (1955). "Tests with acaricides against the brown wheat mite." Journal of Economic Entomology **48**: 157-161.Herron, G. A., K. Powis and J. Rophail (2001). "Insecticide resistance in *Aphis gossypii* Glover (Hemiptera: Aphididae), a serious threat to Australian cotton." Australian Journal of Entomology **40**(1): 85–91.

Herron, G. A. and J. Rophail (2000). Insecticide resistance in cotton aphid linked to field-control failures in Australian cotton. The 10th Australian Cotton Conference, Gold Coast, N.S.W.

Herron, G. A. and L. J. Wilson (2011). "Neonicotinoid resistance in *Aphis gossypii* Glover (Aphididae: Hemiptera) from Australian cotton." Australian Journal of Entomology **50**(1): 93-98.

Herron, G. A., B. J. Langfield, D. R. Bogema and Y. Chen (2013). "Baseline susceptibility and cross-resistance in *Aphis gossypii* Glover (Aphididae: Hemiptera) to phorate and sulfoxaflor." Australian Journal of Entomology **53**(1): 32-35.

Herron, G. A., R. V. Gunning, E. L. Cottage, V. Borzatta and C. Gobbi (2014). "Spinosad resistance, esterase isoenzymes and temporal synergism in Frankliniella occidentalis (Pergande) in Australia." Pestic Biochem Physiol **114**: 32-37.

Higgins, C. F. (1992). "ABC transporters: from microorganisms to man." Annual Review of Cell Biology **8**: 67-113.

Iseli, C., C. V. Jongeneel and P. Bucher (1999). "ESTScan: a program for detecting, evaluating, and reconstructing potential coding regions in EST sequences." Proc Int Conf Intell Syst Mol Biol: 138-148.

Jeschke, P. and R. Nauen (2008). "Neonicotinoids—from zero to hero in insecticide chemistry." Pest Management Science **64**(11): 1084-1098.

Jones, W. A. and L. Zhao (2012). "Expression of heat shock protein genes in insect stress responses." Invertebrate Survival Journal **9**(1): 93-101.

Karunker, I., J. Benting, B. Lueke, T. Ponge, R. Nauen, E. Roditakis, J. Vontas, K. Gorman, I. Denholm and S. Morin (2008). "Over-expression of cytochrome P450 CYP6CM1 is

associated with high resistance to imidacloprid in the B and Q biotypes of *Bemisia tabaci* (Hemiptera: Aleyrodidae)." Insect Biochemistry and Molecular Biology **38**(6): 634-644.

Khan, H. A. A., W. Akram, J. Iqbal and U. Naeem-Ullah (2015). "Thiamethoxam Resistance in the House Fly, *Musca domestica* L.: Current Status, Resistance Selection, Cross-Resistance Potential and Possible Biochemical Mechanisms." PLoS One **10**(5).

Kim, D. H., S.-C. Lee, D.-Y. Kwak and K.-Y. Lee (2008). "Cloning of heat shock protein genes from the brown planthopper, *Nilaparvata lugens*, and the small brown planthopper, *Laodelphax striatellus*, and their expression in relation to thermal stress." Insect Science **15**(5): 415-422.

Kim, J. I., M. Kwon, G.-H. Kim, S. Y. Kim and S. H. Lee (2015). "Two mutations in nAChR beta subunit is associated with imidacloprid resistance in the *Aphis gossypii*." Journal of Asia-Pacific Entomology **18**(2): 291-296.

Koo, H.-N., J.-J. An, S.-E. Park, J.-I. Kim and G.-H. Kim (2014). "Regional susceptibilities to 12 insecticides of melon and cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae) and a point mutation associated with imidacloprid resistance." Crop Protection **55**: 91-97.

Kumar, S., G. K. Christophides, R. Cantera, B. Charles, Y. S. Han, S. Meister, G. Dimopoulos, F. C. Kafatos and C. Barillas-Mury (2003). "The role of reactive oxygen species on Plasmodium melanotic encapsulation in Anopheles gambiae." Proceedings of the National Academy of Sciences of the United States of America **100**(24): 14139-14144.

Li, R., C. Yu, Y. Li, T. W. Lam, S. M. Yiu, K. Kristiansen and J. Wang (2009). "SOAP2: an improved ultrafast tool for short read alignment." Bioinformatics **25**(15): 1966-1967.

Li, X., M. A. Schuler and M. R. Berenbaum (2006). "Molecular Mechanisms of Metabolic Resistance to Synthetic and Natural Xenobiotics." Annual Review of Entomology **52**(1): 231-253

Liu, Z., M. S. Williamson, S. J. Lansdell, I. Denholm, Z. Han and N. S. Millar (2005). "A nicotinic acetylcholine receptor mutation conferring target-site resistance to imidacloprid in *Nilaparvata lugens* (brown planthopper)." Proceedings of the National Academy of Sciences of the United States of America **102**(24): 8420-8425.

Liu, Z., M. S. Williamson, S. J. Lansdell, Z. Han, I. Denholm and N. S. Millar (2006). "A nicotinic acetylcholine receptor mutation (Y151S) causes reduced agonist potency to a range of neonicotinoid insecticides." Journal of Neurochemistry **99**(4): 1273-1281.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Maienfisch, P., M. Angst, F. Brandl, W. Fischer, D. Hofer, H. Kayser, W. Kobel, A. Rindlisbacher, R. Senn, A. Steinemann and H. Widmer (2001). "Chemistry and biology of thiamethoxam: a second generation neonicotinoid." Pest Management Science **57**(10): 906-913.

Markussen, M. D. K. and M. Kristensen (2010). "Low expression of nicotinic acetylcholine receptor subunit Mdα2 in neonicotinoid-resistant strains of *Musca domestica* L." Pest Management Science **66**(11): 1257-1262.

Marshall, K. L., C. Moran, Y. Chen and G. A. Herron (2012). "Detection of kdr pyrethroid resistance in the cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae), using a PCR-RFLP assay." Journal of Pesticide Science **37**(2): 169-172.

Mass, S. (2012). Cotton Pest Management Guide 2011-12. Toowoomba, Australia, Greenmount Press.

Matsuda, K., S. D. Buckingham, D. Kleier, J. J. Rauh, M. Grauso and D. B. Sattelle (2001). "Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors." Trends in Pharmacological Sciences **22**(11): 573-580.

McLoon, M. O. and G. A. Herron (2009). "PCR detection of pirimicarb resistance in Australian field isolates of *Aphis gossypii* Glover (Aphididae: Hemiptera)." Australian Journal of Entomology **48**(1): 65-72.

Miller, W. B., E. Peralta, D. R. Ellis and H. H. Perkins (1994). "Stickiness Potential of Individual Insect Honeydew Carbohydrates on Cotton Lint." Textile Research Journal **64**(6): 344-350.

Mortazavi, A., B. A. Williams, K. McCue, L. Schaeffer and B. Wold (2008). "Mapping and quantifying mammalian transcriptomes by RNA-Seq." Nature Methods **5**(7): 621-628.

Murray, C. L., M. Quaglia, J. T. Arnason and C. E. Morris (1994). "A putative nicotine pump at the metabolic blood-brain barrier of the tobacco hornworm." Journal of Neurobiology **25**(1): 23-34.

Nazir, A., I. Mukhopadhyay, D. K. Saxena and D. Kar Chowdhuri (2001). "Chlorpyrifos-induced hsp70 expression and effect on reproductive performance in transgenic Drosophila melanogaster (hsp70-lacZ) Bg9." Archives of Environmental Contamination and Toxicology **41**(4): 443-449.

Pan, Y., T. Peng, X. Gao, L. Zhang, C. Yang, J. Xi, X. Xin, R. Bi and Q. Shang (2015). "Transcriptomic comparison of thiamethoxam-resistance adaptation in resistant and susceptible strains of *Aphis gossypii* Glover." Comparative Biochemistry and Physiology Part D Genomics and Proteomics **13**: 10-15.

Parsell, D. A. and S. Lindquist (1993). "The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins." Annual Review of Genetics **27**: 437-496.

Pournourmahammadi, S. and M. Abdollahi (2011). Gene Expression. Anticholinesterase Pesticides: Metabolism, Neurotoxicity, and Epidemiology. T. Satoh and R. C. Gupta. Hoboken, New Jersey, John Wiley & Sons**:** 175-188.

Powell, G. F., D. A. Ward, M. C. Prescott, D. G. Spiller, M. R. H. White, P. C. Turner, F. G. P. Earley, J. Phillips and H. H. Rees (2011). "The molecular action of the novel insecticide, Pyridalyl." Insect Biochemistry and Molecular Biology **41**(7): 459-469.

Prasanna, A. R., M. Bheemanna and B. V. Patil (2004). "Evaluation of Thiamethoxam 70WS as Seed Treatment Against Leaf Miner and Early Sucking Pests on Hybrid Cotton." Karnataka Journal of Agricultural Science **17**(2): 238-241.

Puinean, A. M., S. P. Foster, L. Oliphant, I. Denholm, L. M. Field, N. S. Millar, M. S. Williamson and C. Bass (2010). "Amplification of a cytochrome P450 gene is associated with resistance to neonicotinoid insecticides in the aphid *Myzus persicae*." PLoS Genetics **6**(6).

Puinean, A. M., S. J. Lansdell, T. Collins, P. Bielza and N. S. Millar (2013). "A nicotinic acetylcholine receptor transmembrane point mutation (G275E) associated with resistance to spinosad in *Frankliniella occidentalis*." Journal of Neurochemistry **124**(5): 590-601.

Raffa, K. F. and T. M. Priester (1985). "Synergist as research tools and control agents in agriculture." Journal of Agricultural Entomology **2**(1): 27-45.

Reddall, A., A. Ali, J. A. Able, J. Stonor, L. Tesoriero, P. R. Wright, M. A. Rezaian and L. J. Wilson (2004). "Cotton bunchy top: an aphid and graft transmitted cotton disease." Australasian Plant Pathology **33**(2): 197-202.

Roberts, A., C. Trapnell, J. Donaghey, J. L. Rinn and L. Pachter (2011). "Improving RNA-Seq expression estimates by correcting for fragment bias." Genome Biology **12**(3).

Robertson, J. L., R. M. Russell, H. K. Preisler and N. E. Savin (2007). Bioassays with Arthropods 2nd ed. Boca Raton, CRC Press.

Russell, R. J., C. Claudianos, P. M. Campbell, I. Horne, T. D. Sutherland and J. G. Oakeshott (2004). "Two major classes of target site insensitivity mutations confer resistance to organophosphate and carbamate insecticides." Pesticide Biochemistry and Physiology **79**(3): 84-93.

Schepers, A. (1989). Chemical conrol. World Crop Pests: Aphids, Their Biology, Natural Enemies and Control. A. K. Minks and P. Harrewijn. Amsterdam, Elsevier. **C:** 89-122.

Schuler, M. A. (2011). "P450s in plant-insect interactions." Biochimica et Biophysica Acta **1814**(1): 36-45.

Scott, J. G. (1990). Investigating Mechanisms of Insecticide Resistance: Methods, Strategies and Pitfalls. Pesticide Resistance in Arthropods. R. T. Roush and B. E. Tabashnik. Great Britain, Chapman and Hall**:** 39-57.

Shi, X., L. Jiang, H. Wang, K. Qiao, D. Wang and K. Wang (2011). "Toxicities and sublethal effects of seven neonicotinoid insecticides on survival, growth and reproduction of imidacloprid-resistant cotton aphid, *Aphis gossypii*." Pest Management Science **67**(12): 1528-1533.

Shi, X.-B., X.-G. Shi, H.-Y. Wang, X.-M. Xia and K.-Y. Wang (2011). "Cross-resistance of the imidacloprid-resistant population of *Aphis gossypii* Glover (Homoptera: Aphididae) to other neonicotinoid insecticides and changes in activities of related enzymes." Acta Entomologica Sinica **54**(9): 1027-1033.

Shi, X. G., Y. K. Zhu, X. M. Xia, K. Qiao, H. Y. Wang and K. Y. Wang (2012). "The mutation in nicotinic acetylcholine receptor beta 1 subunit may confer resistance to imidacloprid in *Aphis gossypii* (Glover)." Journal of Food Agriculture & Environment **10**(2): 1227-1230.

Škerl, M. and A. Gregorc (2010). "Heat shock proteins and cell death in situ localisation in hypopharyngeal glands of honeybee (*Apis mellifera carnica*) workers after imidacloprid or coumaphos treatment." Apidologie **41**(1): 73-86.

Smith, T., L. Wilson, S. Heimoana, G. Herron and B. Franzmann (2006). Overwinter host plants of cotton aphid (*Aphis gossypii*) and implications for managing abundance and resistance. Proceedings 13th Australian Cotton Growers Conference. Gold Coast. 8-10 August 2006.

Sørensen, J. G., T. N. Kristensen and V. Loeschcke (2003). "The evolutionary and ecological role of heat shock proteins." Ecology Letters **6**(11): 1025-1037.

Sorensen, J. G., P. Michalak, J. Justesen and V. Loeschcke (1999). "Expression of the heat-shock protein HSP70 in *Drosophila buzzatii* lines selected for thermal resistance." Hereditas **131**(2): 155-164.

Sumitomo Chemical Australia Pty Ltd. (2010). "Shield Cotton Brochure." Retrieved March 12th, 2014, from http://www.sumitomo-chem.com.au/sites/default/files/literature/shield_cotton_brochure.pdf.

Wang, K. I., Q. L. Guo, X. M. Xia, H. Y. Wang and T. X. Liu (2007). "Resistance of *Aphis gossypii* (Homoptera: Aphididae) to selected insecticides on cotton from five cotton production regions in Shandong, China." Journal of Pesticide Science **32**(4): 372-378.

Wilson, L. J. (1996). Will current secondary pests become problems in the transgenic era. 'Cotton on to the Future': The Eighth Australian Cotton Conference. Broadbeach, Gold Coast, Queensland, 14 - 16th August 1996**:** 85-96.

Yang, X., W. Xie, S. L. Wang, Q. J. Wu, H. P. Pan, R. M. Li, N. N. Yang, B. M. Liu, B. Y. Xu, X. Zhou and Y. J. Zhang (2013). "Two cytochrome P450 genes are involved in imidacloprid resistance in field populations of the whitefly, *Bemisia tabaci,* in China." Pesticide Biochemistry and Physiology **107**(3): 343-350.

Zhang, Q. R. and D. L. Denlinger (2010). "Molecular characterization of heat shock protein 90, 70 and 70 cognate cDNAs and their expression patterns during thermal stress and pupal diapause in the corn earworm." Journal of Insect Physiology **56**(2): 138-150.

Zhang, Y., X. Wang, B. Yang, Y. Hu, L. Huang, C. Bass and Z. Liu (2015). "Reduction in mRNA and protein expression of a nicotinic acetylcholine receptor alpha8 subunit is associated with resistance to imidacloprid in the brown planthopper, *Nilaparvata lugens*." Journal of Neurochemistry **135**(4): 686-694.

Zidan, L. T. M. (2012). "Bio-Efficacy of Three New Neonicotinoid Insecticides as Seed Treatment Against Four Early Sucking Pests of Cotton." American-Eurasian Journal Agricultural and Environmental Science **12**(4): 535-540.

*Outcomes*

## 6. Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

The planned outcomes of this project were two-fold: (1) to obtain a better understanding of neonicotinoid resistance in *A. gossypii* to under-pin improved resistance management and restore efficacy of neonicotinoid compounds and (2) to train a young scientist in both bioassay and molecular based methodologies for resistance detection in arthropod pests.

Initial study outputs against thiamethoxam resistant *A. gossypii* found significantly higher resistance levels than previously found during the 2007-08 and 2008-09 cotton seasons suggesting selection pressure for resistant genotypes was high in Australian cotton. Indeed, when resistant *A. gossypii* were included into a whole plant efficacy trial, resistant aphids were able to survive and reproduce on cotton treated with varying rates of thiamethoxam. Importantly, outputs here demonstrated that both rates of thiamethoxam provided adequate protection of susceptible *A. gossypii* with the consequent outcome suggesting if the selection pressure for resistant genotypes could be lowered, the utility of either neonicotinoid pre-germination seed treatment against susceptible *A. gossypii* could be preserved.

Outcomes suggest one such way to avoid selection over successive generations is the rotation of insecticides between different MoA classes. At-planting or in-furrow granular insecticides are one possible alternative to neonicotinoid MoA group 4A seed coated treatments but their use must be carefully considered. As phorate is an at-planting side dressing its place in any control strategy is fixed i.e. it will always be used first. As resistance management of *A. gossypii* is based on the alternation of chemical groups after each chemical treatment cycle, the first foliar spray can't comprise the IPM friendly carbamate insecticide pirimicarb (Pirimor®), as pirimicarb-resistant

aphids were capable of completing their development on cotton seedlings grown with phorate. Therefore, if phorate is used to reduce selection pressure against neonicotinoid resistant genotypes, the first foliar spray needs to be from a different chemical group other than group 1A and 1B. The insecticide diafenthiuron (Pegasus®) (group 12A), like pirimicarb, is selective to beneficial insects and predatory mites and is therefore useful in IPM programs. Additionally, sulfoxaflor (group 4C) provides adequate control against *A. gossypii* and has a low toxicity rating to predators, parasitoids and bees on cotton.

Importantly a study output found the R81T point mutation in loop D of the nAChR β1 subunit gene associated with neonicotinoid resistance in field populations of *A. gossypii* overseas was not present in Australian *A. gossypii* implying an alternate mechanism. Subsequent synergist bioassay identified a potential metabolic resistance mechanism when application of PBO in tandem with thiamethoxam reduced RFs from 7- to >1-fold. The outcome suggests that the addition of a synergist to thiamethoxam containing treatments may overcome insecticide resistance in the field and reduce the amount of chemical product necessary to control resistant aphids. Given the ever increasing difficulty in developing novel insecticide chemistries that target new insecticide targets, the development of mixtures of active compounds and their synergists may provide valuable future control strategies for *A. gossypii*.

Lastly, transcriptome analysis between thiamethoxam resistant and susceptible *A. gossypii* identified several candidate resistance genes linked to thiamethoxam resistance in *A. gossypii*. The up-regulation of transcripts CL1190 and CL1418, [putatively identified as cytochrome P450 gene 6K1 (*CYP6K1*)] from my RNA-Seq analysis and subsequent quantitative analysis of transcript expression via qRT-PCR, coupled with the synergistic effects of PBO provide the first direct evidence of metabolic detoxification acting as the primary causal resistance mechanism against thiamethoxam in field strains of *A. gossypii*. Consequently, this study will contribute to future research relating to molecular characterization of insecticide resistance mechanisms of *A. gossypii* and other insect pests.

Most importantly, this project aimed to train a young scientist in both bioassay and molecular genetic methodology used for resistance detection in arthropod pests. This will bridge the gap between these disciplines which are essential to effective resistance management. To that end, Ms Kate Marshall, now a Research Entomologist with the NSW Department of Primary Industries has received proficient training in bioassay and molecular genetic methodologies, as evidenced in the attached PhD thesis outcome (Appendix E).

**Please describe any:-**

**a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.);**

Technical advances included the improved resistance management of neonicotinoid resistant *A. gossypii*. The resistance management strategy was modified to say if phorate is used to control neonicotinoid resistant *A. gossypii* then pirimicarb, or any other chemical associated with *Ace*1 type resistance, should not immediately follow as the first foliar spray. This has been included into the Cotton Pest Management Guide from 2013-14.

**b) other information developed from research (eg discoveries in methodology, equipment design, etc.); and**

This study provided a first step in the development of a molecular based test for neonicotinoid resistance monitoring. Two transcripts (CL1190 and CL1418) putatively annotated as cytochrome P450 gene 6K1 (*CYP6K1*) were found over-expressed in each resistant strain. Further characterisation of this P450 gene by obtaining the full length gene sequence using genomic DNA would be an essential future study to elucidate any potential single nucleotide polymorphism(s) (SNP) which may be conferring the resistant phenotype observed. If a link is confirmed, the development of a molecular diagnostic to reliably associate this mechanism with thiamethoxam resistance would provide a rapid and cost effective assay for monitoring of resistant genotypes arising in the field.

**c)  required changes to the Intellectual Property register.**

Nil

*Conclusion*

**7.  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?**

- *Improved resistance management*: The study found phorate to provide good control against neonicotinoid resistant *A. gossypii* but that potential use pattern was compromised by cross resistance between phorate and the carbamate insecticide, pirimicarb (Pirimor®). To achieve improved neonicotinoid resistance management phorate use would need to be carefully considered if phorate was to be substituted for thiamethoxam containing seed treatments. The study concluded that if phorate is used to control neonicotinoid resistant *A. gossypii* then pirimicarb, or any other chemical associated with *Ace*1 type resistance, should not immediately follow as the first foliar spray.

- *A P450 detoxification mechanism may cause neonicotinoid resistance*: Molecular genetics found a point mutation (R81T) responsible for neonicotinoid resistance in *A. gossypii* populations overseas was not associated with resistance in *A. gossypii* collected off Australian cotton. Alternatively, synergist bioassays coupled with transcriptome analysis (RNA-Seq) between susceptible and resistant *A. gossypii* suggest an alternative resistance mechanism likely mediated, at least in part, by overexpression of a cytochrome P450 gene [putatively annotated as the cytochrome P450 gene 6K1 (*CYP6K1*)]. With further research there is potential to develop a rapid and cost effective DNA based assay for monitoring neonicotinoid resistance in Australian *A. gossypii*. Any such assay would be hugely beneficial to the Australian cotton industry.

- *Training a young insecticide resistance specialist*: The study allowed a young scientist to become proficient in both bioassay and molecular genetic methodologies for resistance detection in arthropod pest species; thus bridging the gap in a single individual between these two (bioassay and molecular genetics) complex but necessary scientific disciplines required to detect and manage resistance. Most importantly it has boosted the human capacity available to the Australian cotton industry to manage the ongoing problem of insecticide resistance.

*Extension Opportunities*

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

   **(a) to further develop or to exploit the project technology.**

- Reference susceptible and resistant strains need to be maintained so they can be used in future cross resistance studies (i.e. dinotefuran)

- A fitness cost analysis to determine the stability of neonicotinoid resistance in *A. gossypii* would contribute to effective resistance management (strains used in this study were noted to revert despite routine pressuring).

- Further characterisation of transcripts CL1190 and CL1418 [putatively annotated as the cytochrome P450 gene 6K1 (*CYP6K1*) and associated with thiamethoxam resistance in *A. gossypii*] by obtaining their full length gene sequence using genomic DNA may elucidate any potential SNP(s) which may be conferring the resistant phenotype observed. Once confirmed a molecular diagnostic can be developed for future neonicotinoid resistance monitoring in field populations of *A. gossypii*.

   **(b) for the future presentation and dissemination of the project outcomes.**

Where available, opportunities to present and further disseminate this research will be undertaken.

- A final thesis presentation will be given at the University of Technology, Sydney in August 2016.

- A second refereed journal publication summarising the transcriptome output (Appendix E: Chapter 5) has been drafted with the intention to submit to *PLoS Genetics*.

- Contributions will be made to the following cotton industry publications: CottonTails; Australian Cottongrower and; Spotlight.

**(c) for future research.**

Dr. Yizhou Chen (NSW DPI) and Ms Kate Marshall (NSW DPI) are preparing a research proposal in conjunction with the University of Sydney for either a PhD or Honours study to continue the work resulting from this PhD study. It would be focused on two main themes: (1) fitness cost analysis to determine if there is any reduced fitness associated with neonicotinoid resistant phenotypes in *A. gossypii*; (2) development of a molecular based assay for detection of neonicotinoid resistance in Australian *A. gossypii*.

9. **A. List the publications arising from the research project and/or a publication plan.**

*Thesis*

- ***Marshall, K.L.** 2016. *Characterisation of Neonicotinoid Resistance in the Cotton Aphid*. Unpublished PhD Thesis, University of Technology Sydney (submitted).

*Refereed journal publications*

- ***Marshall, K.L.,** D. Collins, Y. Chen, G.A. Herron. 2015. Efficacy of two thiamethoxam pre germination seed treatments and a phorate side-dressing against neonicotinoid- and pirimicarb-resistant cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae) <u>Austral Entomology</u> **(54)**:4 351-357.

*Conference Proceedings*

- ***Marshall, K.L.** 2012. Eye on aphid resistance pp. 23. In: *Spotlight on Cotton R&D, Winter 2012*. CRDC, Narrabri.

- ***Marshall, K.L.,** Wilson, L.J. and Herron, G.A. 2012. *Do the neonicotinoid seed treatments Cruiser® and Cruiser Extreme® control resistant aphids*? p. 102. In: 16th Australian Cotton Conference, Cotton Research and Development Corporation, Broadbeach, Australia, 5th-7th August 2012.

- **Marshall, K.L**. 2012. *Sustainable cotton management threatened by new insecticide resistance*. Presented at the: Elizabeth Macarthur Agricultural Institute, Menangle, Sydney, 27th November 2012.

- **Marshall, K.L.** 2013. *Characterisation of Neonicotinoid Resistance in the Cotton Aphid*. Presented at the: University of Technology, Sydney, Australia, 22nd November 2013.

- ***Marshall, K.L,** Herron, GA & Chen, Y. 2014. *Neonicotinoid Resistance in Cotton Aphid from Australia*. In: 17th Australian Cotton Conference. Cotton Research and Development Corporation, Broadbeach, Australia, 14th – 16th August 2014.

- **Marshall, K.L.** 2014. *Characterisation of Neonicotinoid Resistance in the Cotton Aphid*. Presented at the: 17th Australian Cotton Conference, Broadbeach, Australia, 14th – 16th August 2014.

* indicates an output that is given in the Appendix at the end of this report

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

Outputs produce outcomes made quickly available to industry via the annual *Cotton Pest Management Guide* available at http://www.crdc.com.au/publications/cotton-pest-management-guide-2013-14.

## *Part 4 – Final Report Executive Summary*

*Aphis gossypii* is a highly polyphagous pest that inflicts serious damage to a broad range of agricultural, horticultural and greenhouse crops. In Australia, *A. gossypii* is a significant pest of cotton and is difficult to control with insecticides because of its high propensity to develop resistance. Neonicotinoids are among the most effective insecticides used to control *A. gossypii* but the recent detection of resistance threatens their longevity. Consequently, an industry initiative was established that aimed to restore neonicotinoid efficacy against *A. gossypii* through elucidation of underlying resistance mechanism(s).

Bioassay was used to measure thiamethoxam response in three field strains collected from commercial cotton. RFs between 49- and 85-fold were produced and resistance was correlated with potential field control failures via a RCBD glasshouse efficacy trial. Results showed that resistant *A. gossypii* could complete their development on cotton grown from thiamethoxam-treated seed. A second trial investigated the use of phorate (an organophosphate) as an alternative pre-germination treatment to thiamethoxam. Phorate effectively controls neonicotinoid resistant *A. gossypii* but cross resistance between phorate and the carbamate insecticide pirimicarb via *Ace*1 type resistance must be carefully considered as part of any sustainable management strategy.

PCR-Sequencing was employed to identify if mutation R81T known to confer resistance to neonicotinoid compounds was present in Australian *A. gossypii*. Comparative sequence analysis

between susceptible and resistant strains confirmed the absence of mutation R81T. Potential biochemical mechanisms of thiamethoxam resistance in *A. gossypii* were then studied using synergist bioassays. The use of the synergist PBO in tandem with thiamethoxam completely or partially suppressed resistance. This suggests that resistance is at least in part, mediated by overexpression of detoxification enzymes that could subsequently be targeted to achieve improved field control of resistant aphids.

High-throughput sequencing of the *A. gossypii* transcriptome found differences in gene expression associated with thiamethoxam resistance. Two transcripts involved in the detoxification of xenobiotics (putatively annotated as [cytochrome P450 gene 6K1 (CYP6K1)] were found differentially expressed between resistant and susceptible strains. Transcript expression was further validated by qRT-PCR and showed a similar tendency in up-regulation of expression. As such this gene was identified as the strongest candidate for thiamethoxam resistant *A. gossypii.*

In summary, this study has generated a comprehensive transcriptome resource for *A. gossypii* and has provided the first step in the development of a molecular based test for neonicotinoid resistance monitoring. However, further studies to confirm the role of this gene in detoxifying thiamethoxam are required before a test can be developed. Consequently, this study will contribute to future research relating to molecular characterization of insecticide resistance mechanisms in *A. gossypii* and other insect pests.

# **Appendix A:** Spotlight contribution

Marshall, K.L. (2012) Eye on aphid resistance pp. 23. In: *Spotlight on Cotton R&D, Winter 2012*. CRDC, Narrabri.

# EYE ON APHID RESISTANCE

PROTECTING THE INDUSTRY FROM A CONTINUING PROBLEM OF NEONICOTINOID RESISTANCE AMONG APHID POPULATIONS IS A PRIORITY FOR THIS RESEARCHER.

Dr Grant Herron and Kate Marshall are investigating aphids' resistance to neonicotinoids at Elizabeth Macarthur Agricultural Institute.

## PROFILE – ENTOMOLOGIST KATE MARSHALL

From a young age Kate was interested in the field of entomology and during university developed a particular focus for integrated pest management and its application in agriculture. After completing her university honours project investigating pyrethroid resistance in the cotton aphid at Elizabeth Macarthur Agricultural Institute Kate gained further experience working as a technical officer assisting with sustainable resistance management of mites, aphids and mirids. Kate has begun a PhD focused on the characterisation of neonicotinoid resistance in the cotton aphid.

### What's the PhD about?

"Neonicotinoid insecticides have offered a valuable option to control secondary pests, however the growing reliance on this class due to its high specificity and low toxicity to beneficial insect species has led to resistance outbreaks and control failures. The sustainable management of aphids in Australia is therefore at risk and through this PhD I hope to be able to contribute to restoring neonicotinoid efficacy. I aim to identify the causing mecha-

nism of resistance. This knowledge will allow us to investigate any cross resistance implications and provide the first step in development of a molecular based test for neonicotinoid resistance monitoring."

### What are the most interesting science challenges?

"Molecular genetic techniques used to investigate resistance are extremely valuable; they are rapid and can be used to detect several different mechanisms of insecticide resistance in one sample test. However translation of this knowledge to the field is often delayed, meaning growers can still be using insecticides which are no longer providing effective control. I think bridging the time lag so knowledge can be used promptly is one of the most interesting challenges."

### Your future with cotton?

"I am very eager to further my experiences within the industry and I hope during this PhD I will develop the skills and knowledge necessary to support sustainability of cotton production in Australia."

Two pot trials simulating field conditions and testing responses of two individual classes of insecticide against cotton aphid (*Aphis gossypii*) have recently been completed at the Elizabeth Macarthur Agricultural Institute (EMAI) near Camden by PhD student Kate Marshall.

Neonicotinoids are one of the most widely use groups of insecticides and resistance in aphids across the NSW cotton industry increased from five per cent in 2007-08 to 96 per cent in 2010-11. Kate's research aims to help restore neonicotinoid efficacy.

In one pot trial, Kate tested the residual efficacy of Cruiser 350FS and Cruiser Extreme against susceptible and resistant cotton aphid.

"Cruiser Extreme was more effective than Cruiser 350FS against a resistant population but resistant aphids are not always controlled," Kate said.

"Knowing that resistance exists is important, but we need to know the relationship between resistance and product efficacy and whether current insecticide treatments knock out resistant aphids or cease to be effective.

"Of particular concern and perhaps the most important finding of this first study was the low level of control each insecticide seed treatment provided against resistant aphids.

"From week two, resistant aphids were found on both seed treatments, and each week thereafter."

Leader of the research at EMAI, Dr Grant Herron, says restoring neonicotinoid efficacy against aphids should be seen as an industry priority, as part of an integrated program to better manage sucking insect pests in Australian cotton.

Grant and his team established that high level neonicotinoid resistance in Australian cotton posed an emerging threat to the industry.

"Resistance across the NSW industry increased from five per cent in 2007-08 to 96 per cent in 2010-11," Grant said.

World-wide neonicotinoids are one of the most widely used groups of insecticides, due to their high degree of effectiveness and low toxicity to non-target organisms. This group includes Cruiser as the mainstay cotton seed treatment and the cost effective foliar spray is Shield Systemic Insecticide.

Kate is about to start a second pot trial to investigate the effectiveness of Thimet as a pre-germination treatment against a Pirimor and Rogor resistant cotton aphid.

Rogor and Thimet are similar organophosphate insecticides belonging to a subgroup known as the phosphorodithiolates. As their chemical structure is alike cross resistance between the two is possible and Thimet may not control Pirimor resistant strains. However, if the trial shows this not to be the case then Thimet may potentially be used as an alternative to neonicotinoid seed treatments.

Kate has always had an interest in the field of entomology and during her final year at University of Sydney, completing a Bachelor of Animal and Veterinary Bioscience opted to do her honours project at EMAI. This project investigated pyrethroid resistance in the cotton aphid (*Aphis gossypii*) and was completed in the Insecticide Resistance Group, run by Grant Herron.

"I loved the work I did during that year and so was very happy to take on a role as Technical Officer within the same group once I had graduated. I was than very fortunate when a PhD opened up to study neonicotinoid resistance, also in the cotton aphid."

Kate Marshall
Elizabeth Macarthur Agricultural Institute 02 4640 6389
kate.marshall@dpi.nsw.gov.au

email us

50 of 308

# **Appendix B:** Conference Proceeding

**Marshall, K.L.,** Wilson, L.J. and Herron, G.A. 2012. *Do the neonicotinoid seed treatments Cruiser® and Cruiser Extreme® control resistant aphid*? p. 102. In: 16th Australian Cotton Conference, Cotton Research and Development Corporation, Broadbeach, Australia, 5th-7th August 2012.

# Do the neonicotinoid seed treatments Cruiser® and Cruiser Extreme® control resistant aphids?

Kate L. Marshall*[1,2], Lewis J. Wilson[3] and Grant A. Herron[2]
1. The ithree Institute, University of Technology, PO BOX 123, Broadway 2007 Australia
2. New South Wales Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Woodbridge Road, Menangle 2568 Australia
3. CSIRO, ACRI, PO Box 59 Narrabri 2390 Australia
*kate.marshall@dpi.nsw.gov.au

## Summary

Both neonicotinoid seed treatments provided ineffective control against resistant cotton aphid. Further use of these treatments against resistant populations may exacerbate resistance.

## Introduction

Residual efficacy of neonicotinoid pre-germination seed treatments Cruiser® and Cruiser Extreme® were evaluated against susceptible and resistant cotton aphid.

## Trial design

Initially, sixty seeds of Untreated (UN), Cruiser® (CR) and Cruiser Extreme® (CE) were planted into individual pots and monitored in a growth room. At 7 days post planting when dicotyledons had emerged, one pot per treatment was transferred into an aphid proof cage with pot position randomly assigned (Fig. 1). Each plant was challenged with two adult *A. gossypii* aptera. Three replicates were performed against both resistant and susceptible strains and laid out in a complete randomised block design. Aphid numbers were recorded after one week and the pots discarded. This was repeated for seven weeks to highlight residual efficacy between treatments as plants matured.



**Figure 1.** Untreated and treated cotton plants in an aphid proof cage held in an insectary.

## Statistical analysis

A curvature model via a cubic spline function was fitted to the data as a generalised linear mixed model (GLMM) of treatments by age.

A residual maximum likelihood (REML) method was used to estimate all parameters after logarithmic link re-parameterization and the analysis was run using ASREML (Gilmour, 2011). Predicted values and their 95% confidence intervals were calculated.
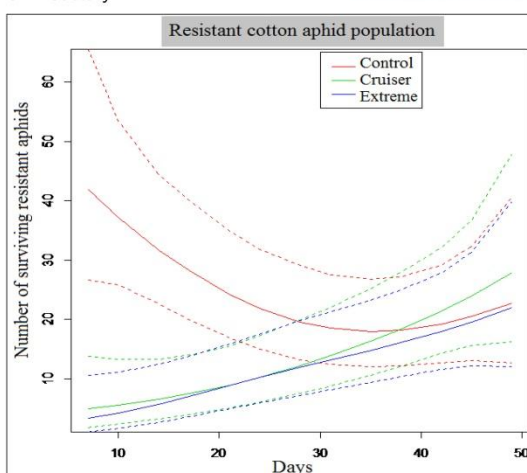


**Figure 2.** Predicted surviving thiamethoxam resistant aphids (solid) and 95% confidence intervals (dashed).

Effects of both treatments on the survivability of aphids were significant (P<0.012). After 21 days the thiamethoxam resistant strain on each thiamethoxam treatment equalled their survival rates to those of the untreated plants (Fig. 2).
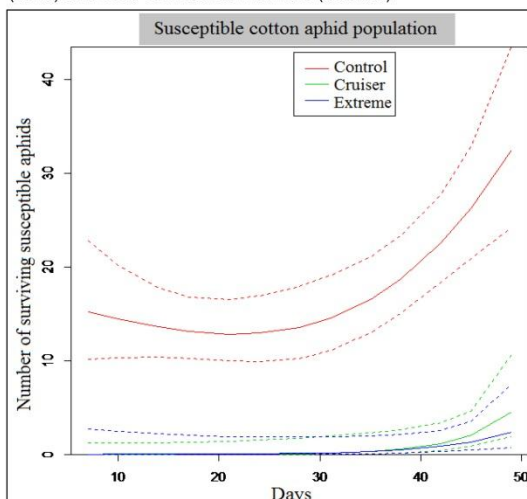


**Figure 3.** Predicted surviving thiamethoxam susceptible aphids (solid) and 95% confidence intervals (dashed).

Effects of both treatments on the survivability of aphids were highly significant (P<0.001). The susceptible strain could not survive on the treated plants (Fig. 3).

16th australian COTTON conference
*Growing Better all the Time*

Australian Government
Cotton Research and Development Corporation

UNIVERSITY OF TECHNOLOGY SYDNEY

NSW GOVERNMENT | Primary Industries

# Appendix C: Conference Proceeding

**Marshall, KL,** Herron, GA & Chen, Y. 2014. *Neonicotinoid Resistance in Cotton Aphid from Australia*. In: 17th Australian Cotton Conference. Cotton Research and Development Corporation, Broadbeach, Australia, 14th – 16th August 2014.

# NEONICOTINOID RESISTANCE IN COTTON APHID FROM AUSTRALIA

AUTHORS Kate.L.Marshall*[1] | Yizhou Chen[1] | Grant. A. Herron[1]
ORGANISATION [1] New South Wales Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Woodbridge Road, Menangle 2568, NSW, Australia

## Summary

**We have shown that target site insensitivity in Australian *Aphis gossypii* via the R81T mutation is not the causal mechanism of neonicotinoid resistance despite overseas studies implicating such. Instead we propose metabolic detoxification as the likely causal mechanism for resistance in Australian *A. gossypii* and we are currently trying to validate that hypothesis via transcriptome analysis. It is not clear why the *R81T* mutation is absent but the difference may relate to limited imidacloprid use in Australian cotton and the progressive nature of the cotton industry itself.**

## Introduction

In Australia, cotton aphid, *Aphis gossypii* Glover is a destructive pest of cotton and cucurbits and is frequently targeted with chemical sprays for its control. Resistance to the organophosphates, carbamates, pyrethroids and more recently the neonicotinoids has been detected in *A. gossypii* in Australian cotton (Herron et al. 2001; Herron & Wilson 2011; Marshall et al. 2012). Insecticide resistance in *A. gossypii* has two primary routes; target site insensitivity and metabolic detoxification. Target site insensitivity is caused from modification/s in the gene of the target site which prevents binding of the insecticide and renders the chemical ineffective. In metabolic detoxification, enzymes which metabolize the insecticide may be over produced (gene amplification) or up-regulated (gene expression), in each case allowing the insect to metabolize the toxin to a level suitable for survival. Alternatively, enzymes may have a greater affinity for binding to the insecticide, allowing it to be slowly sequestered over time. Detoxification and/or sequestration are not mutually exclusive and often occur together in insects whereby metabolic detoxification is the primary mechanism of resistance.

For the three chemical classes; organophosphates, carbmates and pyrethroids, the mechanisms by which *A. gossypii* confers resistance have been elucidated as either target site insensitivity and/or metabolic detoxification. Against the more recent chemical class, the neonicotinoids, the causal mechanism of resistance has not yet been revealed.

Overseas, imidacloprid (a neonicotinoid) resistance in *A. gossypii* has been linked to target site insensitivity via a modification in the predicted binding site of neonicotinoid insecticides in the nicotinic acetylcholine receptor (nAChR) (Koo et al. 2014; Shi et al. 2012). This mutation, termed *R81T* results in an arginine (AGA) to threonine (ACA) base substitution at amino acid position 81 in the loop D region of the $\beta$1 subunit of the nAChR.

To ascertain whether target site insensitivity was responsible for the confirmed resistance in Australian *A. gossypii*, we amplified the mutation site within the loop D region of the $\beta$1 subunit through PCR and compared the DNA sequence of a thiamethoxam resistant *A. gossypii* strain (Carrington) from Australia against a reference imidacloprid resistant *A. gossypii* strain (GenBank Accession number: JQ627836) from China (Shi et al. 2012). Additionally, the cDNA sequences of a neonicotinoid susceptible strain (F 96) and an additional thiamethoxam resistant strain (Glentown) from Australia were included for sequence analysis.

## Methods

### 1. Bioassay

Insecticide susceptible (strain F 96) and thiamethoxam resistant (strains Carrington and Glentown, both collected off commercial cotton) were bioassayed against the neonicotinoid insecticide thiamethoxam (Actara®). Briefly, aphids in batches of thirty were placed onto an excised cotton leaf discs fixed in agar in

a petri dish and sprayed using a Potter spray tower with serial dilutions of the insecticide prepared with distilled water (Herron et al. 2001). Each strain was tested against five serial concentrations, selected to achieve 0 < x < 100% mortality. After spraying, each petri dish was covered with cling wrap with tiny perforations to reduce condensation and placed in an incubator at 25°C for 24 hours. After this period aphids were assessed as dead or alive with the aid of a stereo microscope.

## 2. Data Analysis

Bioassay data was analysed using a stand-alone probit program developed by Barchia (2001), which ensures that variability between replicates is taken into account. Dose response probit regressions were corrected for control mortality (Abbott 1925) and the $LC_{50}$ and $LC_{99.9}$ plus their 95% fiducial-limits were calculated by applying the method of Finney (1971). Resistance factors were calculated by dividing the $LC_{50}$ of the field-collected population by the value of the susceptible strain.

## 3. PCR Amplification

DNA was extracted from a pooled sample of 200 aphids of strain Carrington and used as a template in a polymerase chain reaction (PCR) protocol using primers (Forward primer: CTGTCCAGAACATGACCGAA and Reverse primer: GTGGTAACCTGAGCACCTGT) designed to amplify the mutation site within the loop D region of the β1 subunit of the nAChR. The amplified DNA was purified and sequenced by the Australian Genomic Research Facility (AGRF). Using the sequencing software program CodonCode Aligner® the sequencing data of strain Carrington was aligned to the reference imidacloprid resistant *A. gossypii* strain (GenBank accession number: JQ627836) for comparison.

Additionally, cDNA sequences were produced for susceptible strain F 96 and thiamethoxam resistant strains Carrington, and Glentown for further analysis.

## Results

### Bioassay Results

| Strain | $LC_{50}$ (95% FL[a]) (g/L) | Slope± SE[b] | RF[c](95% CI[d]) |
|---|---|---|---|
| Susceptible | 0.00038(0.00031-0.00046) | 2.4±0.24 | - |
| Carrington | 0.03(0.027-0.039) | 2.2±0.19 | 85.00(65.29-110.66) |
| Glentown | 0.02(0.01-0.03) | 1.2±0.20 | 51.3(30.5-86.2) |

[a]fiducial limits; [b]standard error; [c]resistance factor; [d]confidence interval.

TABLE 1. Full log dose probit regression summary of neonicotinoid susceptible strain F 96 and thiamethoxam resistant strains Carrington and Glentown against thiamethoxam

### Sequencing Results

Sequence alignment between susceptible strain F 96, thiamethoxam resistant strains Carrington and Glentown and the reference imidacloprid resistant *A. gossypii* strain (Genbank accession number: JQ627836) confirmed that the region amplified were the loop D region of the β1 subunit. Comparative sequence analysis identified that all strains sequenced from Australia possessed a nucleotide G at base position 242 in the consensus region of DNA (AGA), whilst the reference imidacloprid resistant *A. gossypii* strain (Genbank accession number: JQ627836) possessed the nucleotide C (ACA), the later resulting in a corresponding codon change at position 81 from arginine to threonine (*R81T*) (Fig.1).



FIGURE 1. Comparative sequence analysis of Aphis gossypii strains susceptible F 96, Carrington (cDNA and gDNA), Glentown and imidacloprid resistant (Genbank accession number JQ627836). (Note: mutation site R81T boxed in red)

## Discussion

Through comparative sequence analysis, our results have illustrated that in Australian *A. gossypii* the causal mechanism of neonicotinoid resistance is not a target site insensitivity via the *R81T* mutation (Fig. 1). Studies overseas which have found the *R81T* mutation in *A. gossypii*, have described it firstly as the putative mechanism for imidaclorpid resistance, and secondly as a cross resistance mechanism to other neonicotinoid insecticides (Koo et al. 2014; Shi et al. 2012). Interestingly, our strains display resistance to thiamethoxam (Table 1) but recent bioassay data has shown they are susceptible to imidacloprid (unpubl.data). Although there is confirmed cross resistance between members of the neonicotinoid mode of action group 4A (Shi et al. 2011; Wang et al. 2007) the spectrum of resistance displayed may be dependent on exposure to each chemical. Infrequent use of imidacloprid in Australian cotton may have increased the susceptibility of *A. gossypii* to this chemical. Additionally, in Australia the majority of cotton seed planted is coated with thiamethoxam as a pre germination seed treatment for the control of all early season insect pests. If the *R81T* mutation develops in response to imidacloprid, *A. gossypii* in Australian cotton may not have had enough exposure to develop the *R81T* mutation. Alternatively, we consider in the absence of imidacloprid a metabolic resistance developed via detoxification to the secondary analogs of imidacloprid and in particular to thiamethoxam. Research to validate a metabolic detoxification theory as the primary mechanism of neonicotinoid resistance in Australian *A. gossypii* from cotton is underway.

## Conclusion

The mutation responsible for imidacloprid resistance (*R81T*) in *A. gossypii* strains overseas is not present in Australia. Thiamethoxam is used widely in Australian cotton as a pre germination seed treatment whilst imidacloprid use is limited. This could explain the reason why the causal mechanism of resistance to neonicotinoids in Australia, in particular to thiamethoxam may develop from a different origin.

## References

Abbott, WS. 1925 A method for computing the effectiveness of an insecticide. *Journal of Economic Entomology* 18, 265-267.

Finney, DJ. 1971. *Probit Analysis*, Cambridge University Press, Cambridge, UK.

Herron, GA, Powis, K & Rophail, J. 2001 Insecticide resistance in *Aphis gossypii* Glover (Hemiptera: Aphididae), a serious threat to Australian cotton. *Australian Journal of Entomology* 40, 85–91.

Herron, GA & Wilson, LJ. 2011 Neonicotinoid resistance in *Aphis gossypii* Glover (Aphididae: Hemiptera) from Australian cotton. *Australian Journal of Entomology* 50, 93-98.

Koo, H-N, An, J-J, Park, S-E, Kim, J-I & Kim, G-H. 2014 Regional susceptibilities to 12 insecticides of melon and cotton aphid, Aphis gossypii (Hemiptera: Aphididae) and a point mutation associated with imidacloprid resistance. *Crop Protection* 55, 91-97.

Marshall, KL, Moran, C, Chen, Y & Herron, GA. 2012 Detection of kdr pyrethroid resistance in the cotton aphid, Aphis gossypii (Hemiptera: Aphididae), using a PCR-RFLP assay. *Journal of Pesticide Science* 37, 169-172.

Shi, X-B, Shi, X-G, Wang, H-Y, Xia, X-M & Wang, K-Y. 2011 Cross-resistance of the imidacloprid-resistant population of *Aphis gossypii* Glover (Homoptera: Aphididae) to other neonicotinoid insecticides and changes in activities of related enzymes. *ACTA ENTOMOLOGICA SINICA* 54, 1027-1033.

Shi, X, Zhu, Y, Xia, X, Qiao, K, Wang, H & Wang, K. 2012 The mutation in nicotinic acetylcholine receptor β1 subunit may confer resistance to imidacloprid in *Aphis gossypii* (Glover). *Journal of Food, Agriculture and Environment* 10, 1227-1230.

Wang, KI, Guo, QL, Xia, XM, Wang, HY & Liu, TX. 2007 Resistance of *Aphis gossypii* (Homoptera: Aphididae) to selected insecticides on cotton from five cotton production regions in Shandong, China. *Journal of Pesticide Science* 32, 372-378.

**Correspondence should be addressed to:**

kate.marshall@dpi.nsw.gov.au

**Australian Government**
**Cotton Research and Development Corporation**

**NSW** GOVERNMENT | **Department of Primary Industries**

**UNIVERSITY OF TECHNOLOGY SYDNEY**

# **Appendix D:** Refereed Journal Publication

**Marshall, K.L.,** Damian Collins, Yizhou Chen, Grant A. Herron (2015). Efficacy of two thiamethoxam pre germination seed treatments and a phorate side-dressing against neonicotinoid- and pirimicarb-resistant cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae) <u>Austral Entomology</u> **(54)**:4 351-357.

# Efficacy of two thiamethoxam pre-germination seed treatments and a phorate side-dressing against neonicotinoid- and pirimicarb-resistant cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae)

**Kate L Marshall,[1]\* Damian Collins,[1] Lewis J Wilson[2] and Grant A Herron[1]**

[1]*New South Wales Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Woodbridge Road, Menangle, NSW 2568, Australia.*
[2]*The Commonwealth Scientific and Industrial Research Organisation (CSIRO) Agriculture Flagship, Locked Bag 59, Narrabri, NSW 2390, Australia.*

**Abstract**    In a glasshouse trial with potted cotton plants grown from thiamethoxam-treated seed, neither 2.76 g a.i./kg seed (Cruiser®) nor 5.52 g a.i./kg seed (Cruiser Extreme®) protected plants from neonicotinoid-resistant *Aphis gossypii* Glover, 1877. Against susceptible *A. gossypii* each treatment was highly effective, providing control of >90% for 42 days. Continued use of either thiamethoxam treatment against resistant *A. gossypii* will select for resistant phenotypes and probably restrict the useful life of the neonicotinoid insecticides against this pest. In a separate trial, side-dressing of cotton seed with phorate 200 g/kg (Thimet®) effectively provided plants with protection from susceptible *A. gossypii*. The insecticidal activity of phorate-treated plants against pirimicarb-resistant *A. gossypii* was not statistically different to untreated plants ($P > 0.05$). To maintain the effectiveness of pirimicarb in the Australian cotton integrated pest management strategy, the use of phorate as an alternative pre-germination treatment to thiamethoxam for aphid control must be managed. We recommend that the first foliar spray applied to cotton treated with phorate at planting should not be pirimicarb or any other insecticide affected by insensitive cholinesterase (ACE1) type resistance.

**Key words**    cotton aphid, Cruiser®, Cruiser Extreme®, resistance management, Thimet®.

## INTRODUCTION

The cotton aphid, *Aphis gossypii*, is a significant pest of cotton, *Gossypium hirsutum* L., and cucurbits both in Australia and worldwide (Blackman & Eastop 2000). In Australian cotton, *A. gossypii* can be found on seedling cotton (October) and typically builds to levels that require control during the mid-late growing season (January–March). *Aphis gossypii* feeding can reduce leaf photosynthesis (Heimoana 2012) and spread plant viruses such as cotton bunchy top virus (CBTV) (Reddall *et al.* 2004; Ellis *et al.* 2013) or cotton leaf roll virus (Corrêa *et al.* 2005) that dramatically reduce the yield potential of affected plants. The excretion of honeydew by aphids (Hequet *et al.* 2000) contaminates the lint of matured fruit (bolls). Damaged lint attracts a lower price and damages the reputation of the region from which it is sourced. Furthermore, it is not economical to clean the lint and contaminated lint binds to machinery during spinning, necessitating shutdown and cleaning.

Economically significant outbreaks of aphids are partially induced by applications of insecticides against other pests (Wilson *et al.* 1999). These pesticides reduce beneficial popu-lations without controlling aphids, which then rapidly increase. Since the advent and widespread uptake of *Bt* cotton, containing the *Cry* proteins to control the primary pests, *Helicoverpa* spp., the use of insecticides has declined dramatically (Wilson *et al.* 2013). However, some species not controlled by the *Cry* proteins have emerged as pests and require targeted control, especially green mirids (*Creontiades dilutus* Stål) (Wilson *et al.* 2013). Insecticides targeted against this pest are generally disruptive to beneficial species but do not control aphids – which consequently have persisted as an important pest. The capacity to control aphids, throughout the crop growing cycle, is consequently important for cotton production.

In Australia, neonicotinoid seed treatments containing thiamethoxam or side-dressings of granular insecticides, such as the organophosphate phorate at planting, are used to control a range of seedling pests such as thrips and wireworms (Elateridae), but also control *A. gossypii* and hence the risk of CBTV transmission. These treatments offer increased selectivity compared with neonicotinoid or organophosphate foliar sprays, which can be highly disruptive to beneficial insect populations (Mass 2013). Cruiser® (thiamethoxam at 2.76 g a.i./kg seed) and Cruiser Extreme® (thiamethoxam at 5.52 g a.i./kg seed) provide early season seedling protection (30–40 days) against *A. gossypii* and several other sucking insect pests

(Maienfisch *et al.* 2001). However, the effectiveness of these products against *A. gossypii* may be threatened because of resistance to neonicotinoid (Herron & Wilson 2011), carbamate and organophosphate insecticides (Herron *et al.* 2001).

Neonicotinoid resistance in *A. gossypii* (Herron & Wilson 2011) was first recorded in the 2007–2008 cotton season and attributed to long-term, widespread use of the neonicotinoid cotton seed treatments. If neonicotinoid-resistant *A. gossypii* are present at the start of the cotton season, the use of neonicotinoid seed treatments may be only partially effective and so could exacerbate resistance. Phorate side-dressing has been suggested as a possible alternative to the neonicotinoid seed treatments, but its suitability as a viable replacement has not been explored, nor has its effectiveness to control carbamate (pirimicarb)-resistant *A. gossypii* been revealed. Similarly, the efficacy of the standard and higher rate thiamethoxam seed treatments against neonicotinoid-resistant aphids *in planta* has not been established. Here we report the results of a glasshouse trial that investigated the effectiveness of these treatments against resistant *A. gossypii*.

## MATERIALS AND METHODS

### Chemicals tested

Cotton seed (variety Sicot 71) treated with thiamethoxam at either 2.76 g a.i./kg seed (Cruiser® Insecticide Seed Treatment) or 5.52 g a.i./kg seed (Cruiser Extreme® Insecticide Seed Treatment) was obtained from Cotton Seed Distributors, Wee Waa, NSW. Phorate 200 g/kg insecticide (Thimet® 200 G Systemic Granular Insecticide) was obtained from Barmac Industries Pty Ltd.

### Aphids

A reference susceptible strain (F 96) was maintained on insecticide-free cotton in an insect-proof cage at $25 \pm 4°C$ under natural light at the Elizabeth Macarthur Agricultural Institute, Camden. Resistant *A. gossypii* strains Glen twn S and Mon P were collected from commercial cotton. Strain Glen twn S was neonicotinoid resistant while strain Mon P was pirimicarb/omethoate resistant (Herron *et al.* 2013). Strain Glen twn S was routinely pressured monthly by exposure to foliar sprays at double the discriminating dose of thiamethoxam (i.e. 0.04 g/L) (Herron & Wilson 2011). Strain Mon P was similarly pressured monthly using a dose 10-fold the discriminating dose of pirimicarb (i.e. 0.1 g/L) (Herron *et al.* 2000). Both strains were pressured a week prior to the initial testing to ensure resistance remained at a high level throughout the trial interval.

### Thiamethoxam trial

Cotton seed treatments were: untreated control (cotton seed variety Sicot 71); 2.76 g a.i./kg seed (Cruiser®) and; 5.52 g

© 2014 State of New South Wales
*Austral Entomology* © 2014 Australian Entomological Society

a.i./kg seed (Cruiser Extreme®). On 7 November 2011, 60 seeds of each treatment group were individually planted into plastic pots (11.5 cm diameter) filled with NativeMix™ premium potting mix and held in a room maintained at $28 \pm 2°C$. Each pot contained only one treatment with all pots planted concurrently (180 treated pots total). At planting and on another three occasions over the following 6 days, 150 mL of water was poured over the soil surface of each pot.

A week after planting the dicotyledons had emerged. At this time (day 0), six pots from each treatment group were transferred onto individual saucers in insect-proof cages maintained at $25 \pm 4°C$ but subject to natural light. Pots were watered by filling their saucers with 200 mL on initial placement into the cages and as necessary during the trial period.

A randomised complete block design was used. Strains were randomised to cages ('whole-plots') and treatments were randomised to three pots within cages ('sub-plots'). Two apterous adult aphids (susceptible or resistant) were placed onto each of the plants within each cage such that three cages contained only susceptible aphids and three contained only resistant aphids. On day 7, all leaves were removed from each plant and final aphid numbers were counted with the aid of a stereo microscope. This process was repeated with new plants at weekly interval until day 49 by which time susceptible aphids could survive on both thiamethoxam treatments.

### Phorate trial

Cotton seed treatments were: untreated control (cotton seed variety Sicot 71) and phorate 200 g/kg (Thimet®) at a dose of 34.4 mg/pot. In a separate trial beginning on 7 July 2013, 60 seeds of each treatment group were planted individually and maintained as above. The dose (34.4 mg/pot) of phorate applied was equivalent to that indicated on the product label for short period protection (3 kg/ha) and assumed a row length equal to the diameter of the pot. Trial design was as above.

### Statistical analysis

Analyses were conducted for each trial using generalised linear mixed models in ASReml (Gilmour *et al.* 2009). The response (number of aphids) was analysed as quasi-Poisson (overdispersed Poisson with log link) for each trial using a mixed model comprising fixed strain, treatment (within strain) and linear day effects and all associated interactions, and random factor day effects and interactions with treatment, strain, strain by treatment, as well as cage, cage by day and position. Wald-type F-tests for fixed terms in the model are reported, as well as contrasts to test for treatment efficacy and interactions between treatment efficacy and (linear) day, for each strain in turn.

The Henderson–Tilton formula (Henderson & Tilton 1955) for treatment control is $100[1 - Ta*Cb/Tb*Ca] = 100[1 - Ta/Ca]$, where Ta and Ca are the number of aphids surviving at the end of the week, and Tb and Cb are the starting number of

aphids used for each pot (2) which cancel out from top and bottom. The ratio Ta/Ca could be estimated, along with an approximate 95% confidence interval, by back-transforming the predicted difference between each treatment and control at each time-point (since a log link was used, and so absolute differences on the log scale correspond to multiplicative effects on the back-transformed scale).

## RESULTS

### Thiamethoxam trial

There were significant ($P < 0.05$) treatment within strain effects and significant interactions of treatment within strain with day (Table 1). The non-zero variance components indicated differences in individual day effects across treatments, and both cage and cage by day effects, as well as residual overdispersion (relative to a Poisson distribution), indicated by a residual variance (3.02) greater than 1 (Table 2). For strain F 96, the interactions of treatment with lin(day) were either non-significant ($P > 0.05$) for the higher rate or just significant ($P < 0.05$) for the lower rate. However, there were statistically highly significant ($P < 0.0001$) treatment within strain effects for both rates of thiamethoxam compared with untreated cotton seed, as expected (Table 1). Both Cruiser® and Cruiser Extreme® provided 100% protection of strain F 96 for 14 days (Table 3). Control of strain F 96 remained very

*Table 1*  Wald-F test statistics for fixed effects of thiamethoxam analysis

|  | DF (num, den) | F-statistic | *P*-value |
|---|---|---|---|
| strain | 1,5.3 | 9.164 | 0.0273 |
| lin(day) | 1,16.4 | 9.669 | 0.0066 |
| strain/trt | 4,29.3 | 13.810 | 0.0000 |
| sus:cruiser vs control | 1,61.7 | 31.250 | 0.0000 |
| sus:extreme vs control | 1,73.7 | 34.470 | 0.0000 |
| res:cruiser vs control | 1,11.5 | 4.740 | 0.0512 |
| res:extreme vs control | 1,12.3 | 8.228 | 0.0139 |
| strain × lin(day) | 1,36.7 | 9.840 | 0.0034 |
| strain/trt × lin(day) | 4,32.2 | 3.778 | 0.0125 |
| sus:{cruiser vs control} × lin(day) | 1,80.1 | 4.110 | 0.0460 |
| sus:{extreme vs control} × lin(day) | 1,79.2 | 3.244 | 0.0755 |
| res:{cruiser vs control} × lin(day) | 1,11.4 | 7.003 | 0.0221 |
| res:{extreme vs control} × lin(day) | 1,14.0 | 9.882 | 0.0072 |

lin, linear; res, resistant strain; sus, susceptible strain; trt, treatment.

*Table 2*  Non-zero variance component and standard error (SE) for random terms of thiamethoxam analysis

|  | Component | SE | Z-ratio |
|---|---|---|---|
| cage | 0.0097 | 0.0309 | 0.3143 |
| cage × fac(day) | 0.1266 | 0.0610 | 2.0738 |
| trt × fac(day) | 0.2708 | 0.1430 | 1.8943 |
| Residual | 3.0163 | 0.5196 | 5.8054 |

fac, factor; trt, treatment.

high (>90%) until day 49 where Cruiser® showed a decrease to 87%. Residual insecticidal activity of Cruiser Extreme® provided greater control at 49 days of 93.3%. Interactions of treatment with day for strain Glen twn S were both significant ($P < 0.05$) for each rate of thiamethoxam when compared with untreated cotton seed, indicating the reduction in treatment efficacy over time. Cruiser Extreme® provided higher initial and residual protection compared with Cruiser® (Table 3 and Fig. 1) but neither treatment adequately controlled resistant *A. gossypii*. From day 28, the effectiveness of Cruiser® against strain Glen twn S was similar to untreated cotton (Table 3).

### Phorate trial

There were statistically highly significant ($P < 0.001$) treatment within strain effects for strain F 96 (and interactions with day), but not for strain Mon P ($P > 0.05$) (Table 4). The non-zero variance components indicated day effects (fac(day)), replicate and replicate by day effects, cage by day effects, treatment, strain by day effects and position effects as well as residual overdispersion (relative to a Poisson distribution), indicated by a residual variance (2.618) greater than 1 (Table 5). Phorate provided robust protection of strain F 96 for the duration of the trial, with control only decreasing below 90% at day 35 (Table 6). From day 42, phorate provided residual control of 80.9%, decreasing to 67.6% control at day 49. Strain Mon P survived well on phorate-treated cotton from day 0 (Fig. 2). Population size of strain Mon P when challenged with phorate showed no statistical significance compared with untreated cotton (Table 4).

## DISCUSSION

The efficacy of two neonicotinoid seed treatments against neonicotinoid susceptible and resistant *A. gossypii* and an organophosphate at-planting treatment against carbamate susceptible and resistant *A. gossypii* were evaluated under simulated field conditions in a glasshouse trial. Raw data produced were transformed via Henderson–Tilton analysis to account for variability seen in *A. gossypii* numbers on untreated controls. We believe that due to the low starting number of two aphids each week, the variability seen in aphid populations from plant to plant was typical. Predicted values were produced for each time-point of the trial which offer a more realistic estimate of the control provided by each treatment.

We have clearly shown that formulated thiamethoxam at either rate (2.76 g a.i./kg seed and 5.52 g a.i./kg seed) is highly effective for protection against neonicotinoid susceptible *A. gossypii* and continues to be a viable option for aphid control. These results are in conformity with previous studies investigating the efficacy of thiamethoxam as a seed treatment against susceptible *A. gossypii*. Maienfisch *et al.* (2001) found that against sucking insect pests of cotton, rates between 105 and 350 g a.i./100 kg seed gave excellent control for 21–45 days. Prasanna *et al.* (2004) also found that thiamethoxam 70WS at a rate of 2.85 g a.i./kg seed effective until 40 days
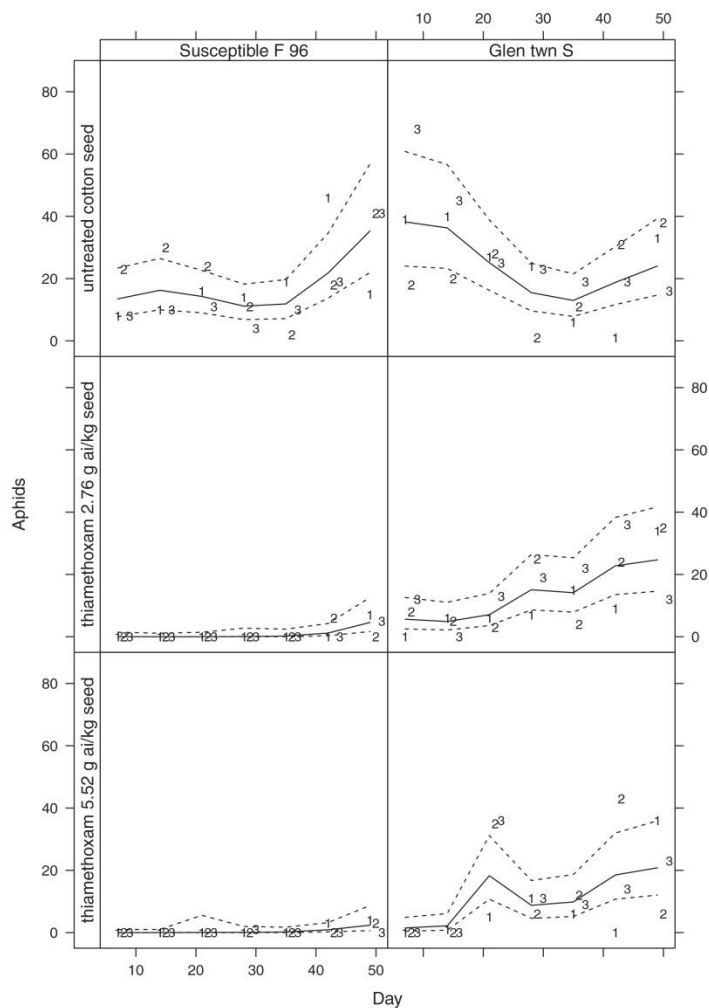
*Table 3*  Estimated treatment efficacies (Est) and approximate 95% confidence intervals of two varying rates of formulated thiamethoxam (g a.i./kg seed) against neonicotinoid susceptible and neonicotinoid-resistant *Aphis gossypii*

| Susceptible strain F 96 | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | Untreated | | 2.76 | | 5.52 | |
| | | Est | 95% CI | Est | 95% CI | Est | 95% CI |
| Day 7 | Aphids | 13.5 | (7.8, 23.4) | 0.0 | (0.0, 1.4) | 0.0 | (0.0, 1.0) |
| | % Control | – | – | 100.0 | (89.3, 100.0) | 100.0 | (92.5, 100.0) |
| Day 14 | Aphids | 16.3 | (10.0, 26.5) | 0.0 | (0.0, 1.1) | 0.0 | (0.0, 1.0) |
| | % Control | – | – | 100.0 | (93.1, 100.0) | 100.0 | (93.8, 100.0) |
| Day 21 | Aphids | 14.3 | (9.0, 22.6) | 0.0 | (0.0, 1.4) | 0.1 | (0.0, 5.6) |
| | % Control | – | – | 99.9 | (89.3, 100.0) | 99.5 | (60.9, 100.0) |
| Day 28 | Aphids | 11.2 | (6.8, 18.2) | 0.1 | (0.0, 2.8) | 0.1 | (0.0, 2.0) |
| | % Control | – | – | 99.4 | (75.0, 100.0) | 99.3 | (82.1, 100.0) |
| Day 35 | Aphids | 11.9 | (7.2, 19.7) | 0.2 | (0.0, 2.4) | 0.2 | (0.0, 1.7) |
| | % Control | – | – | 98.3 | (79.0, 99.9) | 98.3 | (85.1, 99.8) |
| Day 42 | Aphids | 21.7 | (13.7, 34.5) | 1.2 | (0.3, 4.2) | 0.9 | (0.3, 3.2) |
| | % Control | – | – | 94.6 | (80.5, 98.5) | 95.8 | (85.2, 98.8) |
| Day 49 | Aphids | 35.3 | (21.9, 56.8) | 4.6 | (1.7, 12.6) | 2.4 | (0.7, 8.7) |
| | % Control | – | – | 87.0 | (65.2, 95.2) | 93.3 | (75.7, 98.1) |
| Resistant strain Glen twn S | | | | | | | |
| | | Est | 95% CI | Est | 95% CI | Est | 95% CI |
| Day 7 | Aphids | 38.2 | (24.0, 60.8) | 5.6 | (2.5, 12.6) | 1.5 | (0.5, 4.9) |
| | % Control | – | – | 85.3 | (67.6, 93.3) | 96.1 | (87.1, 98.8) |
| Day 14 | Aphids | 36.3 | (23.3, 56.6) | 4.9 | (2.2, 11.1) | 2.2 | (0.8, 6.1) |
| | % Control | – | – | 86.5 | (69.3, 94.1) | 94.0 | (82.9, 97.9) |
| Day 21 | Aphids | 25.1 | (16.2, 38.9) | 7.1 | (3.6, 13.9) | 18.3 | (10.7, 31.2) |
| | % Control | – | – | 71.9 | (43.8, 85.9) | 27.2 | (−23.8, 57.2) |
| Day 28 | Aphids | 15.5 | (9.6, 25.0) | 15.1 | (8.6, 26.4) | 8.8 | (4.6, 16.7) |
| | % Control | – | – | 2.6 | (−79.8, 47.2) | 43.4 | (−14.0, 71.9) |
| Day 35 | Aphids | 13.0 | (7.8, 21.5) | 14.1 | (7.9, 25.3) | 9.8 | (5.2, 18.7) |
| | % Control | – | – | −8.9 | (−110.3, 43.6) | 24.3 | (−54.6, 62.9) |
| Day 42 | Aphids | 18.8 | (11.6, 30.3) | 22.8 | (13.5, 38.3) | 18.5 | (10.7, 32.0) |
| | % Control | – | – | −21.3 | (−109.1, 29.7) | 1.4 | (−74.5, 44.3) |
| Day 49 | Aphids | 24.0 | (14.7, 39.3) | 24.7 | (14.6, 41.7) | 20.8 | (12.1, 35.9) |
| | % Control | – | – | −2.8 | (−72.0, 38.6) | 13.4 | (−48.3, 49.4) |

post seedling emergence, while the higher rate of 4.28 g a.i./kg seed still provided superior control of *A. gossypii* when compared with untreated plants at 60 days, although not statistically significant. Zidan (2012) also found that thiamethoxam 70WS at the recommended rate of 4.9 g a.i./kg seed provided effective control of *A. gossypii*, although when compared with our results provided significantly reduced residual protection.

In contrast to neonicotinoid susceptible *A. gossypii*, our results have revealed that neither rate of thiamethoxam gives adequate control against neonicotinoid-resistant *A. gossypii*. It is likely that ongoing widespread reliance on neonicotinoid seed treatments, at either rate, will continue to select for resistant genotypes. Cross-resistance between members of the neonicotinoid group 4A mode of action (MoA) in *A. gossypii* has been reported elsewhere (Wang *et al*. 2007; Shi *et al*. 2011) and suggests that control of resistant populations is likely to be lost if neonicotinoid use is not managed better. The Insecticide Resistance Management Strategy (IRMS) for control of sucking insect pests of cotton recommends chemical rotation as the primary strategy for control of resistant *A. gossypii* (Mass 2013). Other well-defined

strategies in the IRMS such as use of refugia for control of *Helicoverpa* spp. are limited in their practicality for *A. gossypii* due to a short life cycle and there being no sexual phase of reproduction occurring in Australia (Smith *et al*. 2006). If chemical rotation is maintained over successive generations, then in the absence of selection the resistant population should return to susceptibility. It should be mentioned that this strategy relies on their being an associated fitness cost to the observed resistance. Fortunately, reversion to susceptibility in the absence of insecticide pressure has been noted to occur in laboratory strains of neonicotinoid-resistant *A. gossypii* (KL Marshall unpubl. data 2013). This would suggest that at least in some populations of *A. gossypii*, genes conferring neonicotinoid resistance do not appear to be fixed. Neonicotinoid seed dressings are primarily targeted against other pests where they continue to provide cost-effective control (Mass 2013), so restriction in their use without a viable alternative is impractical. Phorate is registered for the control of *A. gossypii* at planting and has previously been shown to control neonicotinoid-resistant *A. gossypii* as it possesses a distinct MoA to neonicotinoids (Herron *et al*. 2013). However, established cross-resistance

**Fig. 1.** Fitted trend for the thiamethoxam analysis, for each strain by treatment combination (thiamethoxam at 5.52 g a.i./kg seed, Cruiser Extreme® Insecticide Seed Treatment; thiamethoxam at 2.76 g a.i./kg seed, Cruiser® Insecticide Seed Treatment; untreated cotton seed, variety Sicot 71). The solid line represents the fitted trend, with dotted lines representing the 95% confidence interval. The raw data for each replicate is numbered 1 to 3 in each panel (with replicates 1 and 3 shifted slightly left and right, respectively, to avoid overlap).

**Table 4** Wald-F test statistics of fixed effects for phorate analysis

| | DF (num, den) | F-statistic | P-value |
|---|---|---|---|
| strain | 1,5.0 | 3.4440 | 0.1223 |
| lin(day) | 1,4.8 | 0.5352 | 0.4987 |
| strain/trt | 2,5.9 | 10.4000 | 0.0115 |
| sus:{phorate vs control} | 1,23.0 | 18.8700 | 0.0002 |
| res:{phorate vs control} | 1,3.6 | 0.1018 | 0.7675 |
| strain × lin(day) | 1,4.8 | 0.0548 | 0.8245 |
| strain/trt × lin(day) | 2,8 | 3.7490 | 0.0707 |
| sus:{phorate vs control} × lin(day) | 1,40.5 | 7.8890 | 0.0076 |
| res:{phorate vs control} × lin(day) | 1,3.7 | 1.0180 | 0.3746 |

lin, linear; res, resistant strain; sus, susceptible strain; trt, treatment.

**Table 5** Non-zero variance component and standard error (SE) for random terms of phorate analysis

| | Component | SE | z.ratio |
|---|---|---|---|
| fac(day) | 0.3424 | 0.4020 | 0.8475 |
| rep | 0.1070 | 0.1239 | 0.8388 |
| rep × fac(day) | 0.0122 | 0.0553 | 0.2181 |
| cage × fac(day) | 0.0823 | 0.0764 | 1.1067 |
| trt × fac(day) | 0.0806 | 0.0931 | 0.8854 |
| strain × fac(day) | 0.3051 | 0.2512 | 1.213 |
| position | 0.0043 | 0.0227 | 0.1907 |
| Residual | 2.6847 | 0.6312 | 4.0102 |

fac, factor; rep, replicate; trt, treatment.

*Table 6* Estimated treatment efficacies (Est) and approximate 95% confidence intervals of phorate as a side-dressing against pirimicarb susceptible and pirimicarb-resistant *Aphis gossypii*

Susceptible strain F 96

|  |  | Untreated |  | 3 kg/ha |  |
|---|---|---|---|---|---|
|  |  | Est | 95% CI | Est | 95% CI |
| Day 7 | Aphids | 11.6 | (5.6, 23.9) | 0.0 | (0.0, 0.7) |
|  | % Control | – | – | 99.7 | (94.2, 100.0) |
| Day 14 | Aphids | 17.0 | (8.7, 33.0) | 0.2 | (0.0, 1.8) |
|  | % Control | – | – | 99.0 | (89.8, 99.9) |
| Day 21 | Aphids | 12.8 | (6.3, 25.7) | 0.3 | (0.0, 2.0) |
|  | % Control | – | – | 97.6 | (85.7, 99.6) |
| Day 28 | Aphids | 5.3 | (2.3, 12.5) | 0.4 | (0.1, 1.9) |
|  | % Control | – | – | 92.2 | (68.6, 98.0) |
| Day 35 | Aphids | 4.2 | (1.7, 10.4) | 0.4 | (0.1, 1.7) |
|  | % Control | – | – | 89.3 | (67.8, 96.5) |
| Day 42 | Aphids | 11.5 | (5.7, 23.3) | 2.2 | (0.9, 5.5) |
|  | % Control | – | – | 80.9 | (60.9, 90.7) |
| Day 49 | Aphids | 27.0 | (14.6, 50.0) | 8.8 | (4.0, 19.1) |
|  | % Control | – | – | 67.6 | (36.5, 83.4) |

Resistant strain Mon P

|  |  | Est | 95% CI | Est | 95% CI |
|---|---|---|---|---|---|
| Day 7 | Aphids | 7.1 | (3.2, 15.8) | 3.8 | (1.5, 9.6) |
|  | % Control | – | – | 46.0 | (−26.0, 76.9) |
| Day 14 | Aphids | 30.9 | (17.0, 56.4) | 26.6 | (14.4, 49.1) |
|  | % Control | – | – | 14.0 | (−33.1, 44.5) |
| Day 21 | Aphids | 26.8 | (14.6, 49.2) | 27.1 | (14.7, 50.0) |
|  | % Control | – | – | −1.3 | (−59.1, 35.5) |
| Day 28 | Aphids | 10.7 | (5.3, 21.4) | 18.9 | (9.9, 35.9) |
|  | % Control | – | – | −76.8 | (−208.6, −1.3) |
| Day 35 | Aphids | 4.3 | (1.9, 10.1) | 5.5 | (2.4, 12.5) |
|  | % Control | – | – | −26.3 | (−160.1, 38.7) |
| Day 42 | Aphids | 45.0 | (25.3, 80.1) | 53.3 | (30.2, 94.1) |
|  | % Control | – | – | −18.4 | (−68.8, 17.0) |
| Day 49 | Aphids | 22.8 | (12.1, 43.0) | 24.0 | (12.8, 45.1) |
|  | % Control | – | – | −5.3 | (−71.9, 35.5) |

between the organophosphate and carbamate chemical classes via insensitive cholinesterase type resistance (*ACE1*) will select for high level resistance in *A. gossypii* pest populations if used sequentially and may lead to control failures as previously seen (Herron *et al.* 2001; Andrews *et al.* 2004; Benting & Nauen 2004). The IRMS lists the carbamate (pirimicarb) as a favourable first foliar spray for use against *A. gossypii* due to its softness on beneficial insect species (Mass 2013). Herron *et al.* (2013) suggested that pirimicarb-resistant *A. gossypii* would not be controlled by phorate. The results of our glasshouse trial confirm those laboratory findings. If phorate is to successfully substitute for a neonicotinoid seed dressing, the interaction with pirimicarb must be carefully considered. If phorate is used to control neonicotinoid-resistant *A. gossypii*, then pirimicarb, or any other chemical associated with *ACE1*-type resistance, should not immediately follow as the first foliar spray.

## ACKNOWLEDGEMENT

## REFERENCES

Andrews MC, Callaghan A, Field LM, Williamson MS & Moores GD. 2004. Identification of mutations conferring insecticide-insensitive

*Fig. 2.* Fitted trends for the phorate analysis, for each strain by treatment combination (phorate equivalent to 3 kg/ha, Thimet® 200 G Systemic Granular Insecticide; untreated cotton seed, variety Sicot 71). The solid line represents the fitted trend, with dotted lines representing the 95% confidence interval. The raw data for each replicate is numbered 1 to 3 in each panel (with replicates 1 and 3 shifted slightly left and right, respectively, to avoid overlap).

AChE in the cotton-melon aphid, *Aphis gossypii* Glover. *Insect Molecular Biology* **13**, 555–561.

Benting J & Nauen R. 2004. Biochemical evidence that an S431F mutation in acetylcholinesterase-1 of *Aphis gossypii* mediates resistance to pirimicarb and omethoate. *Pest Management Science* **60**, 1051–1055.

Blackman RL & Eastop VF. 2000. *Aphids on the World's Crops*, 2nd edn., John Wiley and Sons, Chichester, UK.

Corrêa RL, Silva TF, Simões-Araújo JL, Barroso PAV, Vidal MS & Vaslin MFS. 2005. Molecular characterization of a virus from the family Luteoviridae associated with cotton blue disease. *Archives of Virology* **150**, 1357–1367.

Ellis MH, Silva TF, Stiller WN *et al*. 2013. Identification of a new Polerovirus (family Luteoviridae) associated with cotton bunchy top disease in Australia. *Australasian Plant Pathology* **42**, 261–269.

Gilmour AR, Gogel BJ, Cullis BR & Thompson R. 2009. *ASReml User Guide Release 3.0*. VSN International Ltd, Hemel Hempstead, HP1 1ES, UK.

Heimoana SC. 2012. The effects of aphids (*Aphis gossypii*) Glover on photosynthesis in cotton (*Gossypium hirsutum*), The University of Queensland.

Henderson CF & Tilton EW. 1955. Tests with acaricides against the brown wheat mite. *Journal of Economic Entomology* **48**, 157–161.

Hequet E, Ethridge D, Cole B & Wyatt B. 2000. How cotton stickiness measurements relate to spinning efficiency. In: Proc. Engineered Fiber Selection System Conference (EFS).

Herron G, Powis K & Rophail J. 2000. Baseline studies and preliminary resistance survey of Australian populations of cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae). *Australian Journal of Entomology* **39**, 33–38.

Herron GA & Wilson LJ. 2011. Neonicotinoid resistance in *Aphis gossypii* Glover (Aphididae: Hemiptera) from Australian cotton. *Australian Journal of Entomology* **50**, 93–98.

Herron GA, Powis K & Rophail J. 2001. Insecticide resistance in *Aphis gossypii* Glover (Hemiptera: Aphididae), a serious threat to Australian cotton. *Australian Journal of Entomology* **40**, 85–91.

Herron GA, Langfield BJ, Bogema DR & Chen Y. 2013. Baseline susceptibility and cross-resistance in *Aphis gossypii* Glover (Aphididae: Hemiptera) to phorate and sulfoxaflor. *Australian Journal of Entomology* **53**, 32–35.

Maienfisch P, Angst M, Brandl F *et al*. 2001. Chemistry and biology of thiamethoxam: a second generation neonicotinoid. *Pest Management Science* **57**, 906–913.

Mass S. 2013. *Cotton Pest Management Guide 2013–14*. Greenmount Press, Toowoomba, Australia.

Prasanna AR, Bheemanna M & Patil BV. 2004. Evaluation of Thiamethoxam 70WS as seed treatment against leaf miner and early sucking pests on hybrid cotton. *Karnataka Journal of Agricultural Science* **17**, 238–241.

Reddall A, Ali A, Able JA *et al*. 2004. Cotton bunchy top: an aphid and graft transmitted cotton disease. *Australasian Plant Pathology* **33**, 197–202.

Shi X-B, Shi X-G, Wang H-Y, Xia X-M & Wang K-Y. 2011. Cross-resistance of the imidacloprid-resistant population of *Aphis gossypii* Glover (Homoptera: Aphididae) to other neonicotinoid insecticides and changes in activities of related enzymes. *Acta Entomologica Sinica* **54**, 1027–1033.

Smith T, Wilson L, Heimoana S, Herron G & Franzmann B. 2006. Over-winter host plants of cotton aphid (*Aphis gossypii*) and implications for managing abundance and resistance. In: Proceedings 13th Australian Cotton Growers Conference, Gold Coast. 8–10 August 2006.

Wang KI, Guo QL, Xia XM, Wang HY & Liu TX. 2007. Resistance of *Aphis gossypii* (Homoptera: Aphididae) to selected insecticides on cotton from five cotton production regions in Shandong, China. *Journal of Pesticide Science* **32**, 372–378.

Wilson L, Downes S, Khan M *et al*. 2013. IPM in the transgenic era: a review of the challenges from emerging pests in Australian cotton systems. *Crop and Pasture Science* **64**, 737–749.

Wilson LJ, Bauer LR & Lally DA. 1999. Insecticide-induced increases in aphid abundance in cotton. *Australian Journal of Entomology* **38**, 242–243.

Zidan LTM. 2012. Bio-efficacy of three new neonicotinoid insecticides as seed treatment against four early sucking pests of cotton. *American-Eurasian Journal Agricutural and Environmental Science* **12**, 535–540.

*Accepted for publication 29 October 2014.*

# **Appendix E:** PhD Thesis

Kate L. Marshall (2016) *Characterisation of Neonicotinoid Resistance in the Cotton Aphid.* Unpublished PhD thesis. University of Technology, Sydney (as submitted).

# Characterisation of neonicotinoid resistance in the cotton aphid, *Aphis gossypii* from Australian cotton

A thesis submitted in (partial) fulfilment of the requirements for the award of the degree

Doctor of Philosophy

*from*

University of Technology

*By*

**Kate L. Marshall**
**Bachelor of Animal and Veterinary Bioscience**
**(Hons)**

**School of Life Sciences**

**MAY 2016**

# Certificate of Original Authorship

This thesis is the result of a research candidature conducted jointly with another University as part of a collaborative Doctoral degree. I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as part of the collaborative doctoral degree and/or fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signature of Student:

Date:

# Refereed Journal Publications

This thesis includes a chapter that has been published as the following journal article:

Chapter 3:

**Marshall**, **KL**, Collins, D, Wilson, LJ & Herron, G. 2014. Efficacy of two thiamethoxam pre-germination seed treatments and a phorate side-dressing against neonicotinoid and pirimicarb resistant cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae). Austral Entomology. **54**(4): 351-357

# Conference Proceedings

**Marshall, K.L.,** Wilson, L.J. & Herron, G.A. 2012. Do the neonicotinoid seed treatments Cruiser® and Cruiser Extreme® control resistant aphid? p. 102. In: *16ᵗʰ Australian Cotton Conference.* Cotton Research and Development Corporation, Broadbeach, Australia, August 14ᵗʰ - 16ᵗʰ.

**Marshall, K.L.,** Herron, GA & Chen, Y. 2014. Neonicotinoid Resistance in Cotton Aphid from Australia. In: *17th Australian Cotton Conference.* Cotton Research and Development Corporation, Broadbeach, Australia, 5ᵗʰ to 7ᵗʰ August.

**Marshall, K.L.** 2014. Characterisation of Neonicotinoid Resistance in the Cotton Aphid. Presented at the: *17th Australian Cotton Conference.* Broadbeach, Australia, 5ᵗʰ to 7ᵗʰ August.

# Acknowledgements

The process of earning a doctorate is often considered a solitary endeavour; however, below is a list of people who have each contributed in their own way, to the successful completion of this doctorate.

First and foremost, I would like to acknowledge Dr. Grant Herron, my primary supervisor at the Elizabeth Macarthur Agricultural Institute. I deeply appreciate all of the guidance, encouragement, and knowledge you bestowed upon me during my graduate studies at the Elizabeth Macarthur Agricultural Institute. For all of your help and direction I will always be indebted. To Drs. Yizhou Chen and Thomas Walsh, thank you both for your ongoing support and valuable contributions, and lastly, thank you to Professor Steven Djordjevic for accepting me as your graduate student at the University of Technology, Sydney.

Without the financial support of the Cotton Research and Development Corporation which offered me a scholarship for graduate studies, this work would not have been possible. Special thanks go to Tracey Leven and Susan Maas for their unwavering support and dedication throughout the course of this project. My appreciation is also extended to the staff of the Elizabeth Macarthur Agricultural Institute, especially those who I have worked alongside in the Insecticide Resistance Unit.

Thank you to my family and friends, especially my sister Jane, brother Luke and best friend Romy, for helping me survive all of the stress, especially from this last year and for the continued encouragement as I neared the end.

To my parents, I would like to acknowledge the tremendous support you have given me throughout my life. Together, you are the inspiration behind what led me to take on this endeavour. Most of all, thank you for both being a constant example of what *'energy well directed'* can achieve: success!

Finally, I would like to thank my fiancé Brendan for providing me with endless love and encouragement throughout the duration of this doctorate. Put simply, I have never met

anyone who believes in me more. I feel incredibly lucky to have met you and look forward to spending the rest of my life with you. I love you always.

# Contents

# List of Tables

# List of Figures

# List of Abbreviations and Acronyms

*Ace:* Acetylcholinesterase gene

**AChE:** Acetylcholinesterase (the target of organophosphate and carbamate insecticides)

**ACh:** Acetylcholine

**CBT:** Cotton Bunchy Top

**CE:** Carboxylesterase

**CLR:** Cotton Leaf Roll

**COG:** Cluster of orthologous groups

**cys-LGIC:** cys-loop Ligand gated ion channel

**CYP:** Family of P450 genes

**DEF:** *S,S,S*-tributyl phosphorotrithioate (a synergist)

**ddNTPs:** di-deoxynucleotidetriphosphates

**DDT:** Dichlorodiphenyltrichloroethane

**DEG:** Differentially expressed genes

**EMAI:** Elizabeth Macarthur Agricultural Institute

**EMS:** Ethyl methanesulfonate

**EST:** Esterase

**GABA:** gamma-aminobutyric acid (the target receptor of organochlorines and Phenylpyrazoles (Fiproles) insecticides)

**GST:** Glutathione-S-transferase

**GO:** Gene Ontology

**IPM:** Integrated Pest Management

**IRAC:** Insecticide Resistance Action Committee

**IRMS**: Insecticide Resistance Management Strategy

*kdr*; **super**-*kdr*: knock down resistance (knock-down resistance traits)

**KEGG**: Kyoto Encyclopedia of Genes and Genomes

**LC$_{50}$**: The dose required to kill half the tested population

**LC$_{99}$**: The dose required to kill 99% of the tested population

**LBD**: Ligand binding domain

**nAChR**: Nicotinic acetylcholine receptor (the target of neonicotinoid insecticides)

**NADPH**: Nicotinamide Adenine Dinucleotide Phosphate

**NHC**: Nitromethylene heterocyclic compounds

**NGS**: Next generation sequencing

**NR**: Non-redundant

**NSW**: New South Wales

*o-Ace*: orthologous gene to *Drosophila* gene *Ace*

*p-Ace*: paralogous gene to *Drosophila* gene *Ace*

**PBO**: Piperonyl butoxide (a detoxification enzyme inhibitor)

**PCR**: Polymerase chain reaction

**P450**: Cytochrome P450-dependent monooxygenase

**QLD**: Queensland

**qRT-PCR**: Quantitative real-time polymerase chain reaction

*Rdl*: Resistance to dieldrin gene

**RF**: Resistance factor

**RFLP**: Restriction fragment length polymorphism

**RNA-Seq**: RNA-Sequencing

**RPKM:** Reads Per Kilobase of transcript per Million mapped reads

**SNP:** Single nucleotide polymorphism

**USA:** United States of America

**USD:** United States dollar

**VGSC:** *para*-type voltage gated sodium channel (the target of pyrethroid insecticides,

pyrethrins and DDT)

**WHO:** World Health Organisation

# Abstract

The cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is a highly polyphagous pest that inflicts serious damage to a broad range of agricultural, horticultural and greenhouse crops. In Australia, *A. gossypii* is a significant pest of cotton and is difficult to control with insecticides because of its high propensity to develop resistance. Neonicotinoids are among the most effective insecticides used to control *A. gossypii* but the recent detection of resistance threatens their longevity. Consequently, I aimed to restore neonicotinoid efficacy against *A. gossypii* through elucidation of underlying resistance mechanism(s).

Bioassay was used to measure thiamethoxam (neonicotinoid) response in three field strains collected from commercial cotton. Resistance factors (RFs) between 49- and 85-fold were produced and resistance was correlated with potential field control failures via a glasshouse efficacy trial. Results showed that resistant *A. gossypii* could complete their development on cotton grown from thiamethoxam-treated seed. A second trial investigated the use of phorate (an organophosphate) as an alternative pre-germination treatment to thiamethoxam. Phorate effectively controls neonicotinoid resistant *A. gossypii* but cross resistance between phorate and the carbamate insecticide pirimicarb must be carefully considered as part of any sustainable management strategy.

PCR-Sequencing was employed to identify if mutation R81T known to confer resistance to neonicotinoid compounds was present in Australian *A. gossypii*. Comparative sequence analysis between susceptible and resistant strains confirmed the absence of mutation R81T. Potential biochemical mechanisms of thiamethoxam resistance in *A. gossypii* were then studied using synergist bioassays. The use of the synergist piperonyl butoxide in tandem with thiamethoxam completely or partially suppressed resistance. This suggests that resistance is at least in part, mediated by overexpression of detoxification enzymes that could subsequently be targeted to achieve improved field control of resistant aphids.

High-throughput sequencing of the *A. gossypii* transcriptome found differences in gene expression associated with thiamethoxam resistance. Two transcripts involved in the detoxification of xenobiotics (putatively annotated as *CYP6K1*) were found differentially expressed between resistant and susceptible strains. Transcript expression was further validated by qRT-PCR and showed a similar tendency in up-regulation of expression. As such I identified this gene as the strongest candidate for thiamethoxam resistant *A. gossypii*.

This study has generated a comprehensive transcriptome resource for *A. gossypii* that has characterized the expression of numerous important transcripts encoding proteins involved in insecticide resistance. Consequently, my study will contribute to future research relating to molecular characterization of insecticide resistance mechanisms in *A. gossypii* and other insect pests.

# Chapter 1.  Review of literature

## 1.1 The cotton aphid, *Aphis gossypii*

*Aphis gossypii* Glover (Hemiptera: Aphididae) is a small soft bodied insect that displays considerable variation in both size and colour, and adults may be winged (alate) or wingless (apterae) (Blackman and Eastop 2000) (Figure 1.1). Extensive phenotypic plasticity results in a distinct number of morphs displaying significant colour variation i.e. "normal" light green apterae, "normal" dark green apterae, "dwarf" yellow apterae and alatae (Paddock 1919, Wall 1933, Wilhoit and Rosenheim 1993, Watt and Hales 1996) (Figure 1.1). Dwarf apterae possess a body size approximately one third of normal apterae and yellow instead of green colouration (Watt and Hales 1996). This phenotype is often observed in warmer conditions and is associated with low intrinsic rates of increase, $r_m$ (an estimate of future population growth rate based on the performance of individual aphids) (Wilhoit and Rosenheim 1993, Watt and Hales 1996). In contrast, dark coloured morphs are observed in cooler, favourable conditions and exhibit high intrinsic rates of increase (Blackman and Eastop 2000). Nymphs developing into alatae are often a greenish blue, or amber and blue colour (Blackman and Eastop 2000). Siphunculi, tube like structures on the posterior part of the abdomen, are the main diagnostic feature of aphids (Dixon 1975). In *A. gossypii*, a distinctive pair of short and darkly pigmented siphunculi are present at their tail end (Blackman and Eastop 2000) (Figure 1.1). The absence of tubercles, small rounded projections on the head between the antenna is also characteristic of *A. gossypii* (Blackman and Eastop 2000).

**Figure 1.1** Colour polymorphism of adult *Aphis gossypii*. A, dwarf yellow apterae; B, light green apterae; C, dark green apterae; D, winged (alate) adult.

*A. gossypii* has a widely distributed host range but is mostly found in tropical and temperate regions such as Australia, North and South America, Hawaii and Europe (Blackman and Eastop 2000). Attributed to its highly polyphagous nature, *A. gossypii* can affect over 92 different plant families, including food and fibre crops, ornamentals and flowers (Elbert and Cartwright 1997). The main agricultural crops include those in the families Cucurbitaceae (watermelons, cucumbers and pumpkin) Rutaceae (genus *Citrus*) and Malvaceae (cotton

and okra) (Elbert and Cartwright 1997, Blackman and Eastop 2000). Moreover, there is an extensive list of non-crop plants that can serve as host plants for *A. gossypii* when primary or secondary host crops are not available (Elbert and Cartwright 1997, Blackman and Eastop 2000). Worldwide, it is the most economically significant aphid species found on cotton (Leclant and Deguine 1994).

### 1.1.1 Life cycle of *Aphis gossypii*

In general, there are basically two types of aphid life cycle: non-host alternating (autoecious, monoecious) and host-alternating (heteroecious) (Dixon 1988, Kundu 1994, Kundu and Dixon 1995). Autoecious aphids use only a single host plant for their entire life cycle whilst heteroecious aphids alternate between two taxonomically different host plants; woody species (primary host) on which they overwinter and a herbaceous plant species (secondary host) on which they spend the summer (Kundu 1994). In addition, most aphids undergo cyclical parthenogenesis in which each generation of sexual reproduction (holocyclic phase) is followed by many generations of asexual reproduction (anholocyclic phase) (Moran 1992, Blackman 2000). Typically, sexual reproduction occurs on the primary host plant during late autumn to produce overwintering eggs. In spring, each egg gives rise to a wingless viviparous and parthenogenetically reproducing female and is followed by several generations of asexual reproduction through spring and summer (Blackman 1987, Blackman and Eastop 2000) (Figure 1.2). These parthenogenetic females may be winged or wingless and in autumn give rise to a single sexual generation of males and females (Wellings et al. 1980, Kundu and Dixon 1995, Blackman and Eastop 2000).
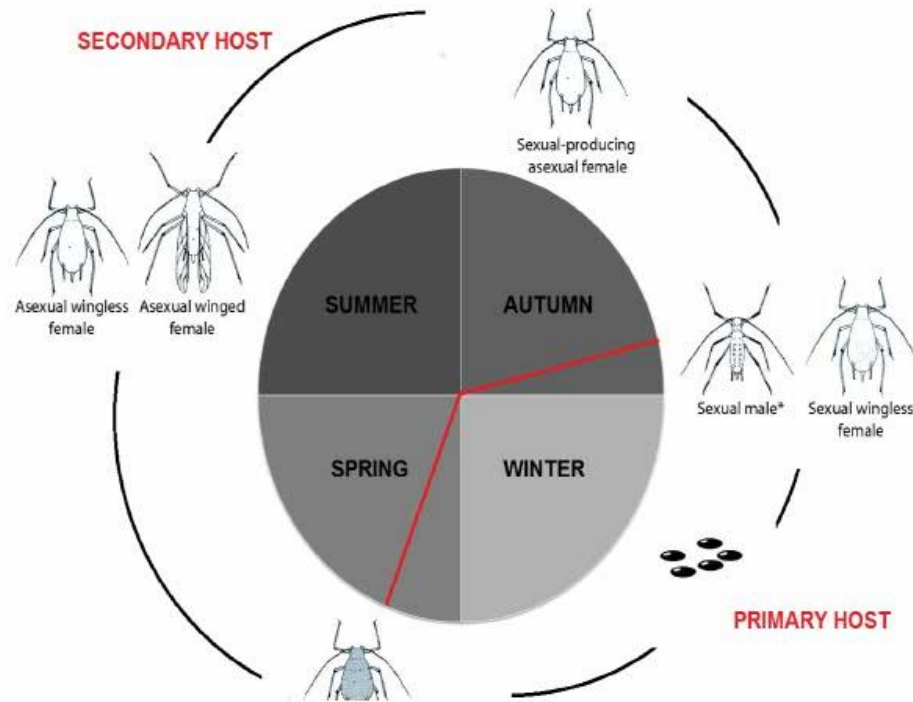
**Figure 1.2** Life cycle of aphids (Shingleton et al. 2003).

In some aphid species, anholocyclicity (where the clone is entirely asexual reproducing by parthenogenesis throughout the year), is triggered by seasonal changes in the environment. For aphids to initiate their annual sexual phase, seasonal changes such as a period of decreasing photoperiod or temperature are required (Blackman and Eastop 2000, Williams and Dixon 2007). Thus, loss of the sexual generation is therefore likely to occur in regions where winter conditions are mild (Williams and Dixon 2007).

In Australia, *A. gossypii* reproduces exclusively via asexual reproduction and does not diapause (Wool and Hales 1997), instead surviving through winter using a range of cultivated and non-cultivated host plants (Smith et al. 2006). Under such conditions, apterous adult females reproduce exclusively via parthenogenesis giving birth to live young that are clones of themselves (asexual lineages). In aphids, parthenogenesis is coupled with

the phenomenon of 'telescoping of generations', whereby offspring at birth contain embryos that also contain embryos (Moran 1992). This can potentially result in billions of individual aphids derived from one individual in a growing season (Dixon 1989, Kersting et al. 1999). Furthermore, 'telescoping of generations' drastically reduces the total development time of an aphid providing them with intrinsic rates of increase normally associated with much smaller organisms, i.e. mites (Leather and Dixon 1984, Dixon 1989). This has implications for resistance management because insects with short generation times can develop resistance more rapidly than insects with longer generation times because more generations can potentially receive insecticide exposure (Roush and McKenzie 1987). Moreover, in a parthenogenetically reproducing resistant population, resistance alleles cannot be diluted via outcrossing to susceptible individuals (Wool and Hales 1997).

Another consequence of parthenogenesis is the production of discrete clonal populations that vary in host preference within a single region (Carletto et al. 2009). Clonal populations associated with Araceae, Asteraceae (chrysanthemum), Cucurbitaceae (cucumber), and Malvaceae (cotton) have all been documented worldwide (Guldemond et al. 1994, Margaritopoulos et al. 2006, Carletto et al. 2010, Agarwala and Choudhury 2013, Chen et al. 2013). This unique relationship with their host plant may contribute to the rapid evolution of insecticide resistance as asexual lineages are subject to strong insecticidal pressure (Furk et al. 1980, Saito 1989). For example in the United Kingdom, *A. gossypii* occurs on chrysanthemum and cucumber (Guldemond et al. 1994) but the strain that occurs on chrysanthemum does not occur on cucumber and vice versa. The strain that occurs on chrysanthemum displayed resistance to organophosphate and carbamates insecticides, whilst the lineage found on cucumber did not (Guldemond et al. 1994). Similarly, ffrench-Constant et al. (1995) found this same host relationship in parthenogenetic lineages from chrysanthemum in glasshouses which showed little or no reproduction on cucumber and vice versa. In Australia, evidence for the occurrence of super-clones was found by genotyping eight microsatellite markers for a collection of *A. gossypii* field isolates (Chen

et al. 2013). A link between host plant and resistance to the insecticide pirimicarb was noted in two multi-locus genotype groups (Chen et al. 2013).

### 1.1.2 Economic damage caused by *Aphis gossypii*

Firstly, direct feeding by *A. gossypii* results in significant yield reduction and economic loss (CABI 2005). Aphids typically feed on the underside of young leaves and on stems where they insert their slender piercing mouthparts (stylet) into the phloem vessel for sap removal (Blackman and Eastop 2000, CABI 2005). The removal of nutritional resources (assimilate) from the phloem results in competition between young shoots and developing fruits for nutrients. If nutrient demands are not met, stunted growth and reduced yield will likely result in the developing plant (Cotton Catchment Communities Cooperative Research Centre 2008).

Indirect damage by *A. gossypii* is caused via the transmission of several debilitating plant viruses and additionally through the production of honeydew (Blackman and Eastop 2000). *A. gossypii* transmits over 50 different plant viruses including non-persistent viruses of beans and peas, cucurbits, peppers, and soybean and the persistent Lily symptomless virus and Lily rosette disease (Blackman and Eastop 2000, Henneberry et al. 2000). *A. gossypii* is the most important vector of Cucumber mosaic virus (CMV) in cucurbits (Blackman and Eastop 2000) and is also a vector of Papaya ringspot virus, transmitting both the P (PRSV-P) and W (PRSV-W) strains (CABI 2005). The former is a disease of papaya, whereas the latter, PRSV-W, also called Watermelon mosaic virus 1 (WMV-1), infects cucurbits and watermelon (CABI 2005). In cotton, *A. gossypii* has been reported as an efficient vector of cotton anthocyanosis virus, cotton curliness virus, cotton blue disease, cotton bunchy top (CBT), cotton leaf roll (CLR) and purple wilt (Kennedy et al. 1978, Brown 1992, Reddall et al. 2004).

As a result of feeding on the phloem sap, aphids excrete a sticky and sugary waste by-product called honeydew (Isley 1946). When in contact with the leaf surface, honeydew can interfere with photosynthetic processes and act as a substrate for fungi, including sooty

moulds which blacken leaves and further reduce photosynthetic activity (Isley 1946). Honeydew contamination of the open boll cotton lint can lead to significant problems during processing and spinning of the fibre (Hequet et al. 2000). Sticky cotton poses a serious problem for ginning and milling because sugars taint equipment and cause the lint to stick to machinery (Miller et al. 1994, Slosser et al. 2002), often necessitating shutdown (Hequet et al. 2000). Efficiency and profitability of the cotton processing industry are ultimately reduced by sticky cotton and so too is the quality of lint produced (Hequet et al. 2000). As an established exporter of high quality cotton fibre, the reputation of Australian cotton could be severely downgraded if such fibre contamination occurs.

### 1.1.3 Management of *Aphis gossypii*

Historically, there has been a general trend towards the use of insecticides for *A. gossypii* control. However, the limits of chemical control were soon realised when their effectiveness and profitability were drastically reduced due to the onset of insecticide resistance in *A. gossypii* to every major insecticide group (Whalon et al. 2008). Today, control strategies are based on the concept of integrated control that includes best management practice and working to economic thresholds as key components (Wilson et al. 2004, Fitt et al. 2009). The best management practices include maintaining good on-farm hygiene i.e. controlling on-farm over-wintering hosts for aphids, conservation of beneficial insect species, and observing control thresholds for aphids before spraying (Wilson et al. 2004, Wilson et al. 2013).

### 1.1.3.1 Cultural and Biological control

Owing to the highly polyphagous nature of *A. gossypii*, good on-farm hygiene is particularly important because it will remove overwintering host plants for aphids to reproduce and feed on during the winter months (Smith et al. 2006). If aphids move from mature cotton where they have been selected for resistance by insecticidal sprays, and harbour on weeds near fallow cotton during the winter months, a reservoir of potentially

96 of 308

resistant aphids is capable of re-colonizing the following year's crop (Schulze and Tomkins 2002).

Insecticide product selection that conserves beneficial insects creates an agro-ecosystem where insect pests in low numbers can be controlled effectively by beneficials, often without further human intervention. Insecticides which have high non-target effects to natural enemies will likely induce the occurrence of secondary pest outbreaks, requiring further insecticide control (Wilson et al. 1999). Selecting an insecticide is very much determined by the development phase of the cotton crop. If *A. gossypii* are present during early growth (post-seedling) and intervention is required then choosing a more selective option to help conserve beneficial populations is desirable (Mansfield et al. 2006).

Use of control thresholds for aphids is particularly important as generally when aphids are present on seedling cotton plants in low numbers they are not considered a problem (Mass 2014). Even when infestation levels are very high (>90%) cotton plants may fully recover if the infestation doesn't persist for too long (<10 days) (Cotton Catchment Communities Cooperative Research Centre 2008). If the infestation continues for 2-3 weeks then significant yield loss can occur (Cotton Catchment Communities Cooperative Research Centre 2008). Thresholds for aphid control are determined by the potential for the aphid population to reduce yield or transmit CBT virus (Cotton Catchment Communities Cooperative Research Centre 2008). To determine aphid numbers and their significance during seedling to first open boll stage a scoring system is used which involves recording the density of aphids on the undersides of main-stem leaves (CottASSIST 2008). Scores of aphid abundance can then be entered into the Aphid Yield Loss Estimator (CottASSIST 2008), which will produce an estimate on the likely yield effect. The Aphid Yield Loss Estimator predicts yield loss as a result of direct aphid feeding and offers a reliability of 85% (CottASSIST 2008). The loss estimator is used between squaring (emergence of developing cotton fruit) and first open bolls as before this time period aphid populations are most likely to be controlled by beneficial predators or parasites (Cotton Catchment Communities Cooperative Research Centre 2008). Chemical intervention is warranted if yield loss is predicted to be at 4% or higher (Mass 2014). Contamination of the open boll

lint with honeydew late in the season poses a serious threat to the quality of lint produced (Slosser et al. 2002); during this phase thresholds for intervention are 50% infested plants or 10% infested plants if trace amounts of honeydew are present (normally 90%) (Mass 2014).

### 1.1.3.2 Chemical control

Insecticides registered for control of *A. gossypii* in Australia span multiple insecticide MoA (mode of action) classes including some twenty different active ingredients (Nauen et al. 2012, Sparks and Nauen 2015) (Table 1.1). A limited range of insecticides may be applied as foliar sprays. Insecticides may also be applied as seed treatments or as granules with the seed at planting. Those that work systemically, by translocation throughout the growing plant are extremely effective against sap feeding insects as they protect all regions of the plant (Elbert et al. 2008). When selecting insecticides, care must be taken as many populations of *A. gossypii* can be resistant to one or many insecticide classes (IRAC 2015).

**Table 1.1** Insecticides registered for control of *Aphis gossypii* in Australian cotton as arranged by their corresponding MoA group (Mass 2014, CottonInfo 2015).

| Group | Mode of Action | Subgroup | Chemical group | Trade names |
|---|---|---|---|---|
| 1 | Acetylcholine esterase inhibitors | A | Carbamates | Pirimicarb |
| | | B | Organophosphates | Dimethoate, omethoate, phorate, chlorpyrifos |
| 2 | gamma-aminobutyric acid (GABA)-gated chloride channel antagonists | A | Cyclodiene organochlorines | Fipronil |
| 3 | Sodium channel modulators | | Pyrethroids, Pyrethrins | Lambda-cyhalothrin, deltamethrin, permethrin |
| 4 | Nicotinic Acetylcholine receptor agonists / antagonists | A | Neonicotinoids | Acetamiprid, clothianidin, imidacloprid, thiamethoxam, |
| | | C | Sulfoxomines | Sulfoxaflor |
| 5 | Nicotinic Acetylcholine receptor agonists (other than group 4) | | Spinosyns | Spinosad |
| 9 | Modulators of chordotonal organs | B | Pyridinecarboxamide | Pymetrozine |
| | | C | | Flonicamid |
| 12 | Inhibitors of mitochondrial ATP synthase | A | | Diafenthiuron |
| 19 | Octopamine agonists | | | Amitraz |
| 22 | Inhibitors of lipid synthesis | | | Spirotetramat |
| 28 | Ryanodine receptor modulators | | Diamides | Cyantraniliprole |

### 1.1.4 History of insecticide resistance in *Aphis gossypii*

Worldwide, the pest status of *A. gossypii* has steadily increased since the 1800s where it was first reported as a relatively minor pest of cotton in South Carolina, USA (United States of America) (Slosser et al. 1989). By the 1880s, it was reported across most of the South-eastern region of the USA (Slosser et al. 1989) and in 1916 was found on cotton in Texas (Paddock 1919). Its pest status remained static for some time until the 1980s where it developed from an occasional secondary pest to a debilitating annual pest of cotton in most growing areas of the world, including USA, Thailand, the former USSR and Sudan (Schepers 1989). Likewise, in Australia, the wide scale adoption of transgenic cotton in the 1990s led to significant outbreaks of *A. gossypii* due to an overall reduction in insecticide sprays used to control the primary insect pests, *Helicoverpa* spp. These sprays targeting *Helicoverpa* spp. were inadvertently controlling secondary pest populations of *A. gossypii* which subsequently increased to levels requiring targeted control.

Since the mid-1960s, widespread resistance by *A. gossypii* has been recorded worldwide against the carbamate (group 1A), organophosphate (group 1B), cyclodiene organochlorine (group 2A), pyrethroid (group 3A) and neonicotinoid chemical (group 4A) classes (Table 1.2). The first record of resistance was documented by Ghong et al. (1964), who confirmed *A. gossypii* resistant to the organophosphate insecticide demeton. Subsequently, resistance to the carbamate pirimicarb, was reported by Furk et al. (1980) and resistance to pyrethroids by Zil'bermints and Zhuravela (1984). Kerns and Gaylor (1992) detected organophosphate (80-fold) and pyrethroid (50-fold) resistance in *A. gossypii* from cotton fields in Texas and Alabama. O'Brien et al. (1992) found carbamate and organochlorine resistance in *A. gossypii* from Mississippi, while in Hawaii, >2000-fold resistance to the organophosphate oxydemeton-methyl was reported (Hollingsworth et al. 1994). In India, >1000 fold resistance to several pyrethroid insecticides has been previously demonstrated in *A. gossypii* collected off cotton (Ahmad et al. 2003). Reported cases of neonicotinoid resistance in *A. gossypii* include the southern USA (Gore et al. 2013), South Korea (Koo et al. 2014), China (Wang et al. 2002), Japan (Matsuura and Nakamura 2014) and Australia (Herron and Wilson 2011). Gore et al. (2013) detected neonicotinoid (thiamethoxam)

resistance in 25 field collected strains of *A. gossypii* off cotton from southern USA and observed resistance ratios up to 562-fold. In Japan, Matsuura and Nakamura (2014) detected 91-fold resistance to thiamethoxam in a field strain collected off cucumber. In South Korea, Koo et al. (2014) tested six neonicotinoid insecticides against six field collected *A. gossypii* strains and observed resistance ratios up to 14,000-fold for clothianidin.

**Table 1.2** Insecticides documented worldwide to which *Aphis gossypii* has developed resistance as a result of field exposure or laboratory selection (Whalon et al. 2008).

| Group | Sub group | Common names |
|---|---|---|
| **1A** | Carbamates | Benfuracarb, carbaryl, carbofuran, carbosulfran, methomyl, pirimicarb |
| **1B** | Organophosphates | Acephate, chlorpyrifos, dichlorvos, dimethoate, malathion, methamidophos, methidathion, omethoate, parathion, phosphamidon |
| | Organothiophosphate | Diazinon, oxydemeton-methyl |
| | Phosphorothioate | Demeton, demeton-S-methyl, |
| **2A** | Cyclodiene Organochlorines | Dichlorodiphenyltrichloroethane (DDT), endosulfan, endrin, lindane |
| **3A** | Pyrethroids, Pyrethrins | Bifenthrin, cyfluthrin, cyhalothrin, lambda-cyhalothrin, cypermethrin, alpha-cypermethrin, deltamethrin, esfenvalerate, fenpropathrin, fenvalerate, |
| **4A** | Neonicotinoids | Acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, thiamethoxam |
| **4C** | Sulfoximines | Sulfoxaflor |
| **9C** | Pyridinecarboxamide | Flonicamid |

In Australia, resistance to *A. gossypii* against the carbamate, organophosphate and pyrethroid insecticide classes has been detected in nearly all cotton growing regions (Herron and Rophail 2000, Herron et al. 2001). During the 1999-00 and 2000-01 cotton

seasons, resistance levels were often high to extreme and for the first time were linked to control failures in all of the major cotton growing regions of Australia (Herron and Rophail 2000, Herron et al. 2001). Cross resistance in *A. gossypii* between the widely used organophosphates (Folimat® and Rogor®) and carbamate (Pirimor®) insecticides (Moores et al. 1996) exacerbated the potential for resistance outbreaks and dramatically reduced available control options (Herron et al. 2000, Herron and Rophail 2000, Herron et al. 2001). Fortunately, introduction of the neonicotinoid group 4A insecticides, in combination with an integrated pest management (IPM) strategy, eventually recovered the widely used carbamate insecticide Pirimor® (Aggs 2011). As a result, the detection of resistance outbreaks against group 1A, 1B and 3A insecticides was drastically reduced.

Widespread reliance on the neonicotinoid chemical class (group 4A) led to the first outbreak of resistance in Australian *A. gossypii* collected off cotton in the 2007-08 cotton season, with control failures reported the following season (Herron and Wilson 2011). At that time cross resistance between members of the MoA group 4A had been previously demonstrated (Wang et al. 2007, Alyokhin et al. 2008, Shi et al. 2011) so it was reasonable to assume for resistance management purposes that Australian populations of *A. gossypii* would also show cross resistance. It became clear with the neonicotinoid failures that the sustainable management of *A. gossypii* in Australian cotton was at risk and the management strategy was modified to reduce neonicotinoid selection (Herron and Wilson 2011). At that time research to restore neonicotinoid efficacy and maintain the class as a viable control option for *A. gossypii* was seen as an industry priority (Herron and Wilson 2011).

## 1.2 The Neonicotinoids

Most commercial insecticides available today are designed to act on ion channels, receptors or enzymes within the insect nervous system (Greenwood et al. 2007). These target sites are often the same as naturally occurring compounds from which a synthetic analogue is produced and used for insect pest control (Isman 2006). The discovery and synthesis of the neonicotinoid chemical class can be attributed to nicotine, a natural insecticide acting as an agonist on postsynaptic acetylcholine (ACh) receptors (Yamamoto 1999). Unfortunately

nicotine also had a high affinity (toxicity) to mammalian ACh receptors and low field persistence, making large scale commercialization for agricultural use impractical (Yamamoto 1999). However, the promise that nicotine showed towards insect nAChRs was realised via the development of synthetic derivatives: the 'neonicotinoids' (Jeschke and Nauen 2008).

### 1.2.1 Development and Structure

In the early 1970's, Shell Development Company's Biological Research Centre in Modesto, California, started screening a number of lead structures from university sources in an effort to discover new crop protection chemicals. The most promising was 2-(dibromo-nitromethyl)-3-methyl pyridine, which exhibited low-level insecticidal activity against house fly *Musca domestica* Linnaeus and pea aphid *Acyrthosiphon pisum* Harris (Soloway et al. 1978, Soloway et al. 1979, Kollmeyer et al. 1999). This find led to the development of a new class of nitromethylene heterocyclic compounds (NHC) that showed specificity for insect nAChRs. After further study of NHC compounds, nithiazine was selected for its rapid knockdown of susceptible insects and low toxicity to mammals (Soloway et al. 1978, Soloway et al. 1979, Kollmeyer et al. 1999). However, the development of nithiazine, that concentrated on the nitromethylene amidine skeleton, was later found to be photo-chemically unstable in field conditions and so was never commercialized for use (Kleier et al. 1985). In the 1980's, continued research on the chemical structure of nithiazine led to the discovery of 1-(6-chloro-3-pyridylmethyl)-2-nitromethyleneimidazolidine (Kollmeyer et al. 1999). The chloropyridlymethyl substituent was found to greatly enhance toxicity towards insect nAChRs and also led researchers to explore other bioisosteric heterocycles (Kagabu 2011). The original nithiazine flaw of photo-lability was found to be the 2-nitromethylene chromophore and that was replaced with a 2-nitroimino chromophore, making compound 1-(6-chloro-3-pyridylmethyl)-2-nitroimino-imidazolidine relatively persistent in the field (Moriya et al. 1993, Kagabu and Medej 1995). This chemical, now known as imidacloprid (Figure 1.3), was commercially released by Bayer in 1991 (Elbert et al. 1991) and marked the beginning of a new class of

chemicals (Group 4: nAChR competitive modulators) which were to become more popular than the widely used synthetic pyrethroids (Jeschke and Nauen 2008). Today, group 4A neonicotinoids include thiamethoxam (Syngenta) (Maienfisch et al. 1999), acetamiprid (Aventis Crop Sciences) (Yamada et al. 1999), dinotefuran (Mitsui chemicals) (Wakita et al. 2003), clothianidin (Takeda and Bayer) (Ohkawara et al. 2002) and thiacloprid (Bayer CropScience) (Jeschke et al. 2001).
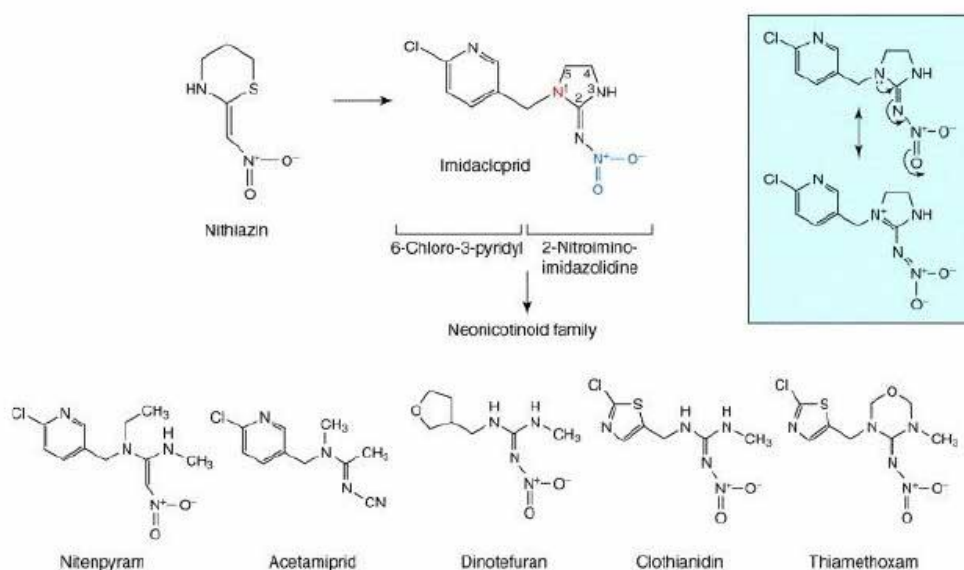


**Figure 1.3** The structure of the synthetic insecticide imidacloprid. Also shown are other synthetic insecticides that are related to imidacloprid: nithiazin, nitenpyram, acetamiprid, dinotefuran, clothianidin and thiamethoxam. The two main moieties of the imidacloprid molecule are shown; the tertiary amine that corresponds to the quaternary ammonium of ACh and the nitro group of imidacloprid are highlighted in red and blue, respectively. Substitution at the 1-position of nithiazin led to the eventual production of imidacloprid, based on which further neonicotinoids have been synthesized (Matsuda et al. 2001).

There are a variety of terms used to subdivide the neonicotinoid chemical class based on structural fragments. If classified by their moieties: compounds with 6-chloro-3-

pyridinylmethyl, 2-chloro-5-thiazolymethyl, and 3-tetrahydrofuranmethyl are referred to as chloronicotinyls, chlorothiazolyls and furanicotinyls, respectively. If classified by their functional group as part of the pharmacophore, then the following terms are used: nitroimines or nitroguanidines (imidacloprid, thiamethoxam, clothianidin and dinotefuran); nitromethylenes (nithizaine and nitenpyram); and cyanoimines (acetamiprid and thiacloprid). Like all group 4 neonicotinoids, the newly developed insecticide sulfoxaflor acts as a nAChR agonist but because of the novel way it interacts with the nAChR, and its lack of insecticidal cross-resistance with group 4A neonicotinoids, sulfoxaflor is placed by the Insecticide Resistance Action Committee (IRAC) separately within group 4 as 4C (4B being nicotine) (Sparks and Nauen 2015).

### 1.2.2 Agricultural and economic importance

Neonicotinoid insecticides were first introduced to the market in 1991 and have rapidly established themselves as the most popular crop protection agents worldwide, with annual global sales in excess of $3.7 billion (Gerwick and Sparks 2014). Registered in more than 120 countries worldwide, neonicotinoid insecticides are available for use on various crops such as cotton, cereals, sorghum, maize and canola (Jeschke et al. 2011, APVMA 2013). Prior to the introduction of the neonicotinoids, the insecticide market was dominated by the organophosphate, carbamate and pyrethroid chemical classes (Elbert et al. 2008). However, by 2008, neonicotinoids held 24% of the global market share for insecticides (Jeschke et al. 2011) and in 2009 had a market value of $2.63 billion USD (United States Dollar) (Simon-Delso et al. 2015). Collectively, three neonicotinoid insecticides (imidacloprid, thiamethoxam and clothianidin) account for 85% of the total neonicotinoid insecticide market (Elbert et al. 2008, Jeschke et al. 2011). Worldwide, imidacloprid is the highest selling neonicotinoid insecticide (Nauen et al. 2008), with a total worth of $1.09 billion USD, accounting for 41.5% of the global market (Jeschke et al. 2011). Thiamethoxam and clothianidin are second and third in terms of total neonicotinoid sales, with values of $0.63 and $0.44 billion USD, respectively (Simon-Delso et al. 2015). The foliar spray formulation of thiamethoxam called Actara® accounts for over half of these sales while the

seed treatment form of thiamethoxam called Cruiser® was used in more than 80 countries on over 20 different crops (Syngenta 2013). The worldwide sales of thiamethoxam reached $1 billion USD in 2011 (Syngenta 2012) and $1.1 USD billion in 2012 (Syngenta 2013). In Australia, compounds imidacloprid and thiamethoxam are routinely implemented for control of hemipteran sap-feeding insects (e.g. aphids and whiteflies), foliar-feeding insects and via seed treatment a range of soil pests (e.g. wireworms) (Jeschke et al. 2011) (Table 1.3). Imidacloprid containing seed treatments include Gaucho® and Genero® (imidacloprid) and Amparo® (imidacloprid plus thiodicarb). Thiamethoxam containing seed treatments (Cruiser® and Cruiser Extreme®) are by far the most popular, accounting for 80% of all cotton seed planted in Australia (Herron and Wilson 2011). Foliar sprays regularly used in Australian cotton include Shield® (clothiandin), Mospilan® (acetamiprid), Actara® (thiamethoxam) and Confidor® (imidacloprid) (Mass 2012) (Table 1.3).

**Table 1.3** List of neonicotinoids and related compounds registered for use in Australian cotton for the control of sucking insect pests (Mass 2014).

| Compound | Developed by | Trade name and treatment type | |
|---|---|---|---|
| | | Foliar spray | Seed treatment |
| **Acetamiprid** | Aventis Crop Sciences | Mospilan® | - |
| **Clothianidin** | Takeda Chemical Industries* & Bayer | Shield® | - |
| **Imidacloprid** | Bayer CropScience | Confidor® | Gaucho® |
| | | | Genero® |
| | | | Amparo® |
| **Thiamethoxam** | Syngenta | Actara® | Cruiser® |
| | | | Cruiser Extreme® |
| **Sulfoxaflor** | Dow AgroSciences | Transform® | - |

## 1.2.3 Target site

Nicotinic acetylcholine receptors are members of the Cys-loop ligand-gated ion channel superfamily (cys-LGIC) whose primary role is to mediate cholinergic synaptic transmission in insect and vertebrate nervous systems (Brejc et al. 2001, Karlin 2002, Lester et al. 2004). Insect nAChRs are confined to the central nervous system only, unlike mammals which also include nAChRs in the peripheral nervous system (Gepner et al. 1978, Breer and Sattelle 1987). These fundamental physiological differences between insects and mammals make the neonicotinoids extremely valuable due to their reduced toxicity to non-target organisms and increased selectivity to insects (Tomizawa and Casida 2003, Matsuda et al. 2009).

Our first understanding of the structure of nAChRs came from initial cloning and sequencing of nAChRs from the electric organs of the Pacific electric ray *Torpedo californica* Ayres (Noda et al. 1982, Noda et al. 1983, Noda et al. 1983, Galzi et al. 1991). The nAChR is composed of a hetero or homo-pentamer subunit combination arranged symmetrically around a central cation selective pore (Celie et al. 2004, Unwin 2005) (Figure 1.4). Neonicotinoid insecticides interact with the orthosteric binding site at each nAChR heteropentamer, occurring at the extracellular ligand binding domain (LBD) at the interface between adjacent α and non-α subunits (Brejc et al. 2001). In heteropentamer nAChRs consisting of two α and three non-α subunits, LBDs contain six loops, and are donated by loops A to C and loops D to E, respectively, to generate the ACh binding site (Corringer et al. 2000, Karlin 2002). In response to agonist binding, a single ion channel is opened allowing an influx of ions into the cell (Breer and Sattelle 1987). This reaction is only temporary and is diffused by the specialized enzyme, acetylcholinesterase (AChE) (Toutant 1989). Neonicotinoid insecticides mimic the action of ACh but are unable to be broken down by AChE, resulting in an irreversible binding to and overstimulation of the receptor (Tomizawa and Casida 2005, Tomizawa and Casida 2009).
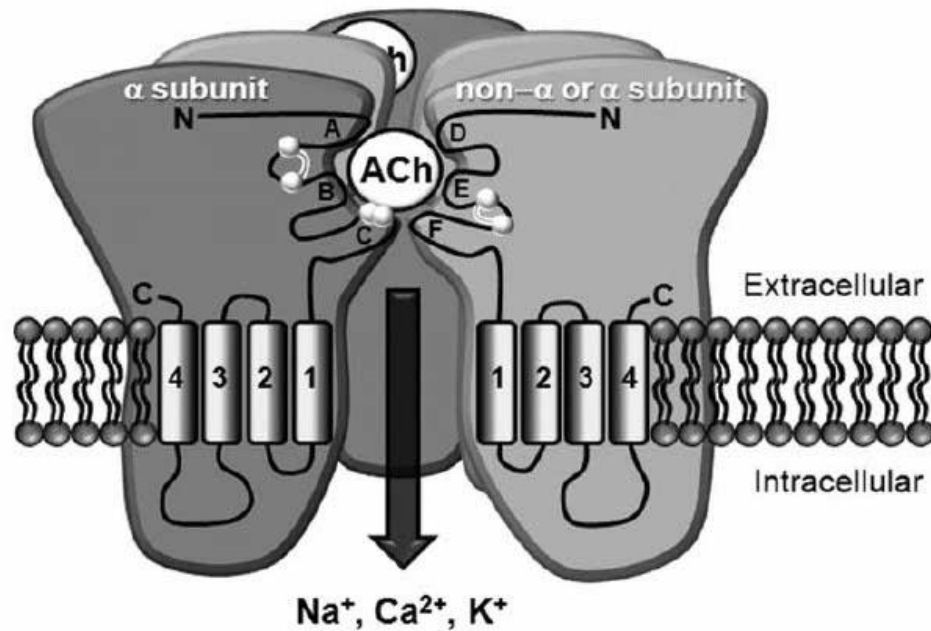
**Figure 1.4** A schematic representation of the arrangement of a heteromeric acetylcholine receptor consisting of two α (dark grey) and three non-α (light grey) subunits arranged around a central cation-permeable channel. Acetylcholine binding sites: ACh; Four transmembrane domains: 1-4; Six binding loops: A-F; Cys-loop: two white circles connected by a white double line (Jones and Sattelle 2010).

Analyses of genome sequences from various insect species such as the ferment fly *Drosophila melanogaster* Meigen (Adams et al. 2000), the malaria mosquito *Anopheles gambiae* Giles (Jones et al. 2005), the honeybee *Apis mellifera* Linnaeus (Jones et al. 2006), silk worm *Bombyx mori* (Linnaeus) (Shao et al. 2007) the rust-red flour beetle *Tribolium castaneum* (Herbst) (Jones and Sattelle 2007) and the pea aphid *A. pisum* (Liu et al. 2013), have revealed the number of nAChR genes in insects is relatively small (10-12 nAChR genes), compared to human (16) (Millar 2003) and the nematode *Caenorhabditis elegans* (Maupas) (29) (Jones et al. 2007). Using *D. melanogaster* as a genetic model, the nAChR gene family was shown to consist of ten subunits, seven of which are α-subunits (Dα1,

Dα2, Dα3, Dα4, Dα5, Dα6, Dα7) and three are non-α (Dβ1, Dβ2 and Dβ3) (Matsuda et al. 2009).

### 1.2.4 Selectivity of neonicotinoid insecticides towards insect nAChRs

Neonicotinoid insecticides, as briefly stated above, are classified based on possession of either a nitroimine, nitromethylene or cyanoimine group, each arising from a common pharmacophore. Possession of either group determines their selectivity for insect nAChRs over mammalian nAChRs and plays a fundamental role in their insecticidal potency (Matsuda et al. 2001, Tomizawa and Casida 2003, Matsuda et al. 2009). Neonicotinoids containing the negatively tipped nitro or cyano group are not ionized and interact with a subsite consisting of cationic amino acid residue(s) in the insect nAChR, while ionized nicotine or the nicotinoids bind at an anionic subsite in the mammalian nAChR (Tomizawa and Casida 2003). Debnath et al. (2003) performed a quantitative structure-activity relationship study using electro-topological state atom indices and demonstrated that nitroimines, nitromethylenes and cyanoimines are more selective to *Drosophila* nAChR, whereas N-substituted imines have affinity for mammalian receptors.

Since the completion of the *D. melanogaster* genome, various nAChR subunits have been implicated as imidacloprid action targets (Millar 2003, Tomizawa and Casida 2003). Functional expression of insect nAChRs using heterologous expression systems has remained elusive due to difficulties in expressing recombinant insect nAChRs (Millar 1999, Sivilotti et al. 2000). Despite this, several *D. melanogaster* nAChR α-subunits can form functional hybrid nAChRs when co-expressed with vertebrate neuronal β-subunits in heterologous expression systems such as African clawed frog *Xenopus laevis* Daudin oocytes (Ihara et al. 2003). For example, replacement of the α-subunit of chicken α4β2 nAChR by either the Dα1 or Dα2 subunit resulted in an increased sensitivity towards the chicken α4β2 receptor by imidacloprid. This illustrates that the α-subunit of *D. melanogaster* possesses structural features that support the selective interaction of neonicotinoid insecticides (Matsuda et al. 1998, Ihara et al. 2003). Furthermore, studies using heterologous expression systems to investigate the role of insect-specific β subunit

loops in neonicotinoid selectivity have elucidated various amino acid residues that confer increased imidacloprid potency (Liu et al. 2008, Yao et al. 2008, Toshima et al. 2009). Two insect-specific amino acid residues located in loop D, T77R/K/N and E79V/R increased neonicotinoid selectivity when introduced into the chicken β2 subunit of Dα2-β2 hybrid receptors (Shimomura et al. 2006). Similarly, Kramer et al. (2001) examined the effects of altering insect-specific loops D-F in hybrid nAChRs containing insect and mammalian subunits. Residues S131Y(R) and D133N in loop E and T191W and P192K in loop F were found to contribute to the neonicotinoid selectivity of insect-specific loops E and F.

## 1.3 Insecticide Resistance Mechanisms

Insecticide resistance is defined by the World Health Organization (WHO) as "the development of an ability in a strain of an organism to tolerate doses of a toxicant which would prove lethal to the majority of individuals in a normal (susceptible) population of the species" (WHO 1957). The evolution of insecticide resistant individuals relies on the presence of naturally occurring resistance genes within a population (Mallet 1989). In insects the genes controlling resistance mechanism(s) are thought present in very low ($10^{-2}$ to $10^{-6}$) frequencies prior to insecticide use (Crow 1957). When a population is sprayed with insecticide the rare resistant genes are favoured and the resistant genotype(s) increase (Roush and McKenzie 1987). This pre-adaptive theory is routinely demonstrated by the generation of resistant lines from laboratory susceptible strains by routine selection pressure over several generations. Insects develop resistance primarily through two major mechanisms: target site insensitivity and metabolic detoxification (Figure 1.5). Target site insensitivity refers to a structural modification in the gene(s) that renders the insecticide ineffective at the target site by changing the binding affinity (Plapp and Wang 1983, Scott 1990). On the other hand, detoxification is achieved by producing more gene copies (gene amplification) or increasing the amount of gene product made (altered gene expression) of gene(s) which detoxify naturally occurring toxins (Scott 1990). A lesser mechanism, penetration resistance, is frequently present alongside other mechanisms whereby it enhances their effectiveness (Raymond et al. 1989, Soderlund and Bloomquist 1990). As a

single mechanism, penetration resistance typically only confers very low resistance (Tabashnik 1989, Bingham et al. 2011).
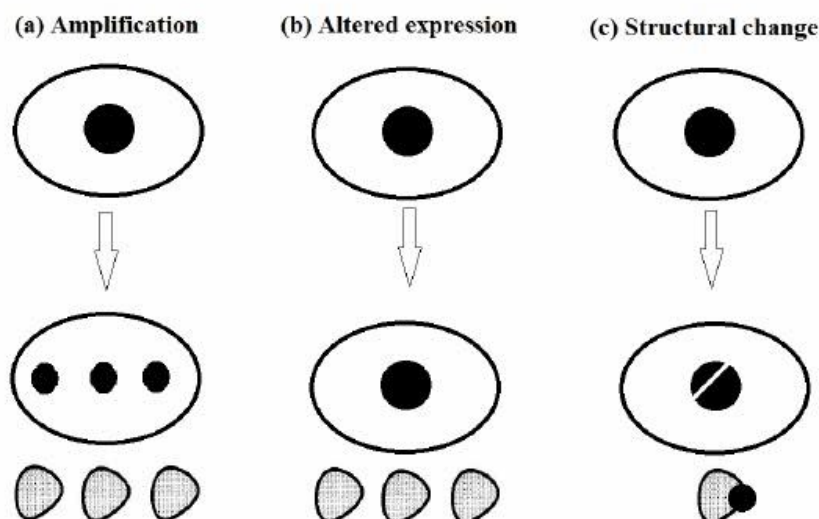
**Figure 1.5** Graphic representation of the types of genetic mutations which occur and cause resistance. (a) the gene is amplified to produce more copies of itself and thus increase the amount of gene product made (b) the regulatory expression of a gene is modified so that the amount of gene product made is increased (c) modification of the gene sequence produces a structurally different product (Scott 1995).

### 1.3.1 Target site insensitivity

Within the insect nervous system there exists many target sites for insecticides where genes can be altered to confer insensitivity (Narahashi 1996, ffrench–Constant et al. 1998). As outlined by Yu (2008), target site insensitivity may be divided into three separate categories: nerve insensitivity, altered AChE and reduction in midgut target site binding.

### 1.3.1.1 Nerve insensitivity

Well documented examples of nerve insensitivity acting as a primary mechanism of resistance in arthropod pest species include point mutations within the voltage gated sodium channel (VGSC), and receptors of nicotinic ACh, gamma-aminobutyric acid (GABA), and AChE (Scott 1990, Li et al. 2007, Thany 2010). Resistance to organochlorine (containing cyclodienes), pyrethroids, neonicotinoids and phenylpyrazole insecticides have been mediated through one of the above point mutations (Williamson et al. 1996, Martinez-Torres et al. 1999, Le Goff et al. 2005).

#### *1.3.1.1.1 Voltage gated sodium channel*

The most widely reported pyrethroid nerve insensitivity is *knockdown resistance* (*kdr*) where a coding sequence mutation in the VGSC gene(s) prevents the proper binding of pyrethroid insecticides (Kundu and Dixon 1995, Davies et al. 2008). This mutation was first documented in *M. domestica* (Williamson et al. 1996) but has since been identified in numerous insect species (Martinez-Torres et al. 1999, Hemingway et al. 2004, Marshall et al. 2012). In Australia, *A. gossypii* is not targeted directly by pyrethroid insecticides but has often received high selection pressure due to indiscriminate spraying against other cotton insect pests (Herron et al. 2001). As a result, the *kdr* mutation has been observed in *A. gossypii* clones collected off Australian cotton. Comparative sequence analysis of the domain II region of the VGSC of *M. domestica* with the orthologous region of pyrethroid resistant *A. gossypii* confirmed the presence of the corresponding *kdr* mutation (L1014F) in Australia (Marshall et al. 2012). Often a second mutation, termed super-*kdr* can exist in combination with *kdr* to provide very high level resistance. The super-*kdr* mutation is located within the intracellular domain II S4-S5 loop and results in a methionine to threonine replacement (M918T) (Kundu and Dixon 1995, Davies et al. 2008). In Northern Cameroon, an *A. gossypii* strain collected off cotton and found to be highly resistant (473-fold) to the pyrethroid cypermethrin was shown to possess both the *kdr* and super-*kdr* mutations (Carletto et al. 2010). In the cotton growing regions of Sudan, cases of *A.*

*gossypii* possessing the super-*kdr* mutation not in conjunction with *kdr* have been reported (Foster et al. 2007).

### 1.3.1.1.2 Receptors of nicotinic acetylcholine

The nAChR is the primary target site of group 4 MoA (nAChR agonists / antagonists), and group 5 MoA (nAChR agonists / antagonists, other than Group 4) insecticides (Sparks and Nauen 2015). In insects, genome analyses using *D. melanogaster* as a model organism have elucidated about 10-12 genes known to encode different subunits of the nAChR (α1-9 and β1-3) (Jones and Sattelle 2010). Mutations within these nAChR subunits have been identified and directly associated with resistance development in several sucking insect species including the green peach aphid *Myzus persicae* (Bass et al. 2011), *A. gossypii* (Shi et al. 2012, Koo et al. 2014), the brown planthopper *Nilaparvata lugens* (Stål) (Liu et al. 2005) *D. melanogaster* (Perry et al. 2008) the western flower thrips *Frankliniella occidentalis* (Pergande) (Puinean et al. 2013) and the diamond back moth *Plutella xylostella* (Linnaeus). In *M. persicae*, radioligand binding assays were employed to assess the interaction of imidacloprid to its binding site of the nAChR. Whole body membrane preparations of imidacloprid susceptible and neonicotinoid (imidacloprid and thiamethoxam) resistant clones (strain FRC) showed a much greater binding affinity of imidacloprid to nAChRs in the susceptible clones compared to FRC clones (Bass et al. 2011). It was found that the high affinity imidacloprid binding site was lost in FRC clones and the low affinity binding site was structurally different when compared to the susceptible clone (Bass et al. 2011). Comparative sequence analysis of the six known nAChR subunit genes of FRC and susceptible clones elucidated a point mutation (R81T) in the loop D region of the nAChR β1 subunit gene, present only in the FRC clone (Bass et al. 2011). Similarly, the R81T mutation has been reported in neonicotinoid resistant South Korean field strains of *A. gossypii* (Koo et al. 2014). Furthermore, in a highly imidacloprid-resistant strain from Korea, a second mutation in the β1 subunit, L80S, which was absent in the susceptible strain, was linked to resistance (Kim et al. 2015). This secondary mutation, in combination the R81T mutation may function as an additional RF in their strain (Kim et al. 2015).

In *N. lugens* a single point mutation (Y151S) located in a conserved position between two nAChR α subunits (Nlα1 and Nlα3) in loop B was demonstrated to be responsible for the reduced binding of imidacloprid at the target site in a resistant strain (Liu et al. 2005). In *D. melanogaster*, mutations in two nAChR subunit genes; Dα1 and Dβ2 produced through ethyl methanesulfonate (EMS) mutagenesis followed by selection with nitenpyram was found to confer resistance (Perry et al. 2008). Further study found the Dβ2 mutation to provided resistance to several neonicotinoids including: imidacloprid, acetamiprid, clothianidin, nitenpyram and dinotefuran, whilst the Dα1 mutation did not provide resistance to dinotefuran (Perry et al. 2012). In *P. xylostella*, a mutation in an intron splice junction of the nAChR α6 subunit was predicted to produce truncated proteins lacking important functional domains leading to insensitivity of nAChR to spinosad (Baxter et al. 2010, Rinkevich et al. 2010). In *F. occidentalis* from Spain, a point mutation (G275E) in the nAChR α6 subunit resulted in insensitivity to spinosad (Puinean et al. 2013), however, in China and the USA populations of *F. occidentalis* resistant to spinosad have been found lacking the G275E mutation (Hou et al. 2014). The widespread elucidation of target-site modifications of nAChR α and β subunits related to resistant phenotypes in insect species has established them as principal targets for resistance detection against neonicotinoid insecticides (Matsuda et al. 2001, Shimomura et al. 2006, Yao et al. 2008).

### 1.3.1.1.3 Receptors of gamma aminobutyric acid

Resistance to cyclodiene insecticides (*Rdl*) in several insect species is due to the same single mutation: a replacement of a single amino acid (alanine 302) in the chloride channel pore of the GABA-gated sodium channel subunit (Margaritopoulos et al. 2006). In *D. melanogaster*, besides directly affecting the binding site, replacement of alanine 302 also destabilizes the preferred confirmation of the receptor (ffrench–Constant et al. 1998, Le Goff et al. 2005). In *M. persicae*, the *Rdl* mutation has been identified in cyclodiene resistant clones. However, unlike other insect species, *M. persicae* possesses up to four different *Rdl* alleles, compared to the standard two (Anthony et al. 1998).

115 of 308

### 1.3.1.1.4 Receptors of altered acetylcholinesterase

Modifications in the gene encoding insect AChE are key determinants of organophosphate and carbamate resistance in insects including *D. melanogaster* (Mutero et al. 1994) and the aphid species *M. persicae* and *A. gossypii* (Andrews et al. 2004, Reddall et al. 2004). AChE is responsible for the hydrolysis of ACh and termination of synaptic transmissions in insects (Toutant 1989). Thus, it makes a primary target for organophosphates and carbamate insecticides which inhibit the action of AChE leading to repeated firing of electrical signals and eventual death (Gunning and Moores 2001). Resistance to the carbamate pirimicarb and to organophosphates generally is caused by two mutant forms of AChE known to confer resistance in *A. gossypii* (Moores et al. 1996, ffrench–Constant et al. 1998). The nomenclature of these two gene variants of AChE varies dependant on literature source but are all classified based on their divergence from the *Drosophila* gene *Ace* (Fournier 2005). The two gene variants of AChE, *o-Ace* (orthologous gene to *Drosophila* gene *Ace*) and *p-Ace* (paralogous gene to *Drosophila* gene *Ace*) possess two point mutations which conferred resistance to carbamates and organophosphates, although they vary in their specificity to each chemical class (Li and Han 2002, Toda et al. 2004). Firstly, an amino acid substitution in the coding sequence of *p-Ace,* Ser431Phe was found to be a primary determinant of pirimicarb resistance in Australian *A. gossypii* (McLoon and Herron 2009), that had previously been identified in pirimicarb resistant strains of *M. persicae* (Nabeshima et al. 2003). A secondary amino acid substitution in the coding sequence of *p-Ace*, Ala302Ser, also provides a less specific insensitivity to a wide range of carbamates and organophosphates (Benting and Nauen 2004, Li and Han 2004, Toda et al. 2004).

### 1.3.1.2 Reduction in midgut binding

Although this type of target site insensitivity is not widely reported, it is the most common resistance to *Bt* insecticidal proteins (Ferre and Van Rie 2002). Here resistance is generally conferred by point mutations in receptor molecules which lead to reduced crystal protein binding to the insect midgut brush border membrane (Heckel et al. 2007).

### 1.3.2 Metabolic detoxification

There are three main enzyme systems involved in metabolic detoxification. These are esterases (ESTs), glutathione-S-transferases (GSTs) and P450s (Feyereisen 1995, Scott 1995, Feyereisen 2005). In most cases but not all, metabolic resistance is characterized by an increased quantity of a metabolic detoxification enzyme (Scott 1995, Hemingway 2000). However, it can also be caused by a point mutation in the gene encoding the enzyme (Hemingway 2000).

### 1.3.2.1 Esterases

The carboxylesterase (CE) family from which ESTs belong to is an extremely versatile enzyme group characterized by an $\alpha$ / $\beta$ hydrolase fold in their three dimensional structures with a nucleophile-acid-histidine catalytic triad (Oakeshott et al. 2010). They use water to hydrolyse ester bonds to generate an acid and an alcohol as metabolites (Testa and Kramer 2007). The vast majority of insecticides used today contain ester bonds so are susceptible to hydrolysis by EST activity; this includes insecticides belonging to either the organophosphate, carbamate and pyrethroid chemical classes (Sogorb and Vilanova 2002, Russell et al. 2011). In most cases, hydrolysis of the ester group leads to a significant reduction in toxicity of the insecticide at the target site (Sogorb and Vilanova 2002).

EST-mediated metabolic resistance is generally divided into two separate mechanisms: those arising from gene amplification and; up-regulation of gene expression (Li et al. 2007). Enhanced sequestration and/or degradation of organophosphate, carbamate and pyrethroid insecticides via gene amplification have been implicated in resistance in the orders Hemiptera and Diptera (Field et al. 1999, Bass and Field 2011). In *M. persicae*, overproduction of E4 and FE4 CE genes through gene amplification is responsible for enhanced degradation and sequestration of specific organophosphate, carbamate and pyrethroid insecticides (Devonshire 1989). Similarly, in *Culex* spp. resistance to organophosphates is most commonly achieved via co-amplification of two types of EST coded at loci Est-3 (A esterase) and Est-2 (B esterase) (Guillemaud et al. 1997). Altered gene expression via up-regulation of CE genes has been repeatedly linked to resistance in

the orders Diptera, Hemiptera and Hymenoptera (Hemingway et al. 2004, Bass and Field 2011).

Additionally, mutations in CE gene-encoding domains have been attributed to resistance in the orders Diptera (Campbell et al. 1998), Hemiptera (Li and Han 2004, Russell et al. 2004), Lepidoptera and Coleoptera (Hotelier et al. 2010) (see Hotelier et al. (2010) for a comprehensive review) against a range of organophosphates and carbamates. For example, in the sheep blowfly, *Lucilia cuprina* Wiedmann, resistance to malathion is attributed to a point mutation, a tryptophan to leucine substitution (Trp251Leu) within the blowfly E3 EST gene (Campbell et al. 1997, Campbell et al. 1998). A second mutation, resulting in a glycine to aspartic acid substitution (Gly137Asp) in the same E3 gene causes a loss in CE activity and increase in phosphatase activity towards the organophosphate, diazinon (Newcomb et al. 1997, Campbell et al. 1998). Elevated expression of ESTs are commonly, associated with resistance to insecticides which contain ester bonds such as organophosphate, carbamate and pyrethroids (Montella et al. 2012). However, in an imidacloprid resistant strain of *A. gossypii* (R-imidacloprid), AChE and alpha-napthyl acetate (α-NA) ESTs were found to be higher in strain R-imidacloprid compared to the susceptible strain (Wang et al. 2002). Elevated ESTs have also been linked to decreased susceptibility to imidacloprid in a field strain of the Asian citrus psyllid *Diaphorina citri* Kuwayama, a serious worldwide pest of citrus (Tiwari et al. 2011, Tiwari et al. 2012).

### 1.3.2.2 Glutathione-S-Transferases

The GSTs are an important family of enzymes best known for their ability to catalyze the conjugation of the reduced form of glutathione to xenobiotic substrates for the purpose of detoxification (Mannervik and Danielson 1988). Elevated GST activity has been found in individuals displaying resistance to the organophosphate, organochlorine, DDT and pyrethroid chemical classes (Ranson and Hemingway 2005, Li et al. 2007). Resistance mechanisms mediated by GSTs include gene amplification and GST overexpression. In organophosphate resistant *M. domestica* and pyrethroid resistant *N. lugens*, resistance is attributed to overproduction of the GST genes *MdGSTD3* and *NlGSTD1* respectively, and

overproduction caused by gene amplification (Syvanen et al. 1996, Vontas et al. 2001, Vontas et al. 2002). Dehydrochlorination of DDT is catalysed by some insect GSTs, causing the elimination of chlorine to generate the non-insecticidal metabolite DDE (1,1-dichloro-2,2-bis-[pchlorophenyl]ethane) (Hemingway et al. 2004). Overexpression of GSTs associated with DDT hydrochlorinase activity (DDTase) has been linked to DDT resistance in the malaria carrying mosquitoes, *A. gambiae* (Ortelli et al. 2003) and *Aedes aegypti* (Linnaeus) (Lumjuan et al. 2005, Lumjuan et al. 2011). In the Cotton leafhopper *Amrasca bigutulla bigutulla* (Ishida) resistance to imidacloprid and acetamiprid was found to be associated with elevated GST levels (Kshirsagar et al. 2012).

### 1.3.2.3 Cytochrome P450-dependent monoxygenases

P450-mediated microsomal electron transport is responsible for oxidative metabolism of endogenous compounds, including fatty acids and steroids and exogenous compounds, including xenobiotics (Hodgson 1985). In insects P450s are involved in many processes including: (i) growth and development and (ii) metabolism of toxic chemicals synthesized by their host plants, and insecticides either by detoxification of substrates or activation of the molecule (Feyereisen 1999). Electron transport is mediated by a multicomponent monoxygenase system in which reducing equivalents from NADPH (Nicotinamide Adenine Dinucleotide Phosphate) are transferred to molecular oxygen (Wang et al. 1997). In its simplest form the monoxygenase system consists of the flavoprotein NADPH-cytochrome P450 reductase and the heme-thiolate protein cytochrome P450 (Guengerich 1996). The overall reaction of P450 mediated metabolism can be expressed as: $S + NADPH + H^+ + O2 \rightarrow SO + NADP^+ + H2O$, whereby the heme-protein in the oxidized form binds the cytochrome P450 substrate. The P450-substrate complex receives two electrons from NADPH via the reductase, used in the reduction of molecular oxygen to water with the co-oxidation of the substrate (Scott and Wen 2001). Depending on the form of P450 involved, cytochrome $b_5$ may be needed to donate the second electron from NADH to P450 (Porter 2002). The number of P450 variants is diverse and may include up to 60 different chemical reactions (Guengerich 2001). Among the variants, hydroxylation,

epoxidation, O-, N-, and S-dealkyation, N- and S-oxidations are the most important with respect to pesticide metabolism (Guengerich 2001).

Nearly all insecticide classes have been shown to express P450 mediated resistance effecting both mite and insect species (Scott and Wen 2001, Li et al. 2006). The exact change(s) behind resistance have been difficult to determine due to the complex nature of the P450 system and the overwhelming diversity of P450 isoforms within and among different species (Scott and Wen 2001, Feyereisen 2005, Wang et al. 2007). In most cases it appears that overexpression of one or more P450 genes are responsible for resistance. Despite these difficulties researchers have employed heterologous expression systems to show that resistance may be mediated by point mutations in the gene(s) encoding P450 enzymes (Amichot et al. 2004). For example, in a laboratory selected strain of *D. melanogaster* resistance to DDT was partially attributed to a point mutation in the P450 gene *CYP6A2* (Amichot et al. 2004). The most widely studied example of neonicotinoid resistance occurred via enhanced P450 detoxification in the Q-type Silverleaf whitefly *Bemisia tabaci* (Gennadius). Initially 1000-fold resistance to imidacloprid was first detected in Q-type *B. tabaci* from intensive horticulture in the Almeria region of Spain (Rauch and Nauen 2003, Nauen and Denholm 2005). Soon thereafter, a B-type *B. tabaci* originating from Israel was also found to possess 1000-fold resistance to imidacloprid (Rauch and Nauen 2003). At this time the use of the synergist PBO provided the first direct evidence of the role of P450s conferring resistance in Q-type *B. tabaci* (Nauen et al. 2002). Over expression of the single P450 gene *CYP6CM1* was later linked to imidacloprid resistance in both B and Q biotypes of *B. tabaci* (Karunker et al. 2008). Interaction studies of imidacloprid with the *CYP6CM1* mediated enzyme revealed hydroxylation at position 5 of the imidacloprid imidazolidine ring system (Karunker et al. 2009). Similarly in China, resistance to imidacloprid in field populations of *B. tabaci* was associated with increased expression of two P450 genes; *CYP6CM1*, previously correlated with imidacloprid resistant *B. tabaci* in Spain, and a newly associated gene *CYP4C64* (Yang et al. 2013).

In *M. persicae* over production of the P450 gene *CYP6CY3* has been linked to decreased susceptibility in aphid clones from varying locations including the United Kingdom and

120 of 308

Greece (Puinean et al. 2010, Bass et al. 2013). Quantitative real-time polymerase chain reaction (qRT-PCR) confirmed that enzyme over-expression in some *M. persicae* was caused by gene amplification with some clones having up to 100 copies of the *CYP6CY3* gene (Puinean et al. 2010).

In the house fly *M. domestica*, three P450 genes *CYP6A1*, *CYP6D1* and *CYP6D3* were found to be overexpressed in imidacloprid resistant strains (Markussen and Kristensen 2010) and similarly in the ferment fly *D. melanogaster*, over-transcription of the P450 gene *CYP6G1* conferred resistance to some neonicotinoid insecticides (Le Goff et al. 2003, Sparks et al. 2012). Further studies utilising the model substrate 7-Ethoxycoumarin O-deethylation confirmed that in resistant whiteflies, microsomal activity was enhanced (Rauch and Nauen 2003). In the Colorado potato beetle *Leptinotarsa decemlineata* Say, synergist studies using PBO reduced the RF of imidacloprid-resistant *L. decemlineata* from 309-fold to just over 100-fold (Mota-Sanchez et al. 2006), providing evidence of P450s conferring resistance. However, the 100-fold RF still persisting despite PBO use may suggest that other resistance mechanisms such as target site insensitivity are also involved (Mota-Sanchez et al. 2006).

There are few studies which have examined the role of P450s in conferring neonicotinoid resistance in *A. gossypii*. Comparative transcriptome analysis between thiamethoxam resistant (ThR) and susceptible (SS) *A. gossypii* by Pan et al. (2015) found a total of 620 significantly differentially expressed genes (DEGs) but no significant difference in the expression of P450 genes. In their study, the ThR strain was established from their SS strain by continuous pressuring with thiamethoxam at varying concentrations to produce a strain which exhibited <20-fold resistance to thiamethoxam when compared with the SS strain (Pan et al. 2015). To date, no transcriptome analyses for thiamethoxam-resistance adaptation in field collected strains of *A. gossypii* have been completed.

### 1.3.2.4 Penetration resistance

Modifications to the insect cuticle to prevent or reduce the penetration or adsorption of a toxin into an insect's body can occur in some resistant insects (Plapp and Hoyer 1968,

Plapp and Wang 1983). This form of resistance is frequently seen alongside other major resistance mechanisms such as target site insensitivity and/or metabolic detoxification. As a single resistance mechanism, reduced cuticular penetration is considered to confer only low levels of resistance. However, in combination it intensifies the effects of those other mechanisms, i.e. reduced cuticular penetration can give detoxifying enzymes more time to metabolize the insecticide before it reaches its target site (Plapp and Hoyer 1968, Raymond et al. 1989, Scott 1990). Examples of reduced cuticular penetration contributing to resistance in insect species include *M. domestica* (Hoyer and Plapp 1968, DeVries and Georghiou 1981), *P. xylostella* (Noppun et al. 1989), *Helicoverpa armigera* (Ahmad and McCaffery 1988), *M. persicae* (Puinean et al. 2010) and in some mosquitoes (Apperson and Georghiou 1975, Pan et al. 2009). Compared to other resistance mechanisms, notably target site insensitivity and metabolic detoxification, the molecular basis of penetration resistance is poorly understood (Pittendrigh et al. 2008). Although, in some insect species genes encoding cuticular proteins have been elucidated and linked to resistance. For example in the bed bug *Cimex lectularius* Linnaeus, Koganemaru et al. (2013) found that resistance was attributed, at least in part to, up-regulation of several transcripts encoding proteins involved in cuticle formation and structure. Laccase, an enzyme with p-diphenol oxidase activity, belongs to a group of proteins known as copper-containing oxidases (Kramer et al. 2001). In insects, laccase is believed to play an important role in insect cuticle sclerotization by oxidizing catechols in the cuticle to their corresponding quinines, which then catalyze protein cross-linking reactions (Kramer et al. 2001, Arakane et al. 2005). In the mosquito *Culex pipiens pallens* Linnaeus, a laccase 2 gene (CpLac2) was found to be significantly overexpressed in the fenvalerate-resistant strain than in the susceptible. This highlights the potential role of *CpLac2* in conferring resistance to fenvalerate via reinforcement of the cuticle and reduced penetration of insecticide (Matsuda et al. 2009). In an imidacloprid resistant strain of *M. persicae*, overexpression of a single P450 gene due to gene amplification was associated with resistance to imidacloprid (Puinean et al. 2010). In the same strain, overexpression of several cuticular protein genes, and penetration assays using radiolabelled insecticide indicated reduced cuticular penetration also contributed to the resistance (Puinean et al. 2010).

## 1.4 Techniques available for resistance detection

Laboratory diagnostic tests for resistance called bioassays are frequently employed to characterize susceptibility in target pests to insecticides and acaricides (Robertson and Preisler 1992). These tests are used initially to detect the phenotypic expression of resistance, but are limited in their ability to elucidate the causal mechanism(s) of resistance. The application of molecular genetics tools such as PCR (polymerase chain reaction) and DNA sequencing have provided a greater understanding of the genetic basis of resistance (ffrench-Constant et al. 1995). There are, however, several constraints in conventional DNA based methods to rapidly and cost effectively identify single candidate gene(s) involved in insecticide resistance when the resistance mechanism is not already known (ffrench-Constant et al. 1995). With the advent of next-generation sequencing techniques, an abundance of genes encoding likely receptors or enzymes involved in resistance can be obtained cost-effectively and in a timely manner (Mardis 2008, Pareek et al. 2011). Molecular genetics techniques will provide an extremely valuable adjunct to bioassay, but both are required for resistance management; bioassay to firstly detect resistance, and molecular genetics to characterize the causal mechanism.

### 1.4.1 Bioassay

Laboratory bioassay is utilized to detect and evaluate the phenotypic expression of resistance in arthropod pest species (Robertson et al. 2007). It refers to any quantitative procedure used to determine the dose-response relationship of an insecticide with its target organism (Busvine 1971, Finney 1971). There are various types of insecticide bioassays used to assess toxic effects on organisms, the most common include: topical applications (Spray tower e.g. Potter spray tower or hand held micro-applicator e.g. Hamilton); leaf-dip methods; and insecticide surface coating assays (leaf, paper, glass or plastic surfaces) (Kranthi 2005). Assessment of insecticide toxicity via bioassay requires initial generation of baseline susceptibility data to define the limits of tolerance within a population. This involves exposing a proven insecticide susceptible standard to serial dilutions of an insecticide (IRAC 1990). The proportion of individuals dying at each concentration is

recorded at a specific post-exposure interval and from this; the level of mortality at known insecticide concentrations can be calculated. Once this single baseline is established, the entire dataset can be subjected to log-dose probit analysis to derive $LC_{50}$ or $LC_{99.9}$ (the dose required to kill 99.9% of the tested population) estimates (Hoskins and Craig 1962). It is important to realize that numerical increase in the $LC_{50}$ estimate is not always consistent with a decline in insecticide efficacy in the field because label application rates are usually conservative enough to kill all but highly resistant individuals (Roush and Miller 1986).

A discriminating dose to distinguish between susceptible and resistant phenotypes for detection and monitoring of resistance can be obtained via the interpretation of the baseline susceptibility data (IRAC 1990). For the discriminating dose to be accurate, a wide range of field strains collected from various geographical regions are required so that population variability in response to the insecticide (i.e. tolerance) can be accounted for (ffrench-Constant and Roush 1990). Ideally, the discriminating dose should be set at a rate that will kill all susceptible insects in the population whilst sparing any resistant insects (ffrench-Constant and Roush 1990, Robertson and Preisler 1992). To calculate a robust discriminating dose, the $LC_{99.9}$ of the baseline susceptibility data is multiplied by a factor of two or three to precisely separate between high level vigour tolerance and low level resistance (Robertson and Preisler 1992). Compared to full dose responses, discriminating doses are useful indicators from a resistance management perspective as they are more efficient for detecting low frequencies of resistance because all individuals are tested at an appropriate dose with no wastage on lower doses (ffrench-Constant and Roush 1990). This is particularly important when resistance is first appearing in the population. To compare between two strains, the $LC_{50}$ of the resistant population may be divided by the $LC_{50}$ of the susceptible population to calculate a RF (Robertson and Preisler 1992, Robertson et al. 2007). A relative potency comparison may only be made if the regression lines of the susceptible and resistant strains are parallel; indicating that genetic variability is absent. As this is generally not the case, a method which includes the $LC_x$ and slope data of both the populations being compared was proposed by Robertson and Preisler (1992). In this way, confidence limits for the ratio may be calculated from the estimates of the intercepts ($\alpha$, $i =$

1, 2) and the slopes ($\varepsilon$, i = 1, 2) of two i i probit (or logit) lines and their variance-covariance matrices (Robertson et al. 2007).

## 1.4.2 Bioassay with synergist

Bioassay involving co-application of the insecticide with a chosen synergist can quickly and cheaply provide a convenient method for investigating potential metabolic resistance in insects (Raffa and Priester 1985). Comparison of the synergised and non-synergised insecticide result is used as an indicator of the synergist interacting with the insecticide being studied (Scott 1990). Synergists are available for the following metabolic detoxification enzymes: ESTs, oxidases and glutathione-S-transferases. The most commonly used synergists are those that cause specific inhibition of certain metabolic-detoxification enzymes so that insecticide detoxification (resistance) in the target insect pest is significantly reduced or removed (Zhu 2008). The insecticide synergist, PBO, has been classified as a potent inhibitor of cytochrome P450-dependent monoxygenases (P450) (Sun and Johnson 1960), one of the largest gene families involved in metabolic detoxification. However, it does not exclusively synergise P450s as it has recently been shown to effectively synergise resistance-associated ESTs linked to pirimicarb (Bingham et al. 2008). Other synergists, including DEF (*S,S,S*-tributyl phosphorotrithioate), sesamex and TPP have been found to inhibit various ESTs associated with resistance to organophosphate, carbamate and synthetic pyrethroid insecticides (Hemingway and Georghiou 1984, Bingham et al. 2008). To adequately attribute resistance to an insecticide detoxifying enzyme, the inclusion of both positive and negative data by different synergists is often required (Raffa and Priester 1985). Moreover, factors such as metabolism of the synergist and differential penetration rates between synergist and insecticide could prevent detection (Raffa and Priester 1985, Scott 1990). For this reason, once an insecticide-synergist combination produces a link to a specific detoxification mechanism, further biochemical or DNA based assays should be employed for confirmation (Scott 1990).

### 1.4.3 DNA sequencing

DNA sequencing is a fundamental component of many insect molecular genetic projects (ffrench-Constant et al. 1995). Simply, DNA sequencing is the process of determining the precise nucleotide sequence within a molecule of DNA. Once the nucleotide sequence is derived, it can be confirmed via sequence analysis to a known protein product of the same origin (Koonin and Galperin 2003). Alternatively, if the sequence is not known, it can be compared to sequences of known genes to elucidate its function (Koonin and Galperin 2003). There are two basic methods which exist for manual DNA sequencing: (1) Maxam-Gilbert sequencing (Maxam and Gilbert 1977) and (2) Chain-termination (also known as Sanger sequencing) (Sanger and Coulson 1975). The most commonly used manual sequencing method is the Sanger method developed in 1977 by Frederick Sanger (Sanger et al. 1977). To synthesize DNA in this manner, a single stranded DNA template, a DNA primer, a DNA polymerase, deoxynucleosidetriphosphates (dNTPs), and modified di-deoxynucleotidetriphosphates (ddNTPs) are required (Sanger and Coulson 1975). DNA strand elongation is terminated at the position where chain-terminating nucleotides, ddNTPs, are incorporated into the DNA chain instead of dNTPs. ddNTPs lack a 3'-OH group essential for polymerase-mediated strand elongation (Sanger et al. 1977). Traditionally, four separate sequencing reactions were required to test all four ddNTPs (ddATP, ddGTP, ddCTP, or ddTTP). Nowadays, dye-terminator sequencing, whereby ddNTPs are radioactively or fluorescently labelled has enabled sequencing to be performed in a single reaction (Smith et al. 1986). Importantly, terminating ddNTPs create a selection of DNA fragments of differing size which can then be separated using conventional agarose gel electrophoresis (Smith et al. 1986). Dyes such as ethidium bromide, SYBR-Green or Gel-Red which bind to DNA are incorporated into the agarose gel so that DNA fragments can be visualized as bands (Yilmaz et al. 2012). The introduction of capillary electrophoresis, which is essentially built on the principles of slab gel electrophoresis resulted in a more efficient process with greater throughput for the separation and analysis of both large and small molecules (Ettre and Guttman 1996).

Applications of the Sanger method include: de novo sequencing whereby the primary genetic sequence of the target organism can be obtained (Chimpanzee Sequencing and Analysis Consortium 2005); targeted sequencing to identify heterozygous point mutations or polymorphisms in genomic DNA (ffrench-Constant et al. 1995, Llaca 2012); validation of mutations in next-generation sequencing output (Llaca 2012); and in gene expression analysis using RNA based assay methods (Velculescu et al. 1995, Mitani et al. 2006).

### 1.4.4 Next generation sequencing

There are several different methods available for next generation sequencing (NGS), but the most popular and widely used is the Illumina platform (Cacho et al. 2015). The concept behind NGS is similar to capillary electrophoresis sequencing but extends the process to perform massive parallel sequencing, whereby millions of small fragments of DNA from a single sample can be sequenced at the same time (Grada and Weinbrecht 2013). For ease, Illumina sequencing technology (IST) can be divided into three main components: 'cluster generation', 'sequencing by synthesis' and 'data analysis' (Bennett 2004, Bennett et al. 2005, Metzker 2010). In cluster generation, IST utilizes a unique solid phase 'bridged' amplification reaction that allows hybridized DNA to form clonal clusters with immediately adjacent primers (Illumina 2010). Firstly, the NGS library is prepared by fragmenting the DNA to be sequenced into about 200 base strands (Bennett et al. 2005). Specialized adapters are ligated onto the ends of DNA fragments and one of these adapters is hybridized on a proprietary flow cell surface (Adessi et al. 2000). With the addition of unlabelled nucleotides and isothermal enzymes, double stranded 'bridges' are formed on the solid-phase substrate (Adessi et al. 2000). Denaturing of the double stranded molecules forms single stranded templates which remain anchored to the substrate (Illumina 2010). Clusters containing up to 1000 identical copies of each single template molecule are then 'sequenced by synthesis' in parallel with four novel fluorescently labelled reversible terminator molecules (Bentley et al. 2008). During each sequencing cycle, a fluorescently labelled reversible terminator is imaged as each 3'-blocked dNTP is added, and then cleaved to allow incorporation of the next base (Bentley et al. 2008). After incorporation,

the fluorescent label is detected using imaging technology and the first base is identified (Illumina 2010). The sequencing cycle is repeated until the precise order of bases in a fragment is determined. During 'data analysis', the newly identified sequence reads are aligned to a reference genome, or *de novo* aligned, where a reference genome isn't available (Grada and Weinbrecht 2013). Compared to traditional Sanger sequencing, NGS offers dramatic increases in cost effective sequence throughput by reducing time and labour inputs (Mardis 2008) although often at the expense of shorter read length (Bentley 2006). For example, the cost of the human genome project in 2004 via Sanger sequencing was $3 billion USD and took 13 years to complete. In contrast, NGS would enable over 45 human genomes to be sequenced in a day for as little as $1000 each (Pareek et al. 2011).

### 1.4.4.1 Applications of *NGS*

Whole genome or targeted resequencing when a reference genome is already available can be used to better understand the genetic basis of phenotypic differences between organisms (Ng and Kirkness 2010). For example, sequence variations such as single nucleotide polymorphisms (SNPs), small indels, copy number variations (CNV) or other structural variants can be detected within individual genomes. Whole genome assembly without a reference genome has greatly facilitated genome sequencing of prokaryotic (Reinhardt et al. 2009) and eukaryotic organisms (Diguistini et al. 2009). In *de novo* assembly, short reads are assembled using assembly software programs to create full-length sequences without a reference genome (Ng and Kirkness 2010).

Additionally, RNA-Seq is able to be performed via NGS platforms such as the Illumina (Nagalakshmi et al. 2010). Compared to traditional Sanger sequencing, microarray or qRT-PCR based methods, these platforms offer unprecedented throughput, cost-effectiveness and sensitivity (Mardis 2008, Wang et al. 2009). Firstly, RNA is converted to a library of cDNA fragments and sequencing adaptors are added to one or both ends of each molecule (Nagalakshmi et al. 2010). Using the Illumina platform, a short sequence is obtained from each cDNA molecule and the resulting reads are either aligned to a reference genome or transcriptome, or where a genomic sequence is unknown, assembled *de novo* (Nagalakshmi

et al. 2010). A transcriptome contains all the genes which have been transcribed from the genomic DNA and converted into mRNA. Therefore, using RNA-Seq, it is possible to elucidate the functional elements of the genome that relate to a specific physiological condition and quantify its expression (Wang et al. 2009). The methodology can be used to study the response of insects to insecticides with comparison of susceptible and resistant insect transcriptomes to identify differential gene expression so giving an indicator to which genes may be contributing to insecticide resistance (Liu et al. 2011, Niu et al. 2012, Zhang et al. 2012). Following sequencing, the resulting reads are either aligned to a reference genome or reference transcripts, or assembled *de novo* without the genomic sequence to produce a genome-scale transcription map that consists of both the transcriptional structure and/or level of expression for each gene (Wang et al. 2009).

# Aims, objectives and thesis format

Research to restore neonicotinoid efficacy against *A. gossypii* was seen as an industry priority as part of an integrated approach to better manage mites and mirids in Australian cotton. To achieve this, the main aim of this project was to develop a greater understanding of neonicotinoid resistance in *A. gossypii* including characterization of its genetic basis. Knowledge of the causal mechanism will simultaneously uncover any underlying cross-resistance implications necessary for effective resistance management. Moreover, the development of a molecular based diagnostic will provide rapid detection of resistant insects and complement current bioassay methodology. Furthermore, research reported in this thesis may be interpreted to yield practical field based management outputs and outcomes for ongoing resistance management of *A. gossypii* in Australian cotton. This will be demonstrated in a series of experimental thesis chapters each consisting of an abstract, introduction, materials and methods, results and discussion.

Chapter 2 was designed to accurately quantify the level of neonicotinoid resistance present in three field collected strains of *A. gossypii* used in this study. Chapter 3 correlates the phenotypic expression of resistance observed in Chapter 2 to potential field control failures via a glasshouse based efficacy trial.

In Chapter 4 I investigate if the target site insensitivity mutation R81T known to cause neonicotinoid resistance in *A. gossypii* from China and Korea (Shi et al. 2012, Koo et al. 2014), and *M. persicae* from Europe (Bass et al. 2011) is responsible for resistance in Australian populations. In the absence of an R81T link, the role of metabolic detoxification is investigated using the monoxygenase inhibitor PBO.

In Chapter 5, Illumina Hi-Seq NGS technology was used to provide high quality gene expression and transcriptome analysis data between a reference susceptible and three thiamethoxam resistant *A. gossypii* strains characterized in Chapter 2. This was initiated to identify and characterize genes encoding detoxification enzymes and insecticide target proteins. Differentially expressed genes between susceptible and resistant strains are

investigated as potential candidates of thiamethoxam resistance. Data presented here can then be used by me or other researchers to further elucidate the genetic basis underlying thiamethoxam resistance in *A. gossypii*.

# Chapter 2. Characterisation and maintenance of three thiamethoxam resistant strains of the cotton aphid *Aphis gossypii* for use in subsequent experimental chapters

## 2.1 Abstract

In the 2007-08 growing season, resistance to the neonicotinoid chemical class was detected for the first time in *A. gossypii* collected off Australian cotton. To detect any changes in the magnitude of neonicotinoid resistance since its initial detection, $LC_{50}$ level RFs against the neonicotinoid thiamethoxam were calculated for three field strains of *A. gossypii* (F 101, Glen twn S and Carr collected during 2011-12). Whilst in laboratory culture, strains were routinely pressured to prevent reversion of resistant phenotypes. Cross resistance profiles to three other major insecticide classes was evaluated using previously established PCR and restriction fragment length polymorphism (RFLP) assays. Against thiamethoxam, discriminating dose assays revealed mortality rates of 47, 67, and 82.5% for strains F 101, Glen twn S and Carr, respectively. Subsequent full log dose probit analysis confirmed $LC_{50}$ level resistance of 49- (65.29-110.66), 51- (30.55-86.19) and 85- (65.29-110.66) fold for strains F 101, Glen twn S and Carr, respectively. No cross resistance between major insecticide classes were detected. Despite routine pressuring, RFs of each strain were reduced to <20-fold whilst strains were maintained in laboratory culture (over a three year period). Despite reversion, heterogeneous populations in this study would still likely lead to control failures if sprayed.

## 2.2 Introduction

*A. gossypii* is a highly polyphagous insect pest of cotton and cucurbits, both in Australia and worldwide (Blackman and Eastop 2000). In cotton, it causes damage via direct feeding and indirectly through the transmission of several debilitating plant viruses (CABI 2005) including CBT (Reddall et al. 2004) and CLR (Corrêa et al. 2005). The excretion of aphid honeydew, a sugary waste by-product of aphid feeding poses a major threat to the quality of

cotton lint produced (Miller et al. 1994). Late season honeydew contamination of the open boll lint causes 'sticky cotton' that leads to problems during spinning as fibres stick to machinery, necessitating shutdown and cleaning (Schepers 1989, Hequet et al. 2000). Historically, *A. gossypii* has rapidly developed resistance to insecticides soon after they are released for commercial use (Devonshire 1989), that is attributed to their high reproductive potential and viviparous parthenogenesis (Wellings et al. 1980). Resistant individuals, once selected by insecticide, produce parthenogenetic clones, with no resistance dilution which would otherwise occur by out-crossing with susceptible insects (Wool and Hales 1997). Consequently, proliferation of insecticide-resistant clones can result in very rapid changes in resistance levels in agricultural systems (Devonshire 1989).

Neonicotinoid insecticides, including imidacloprid (Elbert et al. 1991), thiamethoxam (Maienfisch et al. 2001), clothianidin (Ohkawara et al. 2002) and acetamiprid (Yamada et al. 1999), have become the fastest growing insecticide class since the synthetic pyrethroids (Jeschke and Nauen 2008). These compounds target nAChRs in the insect central nervous system causing paralysis and eventual death (Matsuda et al. 2001). Thiamethoxam and clothianidin are highly effective against a range of chewing and sucking insect pests (Elbert et al. 2008); however, reports of resistance to these insecticides in field populations of *A. gossypii* (Herron and Wilson 2011), and cross resistance between members of the 4A MoA insecticides (Wang et al. 2007, Shi et al. 2011), poses a major risk to the effective life of these insecticides in cotton in Australia. To this end, this study aimed to further characterize the phenotypic expression of neonicotinoid resistance in Australian *A. gossypii* and to investigate any differences in resistance levels already established for *A. gossypii*.

## 2.3 Methods and Materials

### 2.3.1 Collection and maintenance of strains

A reference laboratory susceptible strain (Sus SB) collected from an unsprayed source was maintained under insecticide-free conditions and its susceptibility to several chemicals has been documented (Herron *et al*. 2001). Field strains F 101 and Glen twn S were collected

during the 2010-11 growing season off cotton from St. George (QLD) and Toobeah (QLD), respectively (Figure 2.1). In 2012, a third field strain termed Carr was collected off cotton in Moree (NSW) (Figure 2.1). Aphids were forwarded to the Elizabeth Macarthur Agricultural Institute (EMAI) where they were reared as discrete strains in separate insect proof cages on pesticide free cotton *Gossypium hirsutum* Linnaeus (variety Sicot 71BRF) at $25 \pm 4°C$ and under natural light (Herron et al. 2001). Strains were screened for resistance to the neonicotinoid compounds, thiamethoxam 250 g/kg (Actara[®]) and clothianidin 200 g/L (Shield[®]).
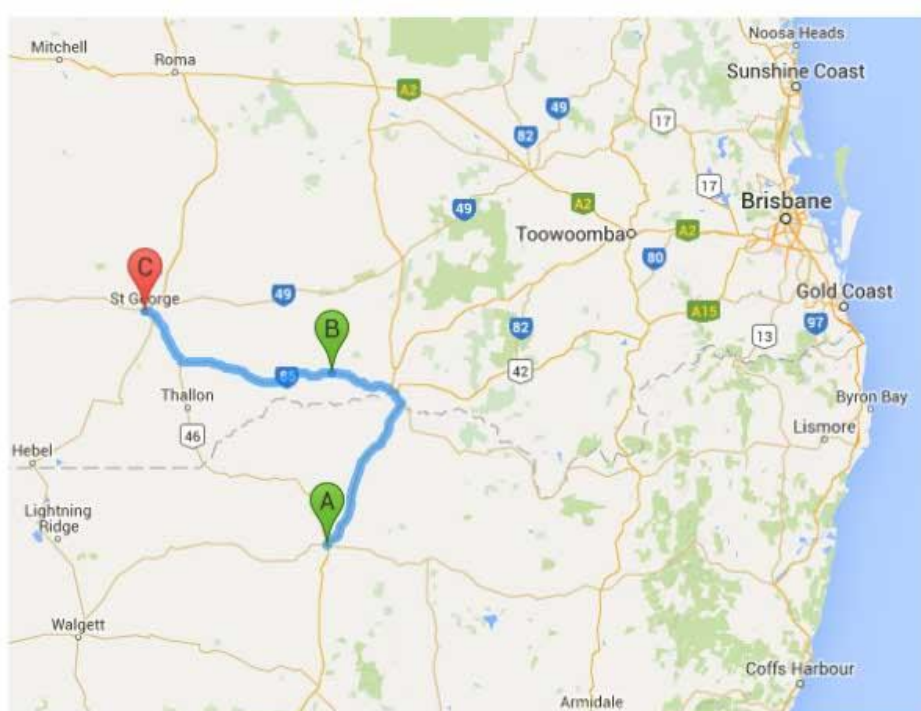


**Figure 2.1** Location of aphid collections: A, Moree; B, Toobeah; C, St George.

## 2.3.2 Plant germination and strain culturing

Weekly, around 30 seeds of pesticide free *G. hirsutum* were planted into a plastic pot (11.5cm diameter) (one pot per strain) filled with NativeMix™ premium potting mix and transferred into a 15×120×60cm tray maintained in a growth room at $28 \pm 2°C$. The tray was filled with enough water to last until the following week, when plants were removed for use in culturing and new plants potted to replace them. This process was repeated weekly whilst strains remained in culture within the insectary. Strains were cultured individually by picking at random 30-40 leaves from the old plant and placing them onto the newly grown plant (one week old). The old plant was subsequently discarded.

## 2.3.3 Discriminating dose tests

Discriminating dose assays were performed via a precision Potter spray tower (Burkhard Scientific, Uxbridge, Middlesex, UK) according to the method described by Herron et al. (2000). Briefly, 30 apterous adult female *A. gossypii* were transferred onto an excised cotton leaf disc set in agar within a small petri dish. Each test (replicate) included three insecticide sprayed batches (discs) and a water only sprayed control. Each petri dish was then sprayed with a single rate of insecticide [0.05 g active ingredient (a.i.)/L clothianidin (200 g/L Shield®) or 0.02 g a.i./L thiamethoxam (250 g/L Actara®)] via the Potter spray tower producing an aqueous deposit of $1.6 \pm 0.007$ mg/cm$^2$. Once sprayed, the dishes were covered in taut plastic cling wrap with tiny (smaller than an aphid) perforations made to prevent condensation. The number of aphids present on each leaf disc was counted and dishes transferred to an incubator maintained at $25°C \pm 0.1°C$ under a light regime of 16:8 L:D for 24 h. Mortality was assessed, with the aid of a stereo microscope, by counting the number of live aphids on the leaf disc and subtracting the number of survivors at test completion. Each test was replicated three times on different days with freshly made solutions (i.e. sequential). The chemical (i.e. thiamethoxam or clothianidin) which produced the highest proportion of resistant individuals was further subject to full log-dose probit analysis.

## 2.3.4 Pressuring

Each resistant strain was routinely pressured (every 8-12 weeks) whilst they were maintained in culture to prevent reversion to the susceptible phenotype. Pressuring required a potted insecticide free *G. hirsutum* plant to be placed into a fume cupboard where it was insecticide sprayed to run off. Using this method, strains F 101 and Glen twn S were exposed to 0.05 g a.i./L of thiamethoxam and strain Carr to 0.1 g a.i./L of thiamethoxam. Once the sprayed plant had dried it was transferred into a cage of the correct chemical / strain combination so that *A. gossypii* could infest it (as was done with routine culturing above). This was achieved by picking at random 30-40 leaves from the old plant and placing them onto the newly sprayed plant. Importantly, when a newly sprayed plant was placed into a cage the old plant was immediately removed so there was no unsprayed harbourage for susceptible aphids.

## 2.3.5 Full log-dose probit tests

Serial concentrations of formulated thiamethoxam selected to achieve $0 < x < 100\%$ were sprayed, using the methods outlined above, to yield full log-dose probit regressions from which RFs could be calculated. Each full log-dose probit regression was replicated three to four times and included a water only sprayed control that was <10% mortality. Results were analysed by probit analysis (Finney 1971) and regressions calculated after correction for control mortality (Abbott 1925). Probit analyses were run using a standalone probit program developed by Barchia (2001) that accounts for variability between replicates. This was achieved by using a $\chi^2$ test and if significant at the 5% level, the variance of the estimated parameter was scaled by the corresponding heterogeneity factor equal to the residual mean deviance. RFs were calculated by dividing the $LC_{50}$ of the resistant strain by the $LC_{50}$ of a reference susceptible strain. The corresponding 95% confidence interval of the calculated $LC_{50}$ ratio was used to determine significance (Robertson et al. 2007).

### 2.3.6 PCR screening of two known mutations: S431F, associated with pirimicarb (carbamate) resistance; and L1014F, associated with pyrethroid resistance

### 2.3.6.1 DNA extraction

Single aphids (n=20) were placed into individually labelled 1.5 mL micro centrifuge tubes containing 80 µL of 5% Chelex-100 resin (Bio-Rad Laboratories, Gladesville, NSW) and ground with a sterile micro pestle. The homogenate was incubated at 56°C for 30 min, followed by 100°C for 5 min. The crude DNA sample was used for PCR (2 µL or 4 µL) or stored at −20°C for later use.

### 2.3.6.2 PCR amplification of L1014F mutation site

Pyrethroid resistance was detected using established methods outlined by Marshall et al. (2012). Each DNA extract was subject to PCR amplification of the L1014F mutation site (*kdr*) within the *para*-type VGSC gene. PCR was conducted in a reaction volume of 50 µL consisting of dNTP's (0.2 mM), primers KDR_DPI1 Forward (TCTTGGCCCACACTTAATCTTT) (0.4 mM) and KDR_DPI4 Reverse (CTCGCCGTTTGCATCTTATT) (0.4 mM) (Table B.1), and Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany; 1 U) in a 10× buffer supplied by the manufacturer and 4 µL crude template DNA. Cycling parameters included an initial 2 min denaturation at 94°C followed by 35 cycles of 94°C for 30 s, 48°C for 1 min, 72°C for 1 min 30 s, and 5 min at 72°C. A *Bst*EII (Genesearch, Australia) restriction enzyme digest was performed by incubating 10 µL of PCR product at 60°C overnight with 1 U of enzyme and the manufacturers supplied buffer in a total reaction volume of 30 µL. [A susceptible individual will generate a single intense band at 325 bp (cut by *Bst*EII), whilst a heterozygous-resistant individual will generate two bands (uncut by *Bst*EII); one for the wildtype susceptible allele (325 bp) and one for the *kdr* allele (410 bp)].

### 2.3.6.3 PCR amplification of the S431F mutation site

Pirimicarb resistance was detected via established methods of McLoon and Herron (2009). Each DNA extraction was subject to PCR amplification of the *Ace1* gene (covering the mutation site (S341F) responsible for resistance) in a final reaction volume of 25 µL consisting of 12.5 µL of iQ™ Supermix (2×) (Bio-Rad Laboratories, Australia), primers AceF (CAAGCCATCATGGAATCAGG) (1 µM) and AceR (TCATCACCATGCATCACACC) (1 µM) (Table B.1), and 2 µL crude template DNA. Cycling parameters included an initial denaturation for 10 min at 95°C followed by 35 cycles of 95°C for 30 s, 53°C for 30 s, 72°C for 1 min 30 s, and 5 min at 72°C. An *Ssp*I (Genesearch, Australia) restriction enzyme digest was performed by incubating 25 µL of PCR product at 37°C overnight with 5 U of enzyme and the manufacturers supplied buffer in a total reaction volume of 35 µL. [A susceptible individual will generate two DNA fragments of the same size (331 bp and 336 bp) which co-migrate on a 2% agarose gel and present as a single intense band half the size of the undigested PCR product (667 bp, pirimicarb resistant). (Note that the *Ssp*I restriction enzyme assay detects resistance to pirimicarb, which would normally also give cross resistance to dimethoate and omethoate (two organophosphate insecticides)].

### 2.3.6.4 Visualisation of PCR products

Agarose gel electrophoresis was performed to visualise the amplified product. Prepared agarose gels, 2% (w/v) molecular-grade agarose (Bio-Rad Laboratories, Australia) in 0.5× Tris-borate ethlenediamine tetraacetic acid buffer (TBE buffer; Bio-Rad Laboratories, Australia) and containing 5 µL of Gel-Red (Jomar Diagnostics, Australia), were transferred to a Bio-Rad Wide MiniSub electrophoresis chamber (Bio-Rad Laboratories, Australia) and overlaid with 0.5× TBE buffer. DNA samples containing 5 µL of loading dye (in the ratio 1 part loading dye to 5 parts DNA sample) were loaded into gel wells and electrophoresed, alongside 3 µL DNA marker (100 bp DNA Ladder; Genesearch, Australia) at a constant 94V for 90 min using a Thermo EC Apparatus (EC250-90 HV) dual mode electrophoresis power supply. DNA was visualised under UV light using a Gel Doc 1000 fluorescent

imaging system (Bio-Rad Laboratories, Australia) and Quantity One software (Bio-Rad Laboratories, Australia).

## 2.4 Results

### 2.4.1 Discriminating dose tests

Initial discriminating dose data showed both thiamethoxam and clothianidin survivors, with thiamethoxam producing the highest proportion of resistant individuals (Table 2.1). Strain F 101 contained the lowest frequency of thiamethoxam susceptible individuals (47%) whilst strain Carr contained the highest frequency of susceptible individuals (82.5%) (Table 2.1). Against clothianidin, strain F 101 also contained the lowest proportion of susceptible individuals (67%), whilst strains Carr and Glen twn S contained 92 and 96%, respectively. Discriminating dose tests with thiamethoxam at pressuring rates of 0.05 g a.i./L for strains F 101 and Glen twn S and 0.1 g a.i./L for strains Carr, confirmed that resistant phenotypes in each strain were maintained (Table A.1). Routine pressuring of each strain over a three year period prevented strain reversion to a susceptible phenotype (Table A.1).

### 2.4.2 PCR restriction enzyme assays

For all four strains tested the *Bst*EII restriction enzyme assay of *kdr* PCR product in each strain resulted in a single intense band at 325 bp, coding for the wild type susceptible allele (Table 2.1). *Ssp*I restriction enzyme assay of *Ace*1 PCR products produced a single intense band at 336 bp (cut by *Ssp*1) in each strain indicating pirimicarb susceptibility.

**Table 2.1** Resistance detection (percent susceptible) in *Aphis gossypii* strains Sus SB, F 101, Glen twn S and Carr using bioassay [Thia (thiamethoxam) and Clo (clothianidin)] and molecular [Pir (pirimicarb) and Pyr (pyrethroid)] based methodology.

| Strain | Pir<br>S431F | Pyr<br>L1014F | Thia<br>0.02%* | Clo<br>0.05%* |
|---|---|---|---|---|
| Sus SB | 100% | 100% | 100% | 100% |
| F 101 | 100% | 100% | 47% | 67% |
| Glen twn S | 100% | 100% | 67% | 96% |
| Carr | 100% | 100% | 82.5% | 92% |

* Dose sprayed in g a.i./L; results control corrected according to Abbott (1925)

### 2.4.3 Full log-dose probit tests

For strains F 101, Glen twn S and Carr full log-dose probit analysis yielded RFs of 49.20- (35.43-68.33), 51.31- (30.55-86.19) and 85.00- (65.29-110.66) fold against thiamethoxam respectively when initially field collected (Figure. 2.2-2.4). As indicated by overlapping 95% confidence intervals at the $LC_{50}$ level no significant differences between strain responses were observed. Strains F 101, Glen twn S and Carr all showed significant heterogeneity (P<0.05) (as indicated by $\chi^2$ values of 33.01, 91.63 and 49.59, respectively) and so were not a good fit to the probit model with excessive heterogeneity accounted for by a scaled fiducial limit calculation (Figure. 2.2-2.4). Pressured strains (denoted by $_P$), F 101$_P$ and Glen twn S$_P$ and were also not a good fit to the model (P<0.05) (indicated by $\chi^2$ values of 41.34 and 35.16, respectively). Regression slope values for strains F 101 (1.59), Glen twn S (1.18) and Carr (2.18) (Figure. 2.2-2.4) were less than that of Sus SB which had the highest slope value recorded at 2.40 (Table 2.2). After routine pressuring regression slope values for strains F 101$_P$, Glen twn S$_P$ and Carr$_P$ were recorded as 1.27, 1.07 and 1.16, respectively (Figure. 2.2-2.4). Calculated $LC_{50}$ values ranged from a low of 0.0019 to a high of 0.0033 g/L in strains F 101 and Carr respectively. Interestingly, strain F 101 with the highest median effective concentration (MEC) to kill all insects tested (0.80 g/L) recorded

the lowest calculated $LC_{50}$ value of 0.0019 g/L (Figure 2.4). Resistance to thiamethoxam decreased to 7.73- (4.82-12.40), 14.25- (8.47-23.98) and 14.56- (10.45-20.30) fold for strains F $101_P$, Glen twn $S_P$ and $Carr_P$, respectively over a three year period (Figure. 2.2-2.4). $LC_{50}$ values of non-pressured strains (F 101, Glen twn S and Carr) when compared to pressured strains (F $101_P$, Glen twn $S_P$ and $Carr_P$) were significantly different as indicated by non-overlapping confidence intervals of the $LC_{50}$ ratio (Figure. 2.2-2.4).

**Table 2.2** Full log dose response data for the reference susceptible *Aphis gossypii* strain Sus SB against formulated thiamethoxam (Actara® 250 g/kg).

| Strain | Chi-square (df) | Slope (se) | LC50*(95% FL) |
|---|---|---|---|
| Susceptible SB | 18.83 (13) | 2.4 (±0.24) | 0.000038 (0.000031-0.000046) |

* g a.i./L; FL, fiducial limit; se, standard error
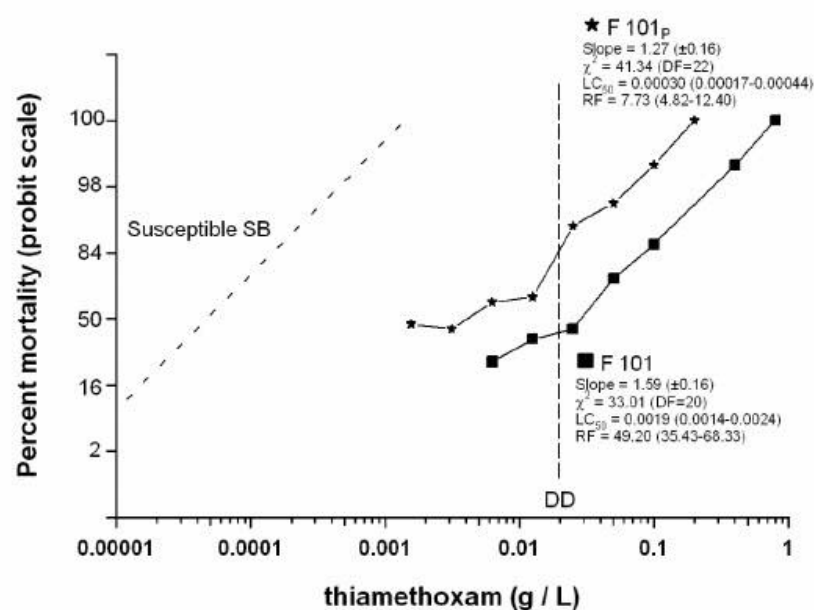
**Figure 2.2** Dose–response for *Aphis gossypii* against thiamethoxam (F 101) and following three years of continual laboratory selection and maintenance (F 101$_P$) (Susceptible SB has been redrawn from Table 2.2 to add clarity).
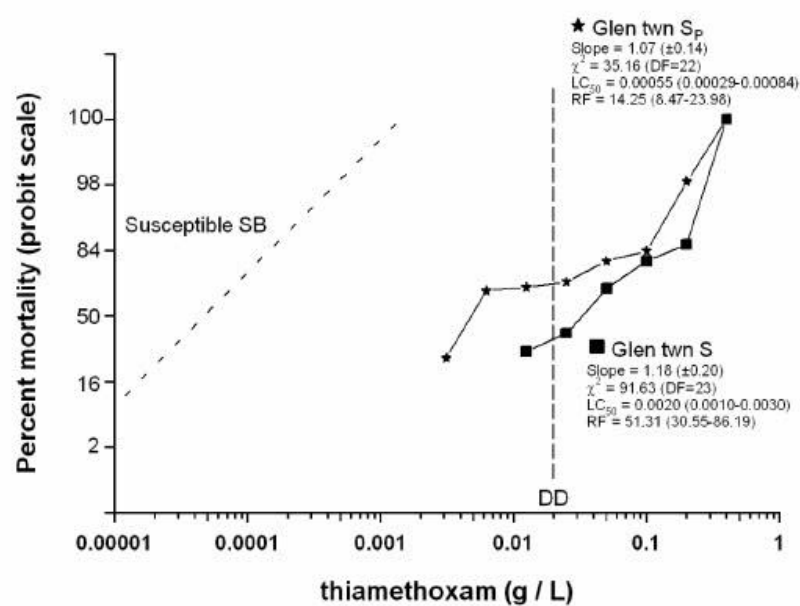
**Figure 2.3** Dose–response for *Aphis gossypii* against thiamethoxam (Glen twn S) and following three years of continual laboratory selection and maintenance (Glen twn $S_P$) (Susceptible SB has been redrawn from Table 2.2 to add clarity).
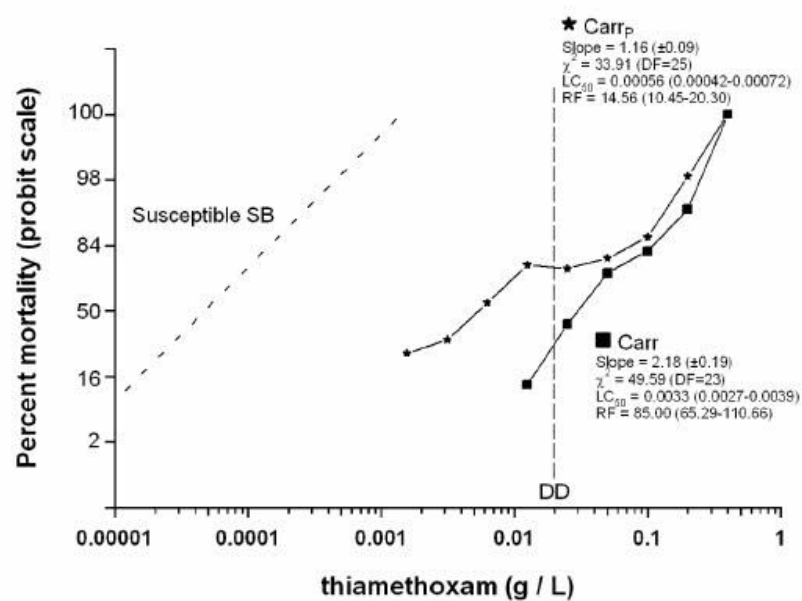
**Figure 2.4** Dose–response for *Aphis gossypii* against thiamethoxam (Carr) and following three years of continual laboratory selection and maintenance (Carr_P) (Susceptible SB has been redrawn from Table 2.2 to add clarity).

## 2.5 Discussion

Results presented here confirm neonicotinoid resistance in the *A. gossypii* used in this study so confirming their suitability for use in the following experimental chapters. Each strain contained a moderate to high frequency of thiamethoxam resistant individuals and demonstrated $LC_{50}$ resistance levels greater than those previously linked to field control failure (Herron and Wilson 2011). Herron and Wilson (2011) produced the highest $LC_{50}$ level RF against thiamethoxam (22-fold) in their field strain (Elra) collected from the Darling Downs (QLD) in the 2007-08 cotton season. In this present study, strain Carr, collected off cotton from Moree, NSW, yielded the highest $LC_{50}$ level RF against thiamethoxam of 85-fold some three seasons later. Each of the strains used were collected from sites where neonicotinoid insecticides had been used either directly for control of *A. gossypii* or used against other insect pests such as *C. dilutus* whereby *A. gossypii* was targeted indirectly. This study indicates that between the 2007-08 and 2011-12 cotton seasons, continued selection pressure for neonicotinoid resistant phenotypes was being placed on *A. gossypii* pest populations. In the 2010-11 cotton season, foliar applications of neonicotinoid compounds, including thiamethoxam and clothianidin rose to 7.5% of the total foliar application in Bollgard II planted cotton (APVMA 2013). This increase was largely attributed to the registration of clothianidin (Shield®) in 2008-09 for control of *C. dilutus* and *A. gossypii* (Sumitomo Chemical Australia Pty Ltd 2010). Furthermore, the percentage of cotton seed planted that was coated with a neonicotinoid insecticide rose from 80 to 92% between seasons 2008-09 and 2011-12 (APVMA 2013). Not surprisingly, in the 2010-11 cotton season, neonicotinoid resistance peaked with 96% of strains tested (via discriminating dose assay) found to contain individuals resistant to thiamethoxam and/or clothianidin (Herron 2012).

After subsequent maintenance in laboratory culture (36 months) and with routine pressuring, the frequency of resistant phenotypes in pressured strains was significantly reduced compared to initial results. This was indicated by non-overlapping 95% confidence intervals of the $LC_{50}$ level RF before and after routine pressuring. Yu (2014) previously stated that resistance may be lost gradually in a strain if it has not been adequately selected

for all the resistance alleles to be homozygous. In such a scenario, remaining resistant individuals will be heterozygous for resistance, whereby some insects may be very sensitive to the insecticide and others comparatively resistant. When bioassayed, this scattered response in the population causes the slope of the dosage-mortality curve to be quite low (Finney 1971) and significant departures from the probit binomial model (i.e. a plateau) may be indicative of a genetically heterogeneous population (Robertson and Preisler 1992). In this study, probit regression slope values became flatter after routine pressuring indicating an increase in the number of heterogeneous or homozygous susceptible individuals. It is likely that the proportion of highly resistant individuals decreased in my laboratory strains due to reduced selection pressure compared to that received in the field (Yu 2014). My results suggest that thiamethoxam resistance could revert in *A. gossypii* in the field if selection pressure is reduced; however, it is unknown how many generations are required for that to occur. One of the operational strategies that can be used to reduce selection pressure is rotation of neonicotinoid treatments with other chemicals that do not have cross-resistance to them. In this study, two PCR based molecular tests for resistance monitoring against *kdr* and *Ace*I type resistance were incorporated to elucidate any potential cross resistance mechanisms between these chemical classes. Results indicated that insecticides which confer resistance by *kdr* or *Ace*1 type mutations may be used as part of a rotational strategy to reduce selection pressure for neonicotinoid resistant phenotypes in *A. gossypii*.

Results presented here, give an indication of the potential for field control failure in *A. gossypii* due to a decline in susceptibility to two neonicotinoid compounds. In spite of reversion to <20-fold ($LC_{50}$ level), field control failure of thiamethoxam containing insecticides is likely to result if resistance is not adequately managed. For instance, previous reports have indicated an $LC_{50}$ level RF of 1.9-fold against clothianidin was linked to loss of field efficacy (Herron and Wilson 2011). Nonetheless, when interpreting susceptibility results, caution is required as they will not always relate directly to field performance. This is due to a complex interaction of factors including, but not limited to, environmental conditions, application equipment and pest pressure, and susceptibility of the

population to be controlled (IRAC 2009). It would therefore be useful to relate resistance quantified via bioassay in this study to a field based situation. This is particularly important for chemicals such as thiamethoxam which are applied predominantly as a seed dressing formulation. Therefore, I recommend that a 'field simulator' experiment be initiated as an adjunct to bioassay data to determine if resistance ratios observed in this or other studies will indeed result in field control failures as hypothesized.

# Chapter 3. Efficacy of two thiamethoxam pre-germination seed treatments and a phorate side-dressing against neonicotinoid and pirimicarb resistant cotton aphid *Aphis gossypii*

## 3.1 Foreword

This chapter is published in *Austral Entomology* (DOI: 10.1111/aen.12136) as "Kate L. Marshall, Damian Collins, Yizhou Chen, Grant A. Herron (2015). Efficacy of two thiamethoxam pre germination seed treatments and a phorate side-dressing against neonicotinoid- and pirimicarb-resistant cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae) Austral Entomology (**54**):4 351-357 (see Appendix C for published version). The formatting and presentation style are consistent with the journal *Austral Entomology*. Although the text and figures are as published, slight editorial changes have been made to enhance continuity of the thesis. The references are not included at the end of the chapter, but integrated into the general reference list at the end of the thesis.

This study was done because resistance in laboratory-based bioassays does not always translate well to field situations. For example, RFs of more than 50-fold to pyrethroids in, *Heliothis virescens* (Fabricus) infesting tobacco in Mexico, were found not to be associated with poor field control (Martinez-Carrillo and Reynolds 1983). Alternatively, resistance can be overlooked when levels are low and interpreted as variation among 'susceptible' strains and not considered indicative of resistance (Denholm et al. 1984, Sawicki 1987). Thus, it is important to establish whether resistance quantified via laboratory bioassay is of practical significance in the field.

Here whole plant efficacy trials provided an opportunity to test whether resistant insects (detected through laboratory-based bioassay) were able to complete their development on insecticide-treated plants. This is particularly important for chemicals such as thiamethoxam which are applied predominantly as a seed dressing so testing the resistance / control relationship speculated in Chapter 2.

## 3.2 Abstract.

In a glasshouse trial with potted cotton plants grown from thiamethoxam treated seed, neither 2.76 g a.i./kg seed (Cruiser®) nor 5.52 g a.i./kg seed (Cruiser Extreme®) protected plants from neonicotinoid resistant *A. gossypii*. Against susceptible *A. gossypii* each treatment was highly effective, providing control of >90% for 42 days. Continued use of either thiamethoxam treatment against resistant *A. gossypii* will select for resistant phenotypes and probably restrict the useful life of the neonicotinoid insecticides against this pest. In a separate trial, side-dressing of cotton seed with phorate 200 g/kg (Thimet®) effectively provided plants with protection from susceptible *A. gossypii*. The insecticidal activity of phorate treated plants against pirimicarb resistant *A. gossypii* was not statistically different to untreated plants (P>0.05). To maintain the effectiveness of pirimicarb in the Australian cotton integrated pest management strategy the use of phorate as an alternative pre-germination treatment to thiamethoxam for aphid control must be managed. I recommend that the first foliar spray applied to cotton treated with phorate at planting should not be pirimicarb or any other insecticide affected by insensitive cholinesterase (*Ace*1) type resistance.

## 3.3 Introduction

The cotton aphid, *A. gossypii* is a significant pest of cotton, *G. hirsutum* L., and cucurbits both in Australia and worldwide (Blackman and Eastop 2000). In Australian cotton, *A. gossypii* can be found on seedling cotton (October) and typically builds to levels that require control during the mid-late growing season (January – March). *A. gossypii* feeding can reduce leaf photosynthesis (Heimoana 2012) and spread plant viruses such as CBT virus (Reddall et al. 2004, Ellis et al. 2013) or CLR virus (Corrêa et al. 2005) that dramatically reduce yield potential of affected plants. The excretion of honeydew by aphids (Hequet et al. 2000) contaminates the lint of matured fruit (bolls). Damaged lint attracts a lower price and damages the reputation of the region from which it is sourced. Furthermore, it is not economical to clean the lint and contaminated lint binds to machinery during spinning, necessitating shutdown and cleaning.

Economically significant outbreaks of aphids are partially induced by applications of insecticides against other pests (Wilson et al. 1999). These pesticides reduce beneficial populations without controlling aphids, which then rapidly increase. Since the advent and widespread uptake of *Bt*-cotton, containing the *Cry* proteins to control the primary pests, *Helicoverpa* spp., the use of insecticides has declined dramatically (Wilson et al. 2013). However, some species not controlled by the *Cry* proteins have emerged as pests and require targeted control, especially *C. dilutus* (Wilson et al. 2013). Insecticides targeted against *C. dilutus* are generally disruptive to beneficial species but do not control aphids – which consequently have persisted as an important pest. The capacity to control aphids, throughout the crop growing cycle, is consequently important for cotton production.

In Australia, neonicotinoid seed treatments containing thiamethoxam or side dressings of granular insecticides, such as the organophosphate phorate at planting, are used to control a range of seedling pests such as thrips and wireworms (Elateridae), but also control *A. gossypii* and hence the risk of CBT virus transmission. These treatments offer increased selectivity compared to neonicotinoid or organophosphate foliar sprays, which can be highly disruptive to beneficial insect populations (Mass 2012). Cruiser® (thiamethoxam at 2.76 g a.i./kg seed) and Cruiser Extreme® (thiamethoxam at 5.52 g a.i./kg seed) provide early season seedling protection (30-40 days) against *A. gossypii* and several other sucking insect pests (Maienfisch et al. 2001). However, the effectiveness of these products against *A. gossypii* may be threatened because of resistance to neonicotinoid , carbamate and organophosphate insecticides (Herron et al. 2001).

Neonicotinoid resistance in Australian *A. gossypii* was first recorded in the 2007-08 cotton season and attributed to long-term, widespread use of the neonicotinoid cotton seed treatments (Herron and Wilson 2011). If neonicotinoid resistant *A. gossypii* are present at the start of the cotton season, the use of neonicotinoid seed treatments may be only partially effective and so could exacerbate resistance. Phorate side-dressing has been suggested as a possible alternative to the neonicotinoid seed treatments but its suitability as a viable replacement has not been explored, nor has its effectiveness to control carbamate (pirimicarb) resistant *A. gossypii* been revealed. Similarly, the efficacy of the standard and

higher rate thiamethoxam seed treatments against neonicotinoid resistant aphids *in planta* has not been established. Here we report the results of a glasshouse trial that investigated the effectiveness of these treatments against resistant *A. gossypii*.

## 3.4 Materials & Methods

### 3.4.1 Chemicals tested

Cotton seed (variety Sicot 71) treated with thiamethoxam at either 2.76 g a.i./kg seed (Cruiser® Insecticide Seed Treatment) or 5.52 g a.i./kg seed (Cruiser Extreme® Insecticide Seed Treatment) was obtained from Cotton Seed Distributors, Wee Waa, New South Wales (NSW). Phorate 200 g/kg insecticide (Thimet® 200 G Systemic Granular Insecticide) was obtained from Barmac Industries Pty Ltd.

### 3.4.2 Aphids

A reference susceptible strain (Sus F 96) was maintained on insecticide free cotton in an insect proof cage at $25 \pm 4°C$ under natural light at EMAI, Camden. Resistant *A. gossypii* strains Glen twn S and Mon P were collected from commercial cotton. Strain Glen twn S was neonicotinoid resistant while strain Mon P was pirimicarb/omethoate resistant (Herron et al. 2013). Strain Glen twn S was routinely pressured monthly by exposure to foliar sprays at a rate just above double the discriminating dose of thiamethoxam (i.e. 0.05 g/L) (Table A.1). Strain Mon P was similarly pressured monthly using a dose 10-fold the discriminating dose of pirimicarb (i.e. 0.1 g/L) (Herron et al. 2000). Both strains were pressured a week prior to the initial testing to ensure resistance remained at a high level throughout the trial interval.

### 3.4.3 Thiamethoxam trial

Cotton seed treatments were: Untreated Control (cotton seed variety Sicot 71); 2.76 g a.i./kg seed (Cruiser®) and; 5.52 g a.i./kg seed (Cruiser Extreme®). Approximately 60 seeds of each treatment group were individually planted into plastic pots (11.5cm diameter) filled

with NativeMix™ premium potting mix and held in a room maintained at $28 \pm 2°C$. Each pot contained only one treatment with all pots planted concurrently (180 treated pots total). At planting and on another three occasions over the following six days 150 mL of water was poured over the soil surface of each pot.

A week after planting the dicotyledons had emerged. At this time (Day 0) six pots from each treatment group were transferred onto individual saucers in insect proof cages maintained at $25 \pm 4°C$ but subject to natural light. Pots were watered by filling their saucers with 200 mL on initial placement into the cages and as necessary during the trial period.

A randomized complete block design was used. Strains were randomized to cages ("whole-plots") and treatments were randomized to three pots within cages ("sub-plots"). On Day 0 a pot from each treatment was placed into one of six insect-proof cages. Two apterous adult aphids (susceptible or resistant) were placed onto each of the plants within each cage such that three cages contained only susceptible aphids and three contained only resistant aphids. On Day 7 all leaves were removed from each plant and final aphid numbers were counted with the aid of a stereo microscope. This process was repeated with new plants at weekly interval until Day 49 by which time susceptible aphids could survive on both thiamethoxam treatments.

### 3.4.4 Phorate trial

Cotton seed treatments were: Untreated Control (cotton seed variety Sicot 71) and phorate 200 g/kg (Thimet®) at a dose of 34.4 mg/pot. In a separate trial, approximately 60 seeds of each treatment group were planted individually and maintained as above. The dose (34.4 mg/pot) of phorate applied was equivalent to that indicated on the product label for short period protection (3 kg/ha) and assumed a row length equal to the diameter of the pot. Trial design was as above.

### 3.4.5 Statistical analysis

Analyses were conducted for each trial using generalised linear mixed models in ASReml (Gilmour et al. 2009). The response (number of aphids) was analysed as quasi-poisson (over-dispersed Poisson with log link) for each trial using a mixed model comprising fixed strain, treatment (within strain) and linear day effects and all associated interactions, and random factor day effects and interactions with treatment, strain, strain by treatment, as well as cage, cage by day and position. Wald type F-tests for fixed terms in the model are reported, as well as contrasts to test for treatment efficacy and interactions between treatment efficacy and (linear) day, for each strain in turn.

The Henderson-Tilton formula (Henderson and Tilton 1955) for treatment control is $100[ 1- Ta*Cb/Tb*C a ]=100[ 1- Ta/Ca ]$ where $Ta$ and $Ca$ are the number of aphids surviving at the end of the week, and $Tb$ and $Cb$ are the number of original number of aphids used for each pot (2) which cancel out from top and bottom. The ratio $Ta / Ca$ could be estimated, along with an approximate 95% confidence interval, by back-transforming the predicted difference between each treatment and control at each time-point (since a log link was used, and so absolute differences on the log scale correspond to multiplicative effects on the back-transformed scale).

## 3.5 Results

### 3.5.1 Thiamethoxam trial

There were significant (P<0.05) treatment within strain effects and significant interactions of treatment within strain with day (Table 3.1). The non-zero variance components indicated differences in individual day effects across treatments, and both cage and cage by day effects, as well as residual over-dispersion (relative to a Poisson distribution), indicated by a residual variance (3.02) greater than 1 (Table 3.2). For strain Sus F 96 the interactions of treatment with lin(day) were either non-significant (P>0.05) for the higher rate or just significant (P<0.05) for the lower rate. However, there were statistically highly significant (P<0.0001) treatment within strain effects for both rates of thiamethoxam compared to

untreated cotton seed, as expected (Table 3.1). Both Cruiser® and Cruiser Extreme® provided 100% protection of strain Sus F 96 for 14 days (Table 3.3). Control of strain Sus F 96 remained very high (>90%) until day 49 where Cruiser® showed a decrease to 87%. Residual insecticidal activity of Cruiser Extreme® provided greater control at 49 days of 93.3%. Interactions of treatment with day for strain Glen twn S were both significant (P<0.05) for each rate of thiamethoxam when compared to untreated cotton seed, indicating the reduction in treatment efficacy over time. Cruiser Extreme® provided higher initial and residual protection compared to Cruiser® (Table 3.3 and Figure 3.1) but neither treatment adequately controlled resistant *A. gossypii*. From day 28 the effectiveness of Cruiser® against strain Glen twn S was similar to untreated cotton (Table 3.1).

**Table 3.1** Wald-F test statistics for fixed effects of thiamethoxam analysis.

| | DF (num, den) | F-statistic | P-value |
|---|---|---|---|
| strain | 1,5.3 | 9.164 | 0.0273 |
| lin(day) | 1,16.4 | 9.669 | 0.0066 |
| strain/trt | 4,29.3 | 13.810 | 0.0000 |
|     sus:cruiser vs control | 1,61.7 | 31.250 | 0.0000 |
|     sus:extreme vs control | 1,73.7 | 34.470 | 0.0000 |
|     res:cruiser vs control | 1,11.5 | 4.740 | 0.0512 |
|     res:extreme vs control | 1,12.3 | 8.228 | 0.0139 |
| strain × lin(day) | 1,36.7 | 9.840 | 0.0034 |
| strain/trt × lin(day) | 4,32.2 | 3.778 | 0.0125 |
|     sus: (cruiser vs control) × lin(day) | 1,80.1 | 4.110 | 0.0460 |
|     sus: (extreme vs control) × lin(day) | 1,79.2 | 3.244 | 0.0755 |
|     res: (cruiser vs control) × lin(day) | 1,11.4 | 7.003 | 0.0221 |
|     res: (extreme vs control) × lin(day) | 1,14.0 | 9.882 | 0.0072 |

**Table 3.2** Non-zero variance component and standard error (SE) for random terms of thiamethoxam analysis.

|  | Component | SE | Z-ratio |
|---|---|---|---|
| Cage | 0.0097 | 0.0309 | 0.3143 |
| cage × fac(day) | 0.1266 | 0.0610 | 2.0738 |
| trt × fac(day) | 0.2708 | 0.1430 | 1.8943 |
| Residual | 3.0163 | 0.5196 | 5.8054 |

**Table 3.3** Estimated treatment efficacies (Et) and approximate 95% confidence intervals (CI) of two varying rates of formulated thiamethoxam (g a.i./kg seed) against neonicotinoid susceptible and neonicotinoid resistant *Aphis gossypii*.

**Susceptible strain F 96**

|  |  | Untreated |  | 2.76 |  | 5.52 |  |
|---|---|---|---|---|---|---|---|
|  |  | Et | 95% CI | Et | 95% CI | Et | 95% CI |
| Day 7 | Aphids | 13.5 | (7.8, 23.4) | 0.0 | (0.0, 1.4) | 0.0 | (0.0, 1.0) |
|  | % Control |  |  | 100.0 | (89.3, 100.0) | 100.0 | (92.5, 100.0) |
| Day 14 | Aphids | 16.3 | (10.0, 26.5) | 0.0 | (0.0, 1.1) | 0.0 | (0.0, 1.0) |
|  | % Control |  |  | 100.0 | (93.1, 100.0) | 100.0 | (93.8, 100.0) |
| Day 21 | Aphids | 14.3 | (9.0, 22.6) | 0.0 | (0.0, 1.4) | 0.1 | (0.0, 5.6) |
|  | % Control |  |  | 99.9 | (89.8, 100.0) | 99.5 | (60.9, 100.0) |
| Day 28 | Aphids | 11.2 | (6.8, 18.2) | 0.1 | (0.0, 2.8) | 0.1 | (0.0, 2.0) |
|  | % Control |  |  | 99.4 | (75.0, 100.0) | 99.3 | (82.1, 100.0) |
| Day 35 | Aphids | 11.9 | (7.2, 19.7) | 0.2 | (0.0, 2.4) | 0.2 | (0.0, 1.7) |
|  | % Control |  |  | 98.3 | (79.0, 99.9) | 98.3 | (85.1, 99.8) |
| Day 42 | Aphids | 21.7 | (13.7, 34.5) | 1.2 | (0.3, 4.2) | 0.9 | (0.3, 3.2) |
|  | % Control |  |  | 94.6 | (80.5, 98.5) | 95.8 | (85.2, 98.8) |
| Day 49 | Aphids | 35.3 | (21.9, 56.8) | 4.6 | (1.7, 12.6) | 2.4 | (0.7, 8.7) |
|  | % Control |  |  | 87.0 | (65.2, 95.2) | 93.3 | (75.7, 98.1) |

**Resistant strain Glen twn S**

|  |  | Et | 95% CI | Et | 95% CI | Et | 95% CI |
|---|---|---|---|---|---|---|---|
| Day 7 | Aphids | 38.2 | (24.0, 60.8) | 5.6 | (2.5, 12.6) | 1.5 | (0.5, 4.9) |
|  | % Control |  |  | 85.3 | (67.6, 93.3) | 96.1 | (87.1, 98.8) |

**(Continued)**

**Table 3.3 (cont'd)** Estimated treatment efficacies (Et) and approximate 95% confidence intervals (CI) of two varying rates of formulated thiamethoxam (g a.i./kg seed) against neonicotinoid susceptible and neonicotinoid resistant *Aphis gossypii*.

Resistant strain Glen twn S

| | | Untreated | | 2.76 | | 5.52 | |
|---|---|---|---|---|---|---|---|
| | | Et | 95% CI | Et | 95% CI | Et | 95% CI |
| Day 14 | Aphids | 36.3 | (23.3, 56.6) | 4.9 | (2.2, 11.1) | 2.2 | (0.8, 6.1) |
| | % Control | | | 86.5 | (69.3, 94.1) | 94.0 | (82.9, 97.9) |
| Day 21 | Aphids | 25.1 | (16.2, 38.9) | 7.1 | (3.6, 13.9) | 18.3 | (10.7, 31.2) |
| | % Control | | | 71.9 | (43.8, 85.9) | 27.2 | (-23.8, 57.2) |
| Day 28 | Aphids | 15.5 | (9.6, 25.0) | 15.1 | (8.6, 26.4) | 8.8 | (4.6, 16.7) |
| | % Control | | | 2.6 | (-79.8, 47.2) | 43.4 | (-14.0, 71.9) |
| Day 35 | Aphids | 13.0 | (7.8, 21.5) | 14.1 | (7.9, 25.3) | 9.8 | (5.2, 18.7) |
| | % Control | | | -8.9 | (-110.3, 43.6) | 24.3 | (-54.6, 62.9) |
| Day 42 | Aphids | 18.8 | (11.6, 30.3) | 22.8 | (13.5, 38.3) | 18.5 | (10.7, 32.0) |
| | % Control | | | -21.3 | (-109.1, 29.7) | 1.4 | (-74.5, 44.3) |
| Day 49 | Aphids | 24.0 | (14.7, 39.3) | 24.7 | (14.6, 41.7) | 20.8 | (12.1, 35.9) |
| | % Control | | | -2.8 | (-72.0, 38.6) | 13.4 | (-48.3, 49.4) |

**Figure 3.1** Fitted trend for the thiamethoxam analysis, for each strain by treatment combination (thiamethoxam at 5.52 g a.i./kg seed, Cruiser Extreme® Insecticide Seed Treatment; thiamethoxam at 2.76 g a.i./kg seed, Cruiser® Insecticide Seed Treatment; untreated cotton seed, variety Sicot 71). The solid line represents the fitted trend, with dotted lines representing the 95% confidence interval. The raw data for each replicate is numbered 1 to 3 in each panel (with replicates 1 and 3 shifted slightly left and right, respectively, to avoid overlap).

160 of 308

### 3.5.2 Phorate trial

There were statistically highly significant (P<0.001) treatment within strain effects for strain Sus F 96 (and interactions with day), but not for strain Mon P (P>0.05) (Table 3.4). The non-zero variance components indicated day effects (fac(day)), replicate and replicate by day effects, cage by day effects, treatment, strain by day effects and position effects as well as residual over-dispersion (relative to a Poisson distribution), indicated by a residual variance (2.618) greater than 1 (Table 3.5). Phorate provided robust protection of strain Sus F 96 for the duration of the trial, with control only decreasing below 90% at day 35 (Table 3.6). From day 42, phorate provided residual control of 81%, decreasing to 67.5% control at day 49. Strain Mon P survived well on phorate treated cotton from day 0 (Figure 3.2). Population size of strain Mon P when challenged with phorate showed no statistical significance compared with untreated cotton (Table 3.4).

**Table 3.4** Wald-F test statistics of fixed effects for phorate analysis.

| | DF (num, den) | F-statistic | P-value |
|---|---|---|---|
| strain | 1,5.0 | 3.4440 | 0.1223 |
| lin(day) | 1,4.8 | 0.5352 | 0.4987 |
| strain/trt | 2,5.9 | 10.4000 | 0.0115 |
| sus:(phorate vs control) | 1,23.0 | 18.8700 | 0.0002 |
| res:(phorate vs control) | 1,3.6 | 0.1018 | 0.7675 |
| strain × lin(day) | 1,4.8 | 0.0548 | 0.8245 |
| strain/trt × lin(day) | 2,8 | 3.7490 | 0.0707 |
| sus:(phorate vs control) × lin(day) | 1,40.5 | 7.8890 | 0.0076 |
| res:(phorate vs control) × lin(day) | 1,3.7 | 1.0180 | 0.3746 |

**Table 3.5** Non-zero variance component and standard error (SE) for random terms of phorate analysis.

|  | Component | SE | Z-ratio |
|---|---|---|---|
| fac(day) | 0.3424 | 0.4020 | 0.8475 |
| Rep | 0.1070 | 0.1239 | 0.8388 |
| rep × fac(day) | 0.0122 | 0.0553 | 0.2181 |
| cage × fac(day) | 0.0823 | 0.0764 | 1.1067 |
| trt × fac(day) | 0.0806 | 0.0931 | 0.8854 |
| strain × fac(day) | 0.3051 | 0.2512 | 1.213 |
| Position | 0.0043 | 0.0227 | 0.1907 |
| Residual | 2.6847 | 0.6312 | 4.0102 |
| fac(day) | 0.3424 | 0.4020 | 0.8475 |

**Table 3.6** Estimated treatment efficacies (Et) and approximate 95% confidence intervals (CI) of phorate as a side dressing against pirimicarb susceptible and pirimicarb resistant *Aphis gossypii*.

**Susceptible strain F 96**

|  |  | Untreated | | 3 kg/ha | |
|---|---|---|---|---|---|
|  |  | Et | 95% CI | Et | 95% CI |
| Day 7 | Aphids | 11.6 | (5.6, 23.9) | 0.0 | (0.0, 0.7) |
|  | % Control |  |  | 99.7 | (94.2, 100.0) |
| Day 14 | Aphids | 17.0 | (8.7, 33.0) | 0.2 | (0.0, 1.8) |
|  | % Control |  |  | 99.0 | (89.8, 99.9) |
| Day 21 | Aphids | 12.8 | (6.3, 25.7) | 0.3 | (0.0, 2.0) |
|  | % Control |  |  | 97.6 | (85.7, 99.6) |
| Day 28 | Aphids | 5.3 | (2.3, 12.5) | 0.4 | (0.1, 1.9) |
|  | % Control |  |  | 92.2 | (68.6, 98.0) |
| Day 35 | Aphids | 4.2 | (1.7, 10.4) | 0.4 | (0.1, 1.7) |
|  | % Control |  |  | 89.3 | (67.8, 96.5) |
| Day 42 | Aphids | 11.5 | (5.7, 23.3) | 2.2 | (0.9, 5.5) |
|  | % Control |  |  | 80.9 | (60.9, 90.7) |
| Day 49 | Aphids | 27.0 | (14.6, 50.0) | 8.8 | (4.0, 19.1) |
|  | % Control |  |  | 67.6 | (36.5, 83.4) |

**Resistant strain Mon P**

|  |  | Et | 95% CI | Et | 95% CI |
|---|---|---|---|---|---|
| Day 7 | Aphids | 7.1 | (3.2, 15.8) | 3.8 | (1.5, 9.6) |
|  | % Control |  |  | 46.0 | (-26.0, 76.9) |
| Day 14 | Aphids | 30.9 | (17.0, 56.4) | 26.6 | (14.4, 49.1) |
|  | % Control |  |  | 14.0 | (-33.1, 44.5) |

**(Continued)**

**Table 3.6 (cont'd)** Estimated treatment efficacies (Et) and approximate 95% confidence intervals (CI) of phorate as a side dressing against pirimicarb susceptible and pirimicarb resistant *Aphis gossypii*.

| Resistant strain Mon P | | | | | |
|---|---|---|---|---|---|
| | | Untreated | | 3 kg/ha | |
| | | Et | 95% CI | Et | 95% CI |
| Day 21 | Aphids | 26.8 | (14.6, 49.2) | 27.1 | (14.7, 50.0) |
| | % Control | | | -1.3 | (-59.1, 35.5) |
| Day 28 | Aphids | 10.7 | (5.3, 21.4) | 18.9 | (9.9, 35.9) |
| | % Control | | | -76.8 | (-208.6, -1.3) |
| Day 35 | Aphids | 4.3 | (1.9, 10.1) | 5.5 | (2.4, 12.5) |
| | % Control | | | -26.3 | (-160.1, 38.7) |
| Day 42 | Aphids | 45.0 | (25.3, 80.1) | 53.3 | (30.2, 94.1) |
| | % Control | | | -18.4 | (-68.8, 17.0) |
| Day 49 | Aphids | 22.8 | (12.1, 43.0) | 24.0 | (12.8, 45.1) |
| | % Control | | | -5.3 | (-71.9, 35.5) |

**Figure 3.2** Fitted trends for the phorate analysis, for each strain by treatment combination (phorate equivalent to 3 kg/ha, Thimet® 200 G Systemic Granular Insecticide; untreated cotton seed, variety Sicot 71). The solid line represents the fitted trend, with dotted lines representing the 95% confidence interval. The raw data for each replicate is numbered 1 to 3 in each panel (with replicates 1 and 3 shifted slightly left and right respectively, to avoid overlap).

## 3.6 Discussion

The efficacy of two neonicotinoid seed treatments against neonicotinoid susceptible and resistant *A. gossypii* and an organophosphate at-planting treatment against carbamate susceptible and resistant *A. gossypii* were evaluated under simulated field conditions in a glasshouse trial. Raw data produced was transformed via Henderson-Tilton analysis to account for variability seen in *A. gossypii* numbers on untreated controls. We believe that due to the low starting number of two aphids each week, the variability seen in aphid populations from plant to plant was typical. Predicted values were produced for each time-point of the trial which offered a more realistic estimate of the control provided by each treatment.

We have clearly shown that formulated thiamethoxam at either rate (2.76 g a.i./kg seed and 5.52 g a.i./kg seed) is highly effective for protection against neonicotinoid susceptible *A. gossypii* and continues to be a viable option for aphid control. These results support previous studies investigating the efficacy of thiamethoxam as a seed treatment against susceptible *A. gossypii*. Maienfisch et al. (2001) found that against sucking insect pests of cotton, rates between 105-350 g a.i./100 kg seed gave excellent control for 21-45 days. Prasanna et al. (2004) also found that thiamethoxam 70WS at a rate of 2.85 g a.i./kg seed effective until 40 days post seedling emergence, whilst the higher rate of 4.28 g a.i./kg seed still provided superior control of *A. gossypii* when compared to untreated plants at 60 days, although not statistically significant. Zidan (2012) also found that thiamethoxam 70WS at the recommended rate of 4.9 g a.i./kg seed provided effective control of *A. gossypii,* although when compared to our results provided significantly reduced residual protection.

In contrast to neonicotinoid susceptible *A. gossypii*, our results have revealed that neither rate of thiamethoxam gives adequate control against neonicotinoid resistant *A. gossypii*. It is likely that ongoing widespread reliance on neonicotinoid seed treatments, at either rate, will continue to select for resistant genotypes. Cross resistance between members of the neonicotinoid group 4A MoA in *A. gossypii* has been reported elsewhere (Wang et al. 2007, Shi et al. 2011) and suggests that control of resistant populations is likely to be lost if neonicotinoid use is not managed better. The Insecticide Resistance Management Strategy (IRMS) for control of sucking insect pests

of cotton recommends chemical rotation as the primary strategy for control of resistant *A. gossypii* (Mass 2012). Other well defined strategies in the IRMS such as use of refugia for control of *Helicoverpa* spp. are limited in their practicality for *A. gossypii* due to a short life cycle and there being no sexual phase of reproduction occurring in Australia (Smith et al. 2006). If chemical rotation is maintained over successive generations, then in the absence of selection the resistant population should return to susceptibility. It should be mentioned that this strategy relies on there being an associated fitness cost to the observed resistance. Fortunately, reversion to susceptibility in the absence of insecticide pressure has been noted to occur in laboratory strains of neonicotinoid resistant *A. gossypii* (Chapter 2). This would suggest that at least in some populations of *A. gossypii*, genes conferring neonicotinoid resistance do not appear to be fixed. Neonicotinoid seed dressings are primarily targeted against other pests where they continue to provide cost-effective control (Mass 2012), so restriction in their use without a viable alternative is impractical. Phorate is registered for the control of *A. gossypii* at planting and has previously been shown to control neonicotinoid resistant *A. gossypii* as it possesses a distinct MoA to neonicotinoids (Herron et al. 2013). However, established cross resistance between the organophosphate and carbamate chemical classes via insensitive cholinesterase type resistance (*Ace*1) will select for high level resistance in *A. gossypii* pest populations if used sequentially and may lead to control failures as previously seen (Herron et al. 2001, Andrews et al. 2004, Benting and Nauen 2004). The IRMS lists the carbamate, pirimicarb as a favourable first foliar spray for use against *A. gossypii* due to its softness on beneficial insect species (Mass 2012). Herron et al. (2013) suggested that pirimicarb-resistant *A. gossypii* would not be controlled by phorate. The results of our glasshouse trial confirm those laboratory findings. If phorate is to successfully substitute for a neonicotinoid seed dressing the interaction with pirimicarb must be carefully considered. If phorate is used to control neonicotinoid resistant *A. gossypii* then pirimicarb, or any other chemical associated with *Ace*1 type resistance, should not immediately follow as the first foliar spray.

# Chapter 4.   Resistance mechanisms associated with the neonicotinoid insecticide thiamethoxam in Australian pest populations of the cotton aphid *Aphis gossypii*

## 4.1 Foreword

Some contents of this chapter have previously been published as a conference paper: Marshall, K.L., Herron, G.A. & Chen, Y. 2014. Neonicotinoid Resistance in Cotton Aphid from Australia. In: *Conference Proceedings of the 17th Australian Cotton Conference*. Cotton Research and Development Corporation, Broadbeach, Australia, 5th to 7th August (see Appendix D for published version). Unlike the previous chapter, here I have significantly re-cast the conference proceedings to fit the requirements of the thesis and included additional results.

## 4.2 Abstract

A point mutation R81T, located in the loop D region of the nAChR β1 subunit, confers resistance to neonicotinoid insecticides in *M. persicae* from Europe and *A. gossypii* from China and Korea. In three thiamethoxam-resistant strains of *A. gossypii* (F 101, Glen twn S and Carr) collected off Australian cotton, the R81T mutation was proposed as the likely causal mechanism of resistance. However, PCR amplification of that mutation site and comparative sequence analysis between susceptible and resistant strains revealed that the R81T mutation was not correlated with the phenotypic expression of resistance in Australian *A. gossypii*. Therefore, metabolic detoxification was investigated as an alternate resistance causing mechanism using the synergist PBO. The use of PBO in tandem with thiamethoxam in bioassays either completely or partially suppressed resistance, suggesting that thiamethoxam resistance in Australian *A. gossypii* from cotton is at least in part, mediated by overexpression of metabolic detoxification enzymes.

## 4.3 Introduction

*A. gossypii* is a significant worldwide insect pest of cotton (Blackman and Eastop 2000) and has demonstrated a high propensity for developing insecticide resistance (Dixon 1992). In Australia, *A. gossypii* has developed resistance to every major insecticide class used against it, including the organophosphate, carbamate, pyrethroid and more recently, the neonicotinoid chemical class (Herron et al. 2001, Herron and Wilson 2011). Target site and/or metabolic detoxification have been identified as mechanisms associated with organophosphate, carbamate and pyrethroid resistance in *A. gossypii* (Wang et al. 2002, McLoon and Herron 2009, Marshall et al. 2012). However, the causal mechanism of neonicotinoid resistance in Australian *A. gossypii* has not yet been revealed. Any information regarding the mechanism of thiamethoxam resistance in Australian *A. gossypii* will be valuable for predicting future cross-resistance spectra and for developing rapid and sensitive molecular based diagnostic assays to detect resistance (Brown and Brogdon 1987, Scott 1990).

Neonicotinoid insecticides target the nAChRs of insects whereby they mimic the agonist action of ACh but are unable to be broken down by AChE (Yamamoto 1999, Matsuda et al. 2001). The result is an irreversible binding to and overstimulation of the receptor, causing paralysis and death of the insect (Matsuda et al. 2001). Their unique MoA makes them highly favourable for control of resistant insect pests as they circumvent many established resistance mechanisms which have evolved to the other major insecticide classes (Jeschke and Nauen 2008). Among the neonicotinoids, imidacloprid and thiamethoxam are the most widely used (Jeschke et al. 2011) as both are extremely effective for the control of many homopteran, coleopteran, lepidopteran and dipteran insect pests of agricultural significance (Elbert et al. 2008). Imidacloprid and thiamethoxam are also used to control a range of piercing-sucking insect pests including aphids, planthoppers and whiteflies (Elbert et al. 2008). As a result of their physiochemical properties, imidacloprid and thiamethoxam may be used in seed / soil treatments and also directly applied to the plant (Elbert et al. 2008).

Target site insensitivity via modifications in some nAChR subunits has been implicated as a causal mechanism of neonicotinoid resistance in *N. lugens* (Liu et al. 2005), *M. persicae* (Bass et al. 2011) and *A. gossypii* (Shi et al. 2012, Koo et al. 2014, Kim et al.

2015). In *N. lugens* a point mutation (Y151S) in two alpha subunits (Nla1 and Nla3) of the nAChR was reported to be associated with imidacloprid resistance in a laboratory-selected strain (Liu et al. 2005). Shortly after, a novel mutation in the β1 subunit of the nAChR, R81T was found to confer imidacloprid resistance in a field population of *M. persicae* (Bass et al. 2011). This same mutation was also detected in field collected strains of *A. gossypii* from Korea (Koo et al. 2014) and China (Kim et al. 2015). Metabolic detoxification via increased expression of P450s has also been correlated with neonicotinoid resistance in *M. persicae* (Puinean et al. 2010, Bass et al. 2011) and *N. lugens* (Zewen et al. 2003, Ding et al. 2013), but not *A. gossypii*.

Previously (Chapter 2 and 3), three *A. gossypii* pest populations (F 101, Glen twn S and Carr) collected from Australian cotton were evaluated for resistance against two neonicotinoid compounds: the cost effective foliar spray Shield® (containing clothianidin); and thiamethoxam which is incorporated as both a foliar spray (Actara®) and a pre germination seed treatment (Cruiser®). These strains were maintained in laboratory culture (with routine pressuring) as reference strains for further resistance characterisation. In this Chapter I investigate the phenotypic expression of thiamethoxam resistance in three field strains of *A. gossypii* using molecular based techniques to detect the presence (or absence) of R81T. As the R81T mutation was not present I subsequently employed synergist bioassays to explore alternate resistance mechanisms to R81T.

## 4.4 Methods and Materials

### 4.4.1 Aphids

Susceptible strain Sus F 96 was collected off commercial cotton in the QLD region of St. George during 2011 and has previously been shown susceptible to a range of chemicals used for *A. gossypii* control (Herron et al. 2013). It was maintained as a reference susceptible strain for this study in isolation. Strains F 101, Glen twn S and Carr are field collected thiamethoxam resistant strains which initially displayed RFs at the $LC_{50}$ level of 49-, 51-, and 85-fold but subsequently reverted (denoted by p) to 8-, 14-, and 15-fold resistance after maintenance in laboratory culture (despite routine pressuring, refer to Chapter 2). Strain Sus F 96 was reared weekly on a potted

insecticide free cotton plant, *G. hirsutum* in a purpose built insect proof cage and held in an insectary maintained at 25°C ± 4°C under natural light. Strains F 101 and Glen twn S were reared similarly except once a month were pressured by exposure to foliar sprays at double the discriminating dose (i.e. 0.05 g a.i./L) of thiamethoxam to maintain resistance (Table A.1). Strain Carr was pressured in the same manner but at the higher dose of 0.1 g a.i./L (Table A.1).

### 4.4.2 Chemicals

The synergist PBO (Endura PB 80 EC-NF, 80% PBO) was kindly supplied by Endura SpA, Italy. Actara® (Thiamethoxam 250 g/kg) was supplied by Syngenta, Australia.

### 4.4.3 Non-synergist and synergist bioassays

In brief, formulated thiamethoxam was prepared in distilled water to appropriate concentrations selected to achieve $0 < x < 100\%$. For synergist bioassays, methodology was the same as insecticide only tests except PBO was prepared in distilled water at a rate of 0.2 mL PBO / 100 mL distilled water and that was used to prepare all insecticide dilutions used in the synergist study (in place of distilled water). A PBO rate of 0.2 mL PBO / 100 mL distilled water was selected as preliminary data had shown it to be the highest rate which did not exceed 10% control mortality. Petri dishes (35 mm diameter) were prepared with cotton leaf discs of cotton on distilled water agar. Twenty to thirty adult *A. gossypii* were transferred to the dishes and allowed to settle before being sprayed. Serial dilutions of PBO and/or thiamethoxam were applied in 2 ml of solution via a Potter spray tower (Burkhard Scientific, Uxbridge, Middlesex, UK) which produced an aqueous deposit of $1.6 \pm 0.007$ mg/cm$^2$. Once sprayed, dishes were covered with taut plastic cling wrap including tiny (smaller than an aphid) perforations made to prevent condensation. The number of aphids present on each disc was counted and dishes transferred to an incubator maintained at 25°C ± 0.1°C under a light regime of 16:8 L:D for 24 h. Tests were replicated three times (on different days) and responses assessed after 24 h. Mortality (unable to walk when prodded) was evaluated with the aid of a stereo microscope by counting the number of live aphids present on the leaf disc and subtracting the number of survivors.

### 4.4.4 Data Analysis

Bioassay data was analysed by probit analysis (Finney 1971) using a stand-alone probit program developed by Barchia (2001) which accounted for variation between replicates. This was achieved by using a $\chi^2$ test and if significant at the 5% level, the variance of the estimated parameter was scaled by the corresponding heterogeneity factor equal to the residual mean deviance. Probit regressions were corrected for control mortality (Abbott 1925) and $LC_{50}$ and $LC_{99.9}$ values plus their 95% fiducial-limits were calculated using the method of Finney (1971). RFs were calculated by dividing the $LC_{50}$ value of the susceptible strain (in the presence or absence of PBO) by the $LC_{50}$ value of the resistant strain (in the presence or absence of PBO). Significance was determined by calculating the ratio (RF) of F $101_P$, Glen twn $S_P$ and $Carr_p$ over strain Sus F 96 and calculating their 95% CI that should not overlap one (Robertson et al. 2007).

### 4.4.5 Primer Design

The forward primer INT1_For (CTGTCCAGAACATGACCGAA) (Table B.2) design was based on GenBank sequence JQ627836.1 (*A. gossypii* nAChR β1 subunit mRNA, complete coding sequence) at position codon<60-240 (Figure D.1). The reverse primer INT2_Rev (GTGGTAACCTGAGCACCTGT) (Table B.2) design was based on GenBank sequence JQ627836.1 (*A. gossypii* nAChR β1 subunit mRNA, complete cds) at position codon<202-345 (Figure D.1). As a complete genome is not available for *A. gossypii*, primers were blasted against the closely related pea aphid, *A. pisum* to check for sequence similarity. Primers were designed to amplify a 350 bp fragment overlapping the R81T mutation site.

### 4.4.6 RNA Extraction and cDNA synthesis

Briefly, 200 adult apterous female aphids per strain were pooled into individually labelled 1.5 mL eppendorf tubes (RNase free) and total RNA extracted from each strain using 500 μL of TriReagent® solution (Sigma-Aldrich, Australia). Samples were homogenised on ice, incubated at room temperature for 5 min and then centrifuged at 13,000 × $g$ for 15 min at 4°C. The supernatant was transferred to a clean microcentrifuge tube and 100 μL of bromochloropropane (Sigma-Aldrich, Australia)

was added. The sample was shaken vigorously, incubated for 5 min at room temperature, and then centrifuged at 13,000 × $g$ for 15 min at 4°C. Following centrifugation, the upper phase was transferred to a new pre-weighed microcentrifuge tube and an equal volume of 75% (v/v) ethanol added. After extraction, aliquots of each sample were added to an RNeasy mini spin column (Qiagen, Australia) and purified according to the manufacturer's protocol. An additional DNase treatment (RNase-free DNase set; Qiagen, Australia) was performed to eliminate potential genomic DNA contamination. RNA samples were quantified using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Australia). RNA (3-5 µg) was transcribed in subsequent cDNA synthesis utilising Superscript III Oligo(dT)$_{12-18}$ primers in a final volume of 20 µL according to the manufacturer's protocol (Thermo Fisher Scientific, Australia).

### 4.4.7 PCR amplification of R81T mutation site

PCR assay mixtures (25 µL) containing primers INT1_For (0.4 mM) and INT2_Rev (0.4 mM) and 2 µL of template DNA (20 ng) were subjected to the following cycling parameters: an initial denaturation for 2 min at 98°C, followed by 35 cycles at 98°C for 30 s, 51°C for 30 s and 72°C for 30 min, and a final extension for 5 min. Amplified PCR products were purified using the Wizard® SV Gel and PCR Clean Up System (Promega, Madison, WI) and quantified using the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Australia). Purified PCR products were sequenced by the Australian Genomic Research Facility (AGRF) (Westmead Millennium Institute, Westmead, NSW, 2145). Sequencing data was aligned to a reference imidacloprid resistant *A. gossypii* strain (GenBank accession number: JQ627836) containing the R81T mutation using Sequencher® (Version 5.3, Gene Codes Corporation).

## 4.5 Results

### 4.5.1 Dose responses with and without synergist

For strains F 101$_P$, Glen twn S$_P$ and Carr$_P$ full log-dose probit analysis yielded RFs of 4.00- (2.53-6.32), 7.37- (4.44-12.23) and 7.53- (5.52-10.27) fold against thiamethoxam (Table 4.1). As indicated by overlapping 95% confidence intervals at the LC$_{50}$ level no

significant differences between strain responses were observed. Calculated $LC_{50}$ values ranged from a low of 0.00030 g/L to a high of 0.00056 g/L in strains F 101$_P$ and Carr$_P$ respectively (Table 4.1). In contrast, $LC_{99}$ values ranged from a low of 0.020 g/L in strain F 101$_P$ to a high of 0.082 g/L in strain Glen twn S$_P$ (Table 4.1). Significant synergism was observed for the P450 inhibitor, PBO, in strains F 101$_P$, Glen twn S$_P$ and Carr$_P$ as indicated by non-overlapping 95% confidence intervals at the $LC_{50}$ level (Table 4.1). The $LC_{50}$ values of strains F 101$_P$ and Carr$_P$ and in comparison to Sus F 96 were lower in the presence of PBO suggesting complete synergism of resistance. In strain Glen twn S$_P$ the $LC_{50}$ of thiamethoxam in the presence of PBO was slightly higher than in strain Sus F 96. In contrast, in strain Sus F 96, the effect of PBO was negligible.

**Table 4.1** Probit mortality data for thiamethoxam + PBO against *Aphis gossypii* strains susceptible F 96 and resistant pressured F 101$_P$, Glen twn S$_P$ and Carr$_P$.

| | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | Thiamethoxam | | | Thiamethoxam + PBO[1] | | |
| **Strain** | No. of aphids treated | LC50 (95% FL) | RR[a] (95% CI) | No. of aphids treated | LC50 (95% FL) | RR[a] (95%CI) |
| **Sus F 96** | 544 | 0.000074 (0.000063-0.000086) | - | 568 | 0.000061 (0.000050-0.000072) | - |
| **F 101$_P$** | 607 | 0.00030 (0.00017-0.00044) | 4.00 (2.53-6.32) | 542 | 0.000052 (0.000014-0.00011) | 0.85 (0.33-2.19) |
| **Glen twn S$_P$** | 598 | 0.00055 (0.00029-0.00084) | 7.37 (4.44-12.23) | 523 | 0.00017 (0.000096-0.00024) | 2.74 (1.68-4.47) |
| **Carr$_P$** | 585 | 0.00056- (0.00042 0.00072) | 7.53 (5.52-10.27) | 601 | 0.000015 (0.000001-0.000053) | 0.24 (0.039-1.51) |

[1]PBO was applied with thiamethoxam, i.e. no pre-treatment. FL, fiducial limits; CI, 95% confidence intervals; [a]RR = LC50 resistant strain/ $LC_{50}$ susceptible strain.

### 4.5.2 PCR amplification of complementary DNA containing the R81T mutation site

Amplification of the loop D region of the nAChR $\beta1$ subunit gene produced 350 bp of quality cDNA sequence in strains Sus F 96, F 101, Glen twn S and Carr (Figure 4.1). Amplification of the loop D region of the nAChR $\beta1$ subunit gene was successfully confirmed by alignment to *A. gossypii* strain IMI-R (Imidacloprid resistant, GenBank accession number: AFH00994.1) Soybean aphid *Aphis glycines* Matsumura (GenBank accession number: JN681174.1) and *M. persicae* (GenBank accession number: AJ251838.1) (Figure 4.1). Unexpectedly, the amino acid substitution at position 81, resulting in an arginine (R) to threonine (T) substitution was present in strain IMI-R (China) but absent in Australian strains Sus F 96, F 101, Glen twn S and Carr (Figure 4.1).

```
Sus F 96      --------MAVFFVCSQFIRGCWCSEDEERLVRDLFRGYNKLIRPVQNMTEKVNVQFGLA 52
F 101         --------MAVFFVCSQFIRGCWCSEDEERLVRDLFRGYNKLIRPVQNMTEKVNVQFGLA 52
Glen twn S    --------MAVFFVCSQFIRGCWCSEDEERLVRDLFRGYNKLIRPVQNMTEKVNVQFGLA 52
Carr          --------MAVFFVCSQFIRGCWCSEDEERLVRDLFRGYNKLIRPVQNMTEKVNVQFGLA 52
IMI-R         MNTPVGLLMAVFFVCSQFIRGCWCSEDEERLVRDLFRGYNKLIRPVQNMTEKVNVQFGLA 60
A. glycines   MNTPVGLLMAVFFVCSQFIRGCWCSEDEERLVRDLFRGYNKLIRPVQNMTEKVNVQFGLA 60
M. persicae   MNTSVGLLMAVFFVCSQFIRGCWCSEDEERLVRDLFRGYNKLIRPVQNMTEKVNVQFGLA 60
                      ********************************************* *****

Sus F 96      FVQLINVNEKSQIMKSNVWLRLVWRDYQLQWDEADYGGIQVLRLPPDKVWKPDIVLFNNA 112
F 101         FVQLINVNEKSQIMKSNVWLRLVWRDYQLQWDEADYGGIQVLRLPPDKVWKPDIVLFNNA 112
Glen twn S    FVQLINVNEKSQIMKSNVWLRLVWRDYQLQWDEADYGGIQVLRLPPDKVWKPDIVLFNNA 112
Carr          FVQLINVNEKSQIMKSNVWLRLVWRDYQLQWDEADYGGIQVLRLPPDKVWKPDIVLFNNA 112
IMI-R         FVQLINVNEKSQIMKSNVWLTLVWRDYQLQWDEADYGGIQVLRLPPDKVWKPDIVLFNNA 120
A. glycines   FVQLINVNEKSQIMKSNVWLRLVWRDYCLQWDEADYGGIQVLRLPPDKVWKPDIVLFNNA 120
M. persicae   FVQLINVNEKSQIMKSNVWLRLVWRDYCLQWDEADYGGIQVLRLPPDKVWKPDIVLFNNA 120
                      *****************       *******************************
                                   Loop D
Sus F 96      DGN----------------------------------------------------- 115
F 101         DGN----------------------------------------------------- 115
Glen twn S    DGN----------------------------------------------------- 115
Carr          DGN----------------------------------------------------- 115
IMI-R         DGNYEVRYKSNVLIRPNGELLWIPPAIYQSSCTIDVTYFPFDQQTCIMKFGSWTFNGDQV 180
A. glycines   DGNYEVRYKSNVLIRPNGELLWIPPAIYQSSCTIDVTYFPFDQQTCIMKFGSWTFNGDQV 180
M. persicae   DGNYEVRYKSNVLIRPNGELLWIPPAIYQSSCTIDVTYFPFDQQTCIMKFGSWTFNGDQV 180
              ***
```

**Figure 4.1** Amino acid alignment of a partial sequence of nAChR β1 subunit from *Aphis gossypii* strains Sus F 96, F 101, Glen twn S, Carr and IMI-R (Imidacloprid resistant, GenBank accession number: AFH00994.1), including partial nAChR β1 subunit gene sequences from two related aphid species *Aphis glycines* (GenBank accession number: JN681174.1) and *Myzus persicae* (GenBank accession number: AJ251838.1) resulting from the ClustalW method. A conserved loop (Loop D) within the ligand binding domain is marked by a red box. A known point mutation site (R81T) in the loop D region of the β1 subunit is marked in bold.

## 4.6 Discussion

The extensive use of neonicotinoid compounds against *A. gossypii* in Australian cotton fields has led to the development of resistance (Herron and Wilson 2011). Target site insensitivity via mutations in nAChR subunits have repeatedly been implicated as causal mechanism(s) of neonicotinoid resistance in many insect species (Liu et al. 2005, Bass et al. 2011, Shi et al. 2012, Puinean et al. 2013, Kim et al. 2015). Those reports demonstrated a reduced binding affinity of neonicotinoid compounds at their target site as one of the main reasons for resistance. Surprisingly then, my sequencing data

presented here shows that the mutation (R81T in loop D of the nAChR β1 subunit) responsible for resistance in overseas aphid species, is not present in my strains. Encouragingly, it is consistent with the recent finding of Pan et al. (2015) who demonstrated a thiamethoxam resistant strain of *A. gossypii*, was also not linked to the R81T mutation (Pan et al. 2015).

It is interesting then that strains of *A. gossypii* where the R81T mutation has been demonstrated, seem to anecdotally show resistance strongly correlated to imidacloprid (Shi et al. 2012, Koo et al. 2014). Although there is confirmed cross resistance between members of the neonicotinoid MoA group 4A (Wang et al. 2007), the spectrum of resistance displayed between members is confounding; some reports show evidence of cross resistance between all group 4A members (Koo et al. 2014) and others demonstrate resistance to one and susceptibility to another (Shi et al. 2011). For example, Shi et al. (2011) demonstrated no cross resistance in an imidacloprid resistant strain of *A. gossypii* to dinotefuran, clothianidin or thiamethoxam. Similarly, preliminary discriminating dose data in my laboratory (obtained via pressuring whole cotton plants with 0.004 g a.i./L imidacloprid (Confidor® 200 g/L) and transferring resistant aphids to the pressured plant once dried) also demonstrated that strains F 101, Glen twn S and Carr were susceptible to imidacloprid..

In Australia, both imidacloprid and thiamethoxam are available as pre germination seed treatments; however, 80% of cotton seed planted is coated with thiamethoxam (Herron and Wilson 2011). For that reason, I consider the limited use of imidacloprid in Australian cotton may be responsible for the metabolic resistance detected rather than target site resistance associated with imidacloprid. Clearly, in my study there is reasonable evidence to suggest that neonicotinoid resistance is likely conferred by metabolic detoxification enzymes. Such a conclusion is consistent with the findings of Khan et al. (2015) who demonstrated that PBO increased toxicity of thiamethoxam against a laboratory selected resistant strain of house fly *M. domestica*, (Khan et al. 2015). In their study Khan et al. (2015) demonstrated via biochemical analyses revealed that mixed function oxidase activity in their thiamethoxam resistant strain was significantly higher than their susceptible strain, suggesting that P450-mediated resistance was involved. Monoxygenase based resistance has also been detected in

thiamethoxam resistant western flower thrips *F. occidentalis* again based on significant PBO synergism (Gao et al. 2014). Furthermore, in the closely related aphid species *M. persicae*, pre-treatment with PBO via topical bioassays substantially synergised the effect of four neonicotinoid insecticides in a neonicotinoid-resistance clone of *M. persicae* (5191A clone) (Puinean et al. 2010). In their resistant 5191A clone, over-expression of a single P450 gene was revealed and attributed at least in part, to gene amplification (Puinean et al. 2010). In complete agreement then to the above studies, I observed thiamethoxam survival times of resistant aphids to be at least decreased in the presence of PBO, and in two strains complete susceptibility was restored.

It should be noted in this study that aphids were not pre-treated with PBO and control mortality did not exceed 10%. I found in preliminary testing the use of two separate sprays (one for PBO and one for thiamethoxam) ultimately doubled the amount of aqueous deposit present on the leaf surface and in some instances aphids were found drowned. As such, I decided all testing should comprise one simultaneous application of PBO and thiamethoxam. It is well known that synergists can be reliably used simultaneously with the application of insecticide (Scott 1990) as I have done here but the potential synergistic effect of PBO may be underestimated. Consequently it is not unreasonable to speculate that thiamethoxam resistance may be fully suppressed (rather than just two of three strains) by PBO with further experimentation, i.e. by employing PBO + insecticide time release formulations.

Overall, this study demonstrated the potential of synergists to reverse resistance in some instances. However, when using synergists alone, the inclusion of positive data only is often not enough to attribute resistance to a specific detoxifying enzyme (Raffa and Priester 1985). This is because synergists are often capable of detoxifying more than one resistance associated enzyme. For instance, past studies have shown that PBO does not exclusively synergise P450s and instead has been shown to effectively synergise resistance-associated ESTs linked to pirimicarb in *A. gossypii* (Bingham et al. 2008) and spinosad resistance in *F. occidentalis* (Herron et al. 2014). Thus, in the future, extending this study to the transcriptome level to identify any changes in gene expression of transcripts relating to metabolic detoxification through comparative

transcriptome analysis of susceptible and resistant aphids would be extremely beneficial.

# Chapter 5. Characterisation of the cotton aphid *Aphis gossypii* transcriptome under thiamethoxam stress identifies transcripts associated with insecticide resistance

## 5.1 Abstract

The neonicotinoid insecticide, thiamethoxam is an agonist of nAChRs and is effective at controlling sucking insect pests such as the cotton aphid *A. gossypii*. Despite reports of target site insensitivity acting as a primary mechanism of neonicotinoid resistance in *A. gossypii*, recent Australian research (see Chapter 4) suggests detoxification can play a major role. For that reason Illumina NGS technology was employed to identify differentially expressed genes (DEGs) in response to thiamethoxam stress, by comparing the transcriptomes of three thiamethoxam resistant *A. gossypii* strains (F 101, Glen twn S and Carr) to a known reference susceptible (Sus F 96). Bioinformatics analysis revealed a number of significantly DEGs in resistant strains as candidates for a role in thiamethoxam resistance (P≤0.001). Expression levels of heat shock proteins (Hsps), P450s, and proteins with choline or glucose dehydrogenase activity were significantly up-regulated in the resistant strains compared to the susceptible. Genes encoding nAChR subunits and multidrug resistance-associated proteins were dramatically decreased. Significant DEGs were subsequently assigned to known Gene Ontology (GO) categories to predict their functional roles and associated biological processes. Transcripts (CL1190 and CL1418) similar to cytochrome P450 *CYP6K1* from *A. pisum* represented the only P450 up-regulated in all three resistant strains, but not significantly in strain F 101. Transcript expressions (CL1190 and CL1418) were confirmed by qRT-PCR and the trends in gene expression observed by qRT-PCR matched those of the Illumina expression profiles. Cytochrome P450 *CYP6K1* emerged as the strongest candidate for further investigation into a role in conferring resistance to thiamethoxam in *A. gossypii*.

## 5.2 Introduction

*A. gossypii* is a highly destructive and polyphagous sucking-insect pest with a worldwide distribution (Blackman and Eastop 2000). It effects a broad range of host

plants belonging to Cucurbitaceae (melon, watermelon and pumpkin) Malvaceae (cotton and okra) and Solanaceae (pepper and zucchini) (Blackman and Eastop 2000). It causes damage both directly and indirectly by feeding on the phloem sap of young plants and by acting as a viral vector (Leclant and Deguine 1994). If *A. gossypii* is present in high numbers late in the cotton growing season, honeydew contamination of the open boll lint can severely impact the quality of cotton fibre produced (Schepers 1989). *A. gossypii* has demonstrated a high propensity for developing insecticide resistance and routinely develops resistance to insecticides soon after they are released for its control (Whalon et al. 2008).

Since their commercial, neonicotinoid insecticides (group 4A) have become the most widely used chemical class for the control of sucking and chewing insect pests on cotton, including *A. gossypii* (Jeschke and Nauen 2008). This group is classified according to the IRAC as nAChR agonists (Sparks and Nauen 2015) and includes the insecticides acetamiprid (Yamada et al. 1999), clothianidin (Ohkawara et al. 2002), dinotefuran (Wakita et al. 2003), imidacloprid (Elbert et al. 1991) and thiamethoxam (Maienfisch et al. 2001). Neonicotinoid insecticides are extremely valuable as they circumvent already established resistance mechanisms which have evolved in *A. gossypii* to insecticides belonging to the organophosphate, carbamate and pyrethroid classes (Jeschke and Nauen 2008).

Recently, in Australian cotton, resistance to several neonicotinoid compounds has been demonstrated in field populations of *A. gossypii* (Herron and Wilson 2011). Since this initial detection of resistance, use of neonicotinoid insecticides in Australian cotton has remained high. In fact, between the 2008-09 and 2010-11 cotton seasons, the percentage of cotton seed planted that was coated with a neonicotinoid insecticide rose from 80 to 92% (APVMA 2013). Unsurprisingly, in the 2010-11 cotton season, routine monitoring of Australian *A. gossypii* field populations identified neonicotinoid resistance in 96% of strains tested (Herron 2012). Three of these strains (F 101, Glen twn S and Carr) were selected for further full log dose probit analysis to reveal any changes in the magnitude of resistance since field failures were first reported (Chapter 2). RFs associated with these strains were considerably higher than those documented by Herron and Wilson

(2011) in the 2008-09 cotton season and suggested field control failures would result if selection pressure wasn't reduced.

Molecular tools offer a cost effective approach for large scale resistance monitoring that underpins successful resistance management. Previous work by others implicated target site insensitivity via a point mutation (R81T) in the nAChR β1 subunit as the causal mechanism of neonicotinoid resistance in *A. gossypii* (Shi et al. 2012, Koo et al. 2014, Kim et al. 2015). However, an alternative resistance mechanism to neonicotinoids is enhanced oxidative detoxification via overexpression of P450s (see Chapter 4). The P450s are a diverse enzymatic system capable of many functions that range from the synthesis and degradation of endogenous compounds to the metabolism of xenobiotic compounds (Guengerich 2001, Feyereisen 2005). In two biotypes (B and Q) of *B. tabaci*, overexpression of two P450 genes, *CYP6CM1* and *CYP4C64* have been strongly correlated to imidacloprid resistance (Karunker et al. 2008, Yang et al. 2013). In *M. persicae*, metabolic detoxification via gene amplification of a single P450 gene (*CYP6CM1*) has been attributed to neonicotinoid resistance (Stern et al. 2010, Bass et al. 2011). Therefore, overexpression of P450 gene(s) may be a route of neonicotinoid resistance in Australian populations of *A. gossypii*.

Here I employ Illumina NGS technology to identify DEGs in response to thiamethoxam stress, by comparing the transcriptomes of three thiamethoxam resistant *A. gossypii* strains (F 101, Glen twn S and Carr) to a known reference susceptible (Sus F 96).

## 5.3 Methods and Materials

### 5.3.1 Aphids

A reference susceptible strain (Sus F 96) was maintained on insecticide free cotton in an insect proof cage at $25 \pm 4°C$ under natural light at the EMAI, Camden. Three resistant *A. gossypii* strains F 101, Glen twn S and Carr were collected from commercial cotton and produced $LC_{50}$ level resistance of 49-, 51-, and 85-fold against thiamethoxam (refer to Chapter 2). Strains F 101 and Glen twn S were routinely pressured monthly by exposure to foliar sprays at double the discriminating dose (i.e. 0.05 g a.i./L) of

thiamethoxam to maintain resistance. Strain Carr was pressured in the same manner but at the higher dose of 0.1 g a.i./L (Table A.1).

### 5.3.2 *Aphis gossypii* cDNA library construction and sequencing

Total RNA was extracted using Tri Reagent® solution (Sigma-Aldrich, NSW, Australia) following the manufacturers protocol. Per strain, 200 adult female apterous aphids were pooled into individually labelled 1.5 mL microcentrifuge tubes (Rnase free) and each sample homogenised on ice in 500 µl of Tri Reagent® (3:1 Tri Reagent to sample ratio). Samples were allowed to incubate for 5min at room temperature and then centrifuged at 13,000 × $g$ for 15 min at 4°C. Following centrifugation the resulting supernatant was transferred to a clean microcentrifuge tube and one hundred microliters of bromochloropropane was added. The sample was shaken vigorously and allowed to incubate for 5 min at room temperature (25°C), after which the samples were centrifuged a second time at 13,000 × $g$ for 15 min at 4°C. Following centrifugation, the upper phase was transferred to a new pre-weighed microcentrifuge tube and an equal volume of 75% (v/v) ethanol added. After extraction, aliquots of each sample were then added to an RNeasy mini spin column (Qiagen, Victoria, Australia) and the manufacturer's protocol followed. An additional DNase treatment (RNase-free DNase set, Qiagen) was performed to eliminate potential genomic DNA contamination. Aliquots of each sample were then added to an RNeasy mini spin column (Qiagen, Australia) and treated with RNase-free DNase I (Qiagen, Australia) following the manufacturers protocol. RNA integrity was determined by gel electrophoresis and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Australia).

Approximately 10-20 µg total RNA per strain were sent to the Beijing Genomics Institute, Shenzhen, China for cDNA library construction and Illumina sequencing. To isolate mRNA, magnetic beads with Oligo (dT) were used and mRNA was fragmented using a fragmentation buffer. Using the cleaved shorter mRNA fragments as templates, random hexamer primers were used to synthesize first strand cDNA. Second strand cDNA was generated using DNA polymerase I and RNaseH. The double stranded cDNA fragments, after end repair using T4 DNA polymerase and adaptor ligation, were

amplified by PCR and used as templates. The cDNA libraries were sequenced using the Illumina HiSeq 2000 (see Figure E.1 for a schematic)

### 5.3.3 Assembly and functional annotation

Transcriptome de novo assembly was carried out using the Trinity short reads assembling program (Grabherr et al. 2011). Unigenes larger than 150 bp were firstly aligned to the protein databases of non-redundant (NR), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), COGs and GO using blastx (e-value<0.00001) and then aligned by blastn to nucleotide databases (NT) (e-value<0.00001). The BLAST results were used to perform a tentatively functional annotation of the unigenes. The sequence orientations of the resulting unigenes were determined based on the best match in each protein database. In the case of conflicting results from different databases, the sequence annotation of the unigenes was resolved according to the following priorities: nr > Swiss-Prot > KEGG > COG. ESTScan software was also used to determine the annotation of sequences that were not aligned to any of the databases mentioned above (Iseli et al. 1999). The Blast2GO program (Conesa et al. 2005) was used for GO annotation of the transcripts and the WEGO software (Ye et al. 2006) to plot the GO annotation results.

### 5.3.4 Analysis of transcript expression differences between resistant and susceptible transcriptomes

Gene expression levels were calculated by mapping clean reads to the reference transcriptome using SOAPaligner / SOAP2 (http://soap.genomics.org.cn/soapaligner.html) (Li et al. 2009). No more than 2 mismatches of bases were allowed in the alignment. Then the Reads Per Kilobase of transcript per Million mapped reads (RPKM) value for each transcript was measured in reads per kilobase of transcript sequence per million mapped reads using the formula: RPKM (A) = (1,000,000*C) / (N*L*1,000), where RPKM (A) denotes the expression of gene A, C is the number of reads that aligned uniquely to gene A, N is the total number of reads that aligned uniquely to all genes and L is the number of base pairs in gene A (Mortazavi et al. 2008). Using this method, I was able to eliminate the influence

of gene length and sequencing discrepancy on the calculation of gene expression level, thus allowing comparison of gene expression between samples.

To identify DEGs between samples, an algorithm derived from "The significance of digital gene expression profiles" was used (Audic and Claverie 1997). A Benjamini multiple-testing correction of the p-value was performed using the false discovery rate (FDR) (Benjamini and Yekutieli 2001). In my analysis, FDR≤0.001 and the absolute value of the $\log_2$ Ratio≥1 were thresholds for determining the significance of gene expression differences (Benjamini and Yekutieli 2001).

### 5.3.5 Quantitative RT-PCR

Four differentially expressed transcripts between thiamethoxam resistant and susceptible strains of *A. gossypii* from RNA-seq were selected for independent validation of their gene expression via qRT-PCR. Approximately 200 adult female apterous *A. gossypii* per strain were transferred into individually labelled 1.5 mL microcentrifuge tubes (RNase free) for RNA extraction and subsequent cDNA synthesis. RNA was extracted using Tri Reagent (Sigma-Aldrich, Australia) following the manufacturer's protocol and as described above. After extraction, aliquots of each sample were then added to an RNeasy mini spin column (Qiagen, Australia) and the manufacturer's protocol followed. An additional DNase treatment (RNase-free DNase set, Qiagen) was performed to eliminate potential genomic DNA contamination. RNA purity was determined using a 2100 Bioanalyser (Agilent Technologies, Integrated Sciences, Australia). Approximately 25 µg DNaseI treated total RNA isolated from each strain was reverse transcribed to cDNA using 0.5 µg of oligo(dT)$_{12\text{-}18}$ primer in a 10 µL reaction (Invitrogen Pty Ltd., Australia). The synthesized cDNAs were used as templates for qRT-PCR in a 7500 Real-Time PCR Detection System (Applied Biosystems, Australia). qRT-PCR reactions were performed in triplicate and fold changes in gene expression between resistant and susceptible strains were derived by the comparative CT method using the endogenous control β-actin to standardize expression. Gene specific primers were designed using Primer3Plus (Untergasser et al. 2007) and synthesized by Sigma Aldrich®, Australia. Primer sequences are listed in Table B.3. The reaction mixture (20 µL) contained 10 µL of SYBR® Select master mix

(Life Technologies, Australia), 1.8 μL each of forward and reverse primers (400 mM total), and 2 μL of cDNA template (equivalent to 50 ng of total RNA). The amplification was conducted using the following cycling parameters: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Upon completion of every run, a dissociation protocol (melt curve analysis) was generated to assess the purity of the amplified products. The expression levels were calculated according to the 2-$\Delta\Delta$CT method (Livak and Schmittgen 2001). The fold change in the target gene was normalized to the internal control gene and relative expression levels were $\log_2$ transformed (Livak and Schmittgen 2001).

## 5.4 Results

### 5.4.1 Illumina sequencing and sequence assembly

A total of 39, 33, 31, and 29 million raw reads were obtained from strains Carr, Sus F 96, F 101, and Glen twn S, respectively after filtering out dirty raw reads (Table 5.1). When pooled, the total number of raw reads obtained from the four individual transcriptomes totalled 143,723,328 and 132,159,760 clean reads de novo assembled. A total of 37,167 contigs were assembled with an $N_{50}$ length of 906 bp. The contigs were further assembled into 31,042 unigenes with an $N_{50}$ of 1337 bp. The size distributions of the unigenes and contigs are shown in Figures E.2 and E.3 Of these assembled unigenes, 13434 (43.28%) unigenes were >N500 bp in length and 7107 (22.89%) unigenes were >N1000 bp.

**Table 5.1** Summary of reads and assembly from Illumina sequencing for *Aphis gossypii* strains: reference susceptible Sus F 96 and thiamethoxam resistant F 101, Glen twn S and Carr.

|  | F 101 | Glen twn S | Carr | Sus F 96 | Combined |
|---|---|---|---|---|---|
| **Total base pairs** | 1,512,190,960 | 1,424,215,968 | 1,913,637,964 | 1,625,783,348 | |
| **Total number of reads** | 30,861,040 | 29,065,632 | 39,053,836 | 33,179,252 | 132,159,760 |
| **GC percentage** | | | | | 42.70% |
| **Q20 percentage** | | | | | 98.50% |
| **Total number of all contigs** | | | | | 37,167 |
| **Mean length of all contigs** | | | | | 506 |
| **The number of all unigenes** | | | | | 31,042 |
| **Mean length of all unigenes** | | | | | 765 |

## 5.4.2 Gene ontology (GO) and Clusters of orthologous groups (COGs) classification

A total of 23,372 (89.75% of all distinct sequence), 16506 (63.38%) and 15460 (59.37%) transcripts were annotated by NR, Swiss-prot, and KEGG, respectively. The identified *A. gossypii* unigenes were most similar to *A. pisum* and a high degree of sequence homology (91.6%) between these species was revealed (Figure 5.1).

## (A) E-value Distribution

## (B) Similarity Distribution

## (C) Species Distribution
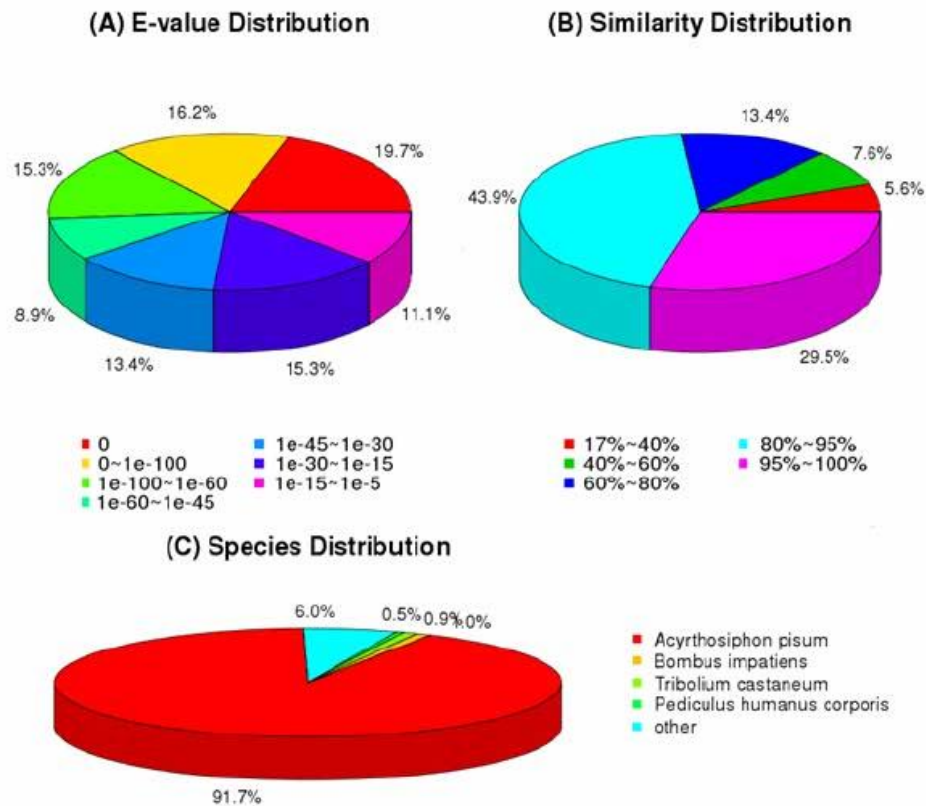
**Figure 5.1** Pie-charts showing distributions from BLASTx matches of pooled *Aphis gossypii* transcriptome unigenes with respect to (A) E-values (B) gene identity and (C) insect species from which the homologous genes were matched to.

COG analysis identified a total of 7,633 transcripts (29.31%) classed into 25 functional categories (Figure 5.2), the largest five being "general function prediction only" (2572 genes), "transcription" (1249 genes), "replication, recombination and repair" (1247 genes), "translation, ribosomal structure and biogenesis" (1014 genes) and "carbohydrate transport and metabolism" (987 genes). "RNA Processing and Modification" (83 genes), "Extracellular structures" (6 genes) and "Nuclear transport" (4 genes) represented the smallest categories.
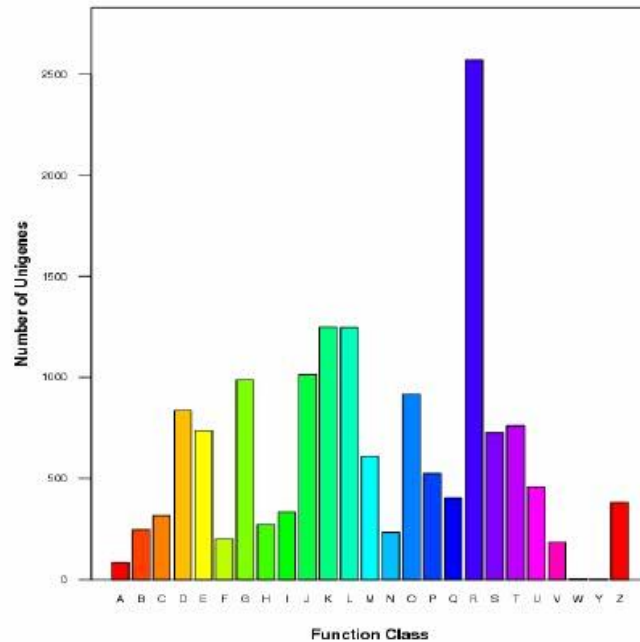
**Figure 5.2** Orthologous Groups (COG) of protein function classification of *Aphis gossypii* unigene sequences (a total of 7633 unigenes were grouped into COG function classifications). A: RNA processing and modification, B: Chromatin structure and dynamics, C: Energy production and conversion, D: Cell cycle control, cell division, chromosome partitioning, E: Amino acid transport and metabolism, F: Nucleotide transport and metabolism, G: Carbohydrate transport and metabolism, H: Coenzyme transport and metabolism, I: Lipid transport and metabolism, J: Translation, ribosomal structure and biogenesis, K: Transcription, L: Replication, recombination and repair, M: Cell wall/membrane/envelope biogenesis, N: Cell motility, O: Posttranslational modification, protein turnover, chaperones, P: Inorganic ion transport and metabolism, Q: Secondary metabolites biosynthesis, transport and catabolism, R: General function prediction only, S: Function unknown, T: Signal transduction mechanisms, U: Intracellular trafficking, secretion, and vesicular transport, V: Defence mechanisms, W: Extracellular structures, Y: Nuclear structure, Z: Cytoskeleton.

GO analysis identified 10,488 transcripts (40.27%) which were categorized into 48 GO terms consisting of three domains: "biological process", "cellular component" and

"molecular function" (Figure 5.3). Of the 48 terms, "cellular process", "metabolic process", "cell", "binding" and "catalytic activity" were over-represented, whilst "extracellular matrix part", "antioxidant activity" and electron carrier activity" were under-represented. The terms "cell killing", "virion", "virion part" and "channel regulator activity" were absent.
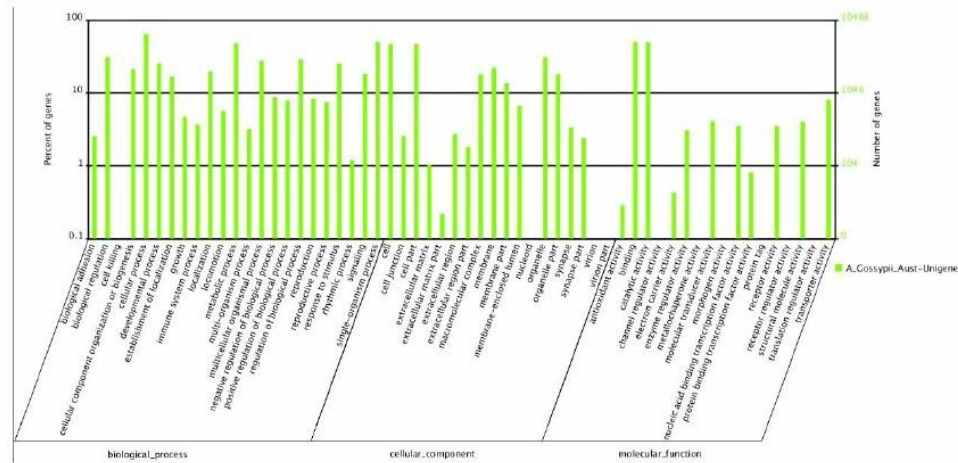
**Figure 5.3** GO annotations of all combined unigenes and DEG sequences. GO categories shown in the x axis are grouped into three main ontologies: biological process, cellular component and molecular function. The right y-axis indicates the number of genes in each category, while the left y-axis indicates the percentage of total genes in that category. The 'A_Gossypii_Aust_unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptome of all strains.

### 5.4.3 Network of unigene

31042 unigenes were mapped to the reference canonical pathways in the KEGG database and 15460 of them obtained KEGG annotation and assigned to 255 pathways (Table E.1). Among them, the "metabolic pathway" was the largest group (2109 unigenes, 13.64%), followed by "RNA transport" (549, 3.55%), "focal adhesion" (516, 3.34%) and "regulation of actin cytoskeleton" (491, 3.18%). In contrast, the following pathways contained <10 unigenes: "Phenylalanine, tyrosine and tryptophan biosynthesis" (9, 0.06%), "Vitamin B6 metabolism" (8, 0.05%), "D-Arginine and D-ornithine metabolism" (6, 0.04%), "Lipoic acid metabolism" (5, 0.03%), "Thiamine metabolism" (5, 0.03%), "Lysine biosynthesis" (4, 0.03%), "D-Glutamine and D-glutamate metabolism" (2, 0.01%) and finally "Caffeine metabolism" with only one unigene (1, 0.01%).

### 5.4.4 Differential expression and pathway analyses in resistant vs susceptible strain combinations

The results revealed 24,299 genes with significantly differential expression levels between Sus F 96 and F 101 (Figure 5.4). Among them, 24,265 (99.86%) and 34 (0.0014%) genes were down-regulated and up-regulated, respectively, in strain F 101 compared to Sus F 96.
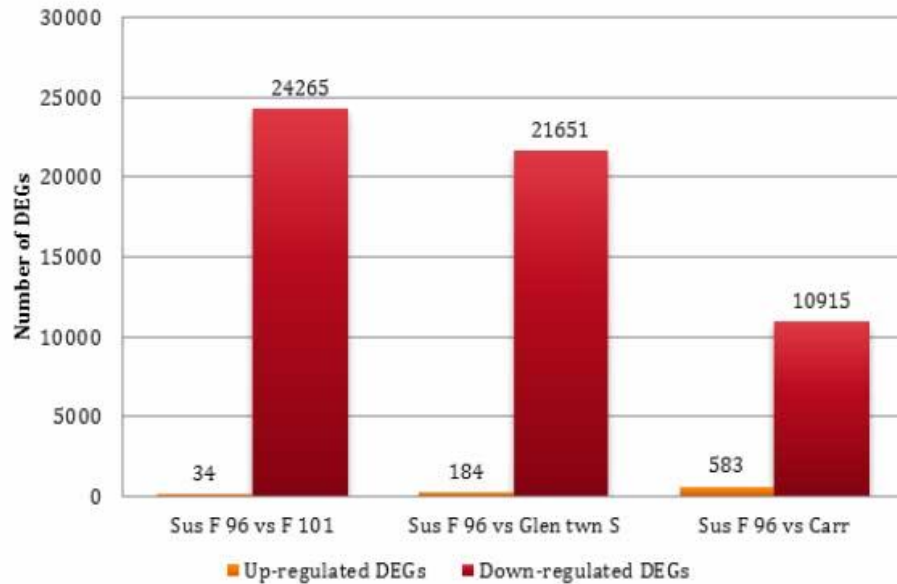
**Figure 5.4** Number of DEGs expressed between susceptible (Sus F 96) and thiamethoxam-resistant (F 101, Glen twn S and Carr) *Aphis gossypii* strains.

Providing further insights into twenty of the most differentially up-regulated genes between strain Sus F 96 and F 101 (Table E.2), significant matches included the gene homologous to one that encodes a hypothetical protein in the red imported fire ant *Solenopsis invicta* Buren; hypothetical protein in the Florida carpenter ant *Camponotus floridanus* (Buckley); replicase polyprotein of *Ascaris suum* (Goeze) (large roundworm of pigs); polyprotein-like protein of a Tsetse fly sp., *Glossina morsitans morsitans* Westwood; and ten predicted functional genes, including five heat shock protein 68-like (*A. pisum*), partial; two heat shock protein 70 B2-like (*A. pisum*) and three similar to gag-pol polyprotein (*T. castaneum*). The six remaining genes had no functional annotation. The top ten down-regulated genes in strain F 101 compared to Sus F 96 (Table E.2) included one gene homologous to exoribonuclease 1 (*A. pisum*) and eight predicted functional genes (major facilitator superfamily domain-containing protein 6-like isoform 1 (*A. pisum*); oligopeptidase A-like (*A. pisum*); protein msta, isoform A-like (*A. pisum*); two genes encoding probable multidrug resistance-associated protein lethal(2)03659-like (*A. pisum*); hypothetical protein LOC100159424 (*A. pisum*); ATP synthase subunit alpha-like (Common eastern bumblebee, *Bombus impatiens* Cresson)

and protein toll-like (*A. pisum*)). In total, twelve down-regulated genes had no functional annotation.

DEGs between Sus F 96 and F 101 were characterized into three groups from the GO classification: cellular component; molecular function; and biological process. The results showed that 3843, 6112 and 5472 DEGs were annotated to 299, 570 and 2054 GO terms of cellular component, molecular function, and biological process, respectively (corrected P-value ≤1) (Figure E.4). To further categorize, the DEGs were significantly enriched to fourteen cellular components, in which "ribonucleoprotein complex" was most strongly presented and the category, "cell" was the largest represented with 3010 DEGs (78.3%). The DEGs were significantly enriched to ten molecular functions, two of which contained the majority of DEGs: "catalytic activity" (3775, 61.8%) and "binding" (3598, 58.9%). Under the umbrella of "biological process", twenty three significantly enriched GO terms were reported between Sus F 96 and F 101. Those associated with "metabolic process" (3438, 62.8%) and "cellular process" (3979, 72.7%) accounted for the two largest represented terms, although the term "carboxylic acid metabolic process" (p-value 0.00570) was strongly presented. KEGG enrichment analysis revealed 11716 DEGs between Sus F 96 an F 101 which were assigned to 254 pathways. DEG enrichment analyses showed that the first four pathways that involved up- or down-regulated genes in response to thiamethoxam stress were "Ribosome" (104 unigenes), "Protein processing in endoplasmic reticulum" (257 unigenes), "Metabolic pathways" (1766 unigenes) and "RNA transport" (366 unigenes).

The comparison between Sus F 96 and Glen twn S also revealed significant variations in expression. A total of 21,835 genes, including 184 (0.0084%) up-regulated genes and 21,651 (99.16%) down-regulated genes were identified (Figure 5.4). Among the twenty most up-regulated genes (Table E.2), matches included the gene homologous to that which encodes GL24774 of ferment fly *Drosophila persimilis* Dobzhansky and Epling (N-glycan biosynthesis) and glutaredoxin-like (glutathione dependent reductase of *A. pisum*) and also eight predicted functional genes (maltase 2-like (*A. pisum*); *A. pisum* hypothetical proteins LOC100575926, LOC10056912, LOC100574103; centrosomal protein of 78 kDa-like (*A. pisum*); similar to SET domain and mariner transposase fusion of Hydra spp., *Hydra magnipapillata* Linnaeus; and deoxynucleotidyltransferase

terminal-interacting protein 1-like (*A. pisum*)). Ten highly up-regulated genes had no functional annotation. Of the top twenty down-regulated genes (Table E.2), nine had no functional annotation and eleven were predicted functional genes (*A. pisum* hypothetical proteins LOC100571774 partial, LOC100162722, LOC100574363, LOC100163439 and LOC100164810; nose resistant to fluoxetine protein 6-like (*A. pisum*); bifunctional purine biosynthesis protein purH-like (*B. impatiens*) (protein coding gene of IMP cyclohydrolase activity); x-ray radiation resistance-associated protein 1-like (*A. pisum*); probable multidrug resistance-associated protein lethal-like (*A. pisum*) (ATP-binding cassette, subfamily C (CFTR/MRP), member 4) and phosphatidylinositol 3-kinase catalytic subunit type 3-like (*A. pisum*) (enzymes involved in cellular functions)).

GO analysis between Sus F 96 and Glen twn S revealed 3543, 5652 and 5064 DEGs annotated to 294, 556 and 1975 terms of cellular component, molecular function and biological process, respectively (corrected P-value ≤1) (Figure E.5). DEGs were significantly enriched to fourteen cellular components, in which "cell" represented the largest with 2792 genes (78.8%). The terms "intracellular" with 2479 (70%) and "ribonucleoprotein complex" with 267 genes (7.5%) were the strongly presented with p-values of 2.85e-08 and 6.41e-08, respectively. Of those DEGs enriched to ten molecular functions, "binding" and "catalytic activity" were again the largest represented terms with 3323 (58.8%) and 3488 (61.7%) genes, respectively. Twenty three significantly enriched terms of the category biological process were reported between Sus F 96 and Glen twn S. Similarly, the terms "cellular process" (3677, 72.6%) and "metabolic process" (3203, 63.3%) were the largest. "Metabolic process" was also very strongly presented (p-value 0.00273), along with "translation" (176, 3.5%) which was slightly stronger (p-value 0.00031) than "metabolic process". Lastly, 10764 DEGs were assigned to 254 KEGG pathways. DEG enrichment analyses showed that the first four pathways that involved up- or down-regulated genes in response to thiamethoxam stress were "Ribosome" (104 unigenes), "Metabolic pathways" (1637 unigenes), "Herpes simplex infection" (159 unigenes) and "Shigellosis" (88 unigenes).

Finally, comparative analysis between Sus F 96 and Carr revealed 11,498 genes with significant expression profile changes, including 583 (0.05%) up-regulated genes and 10,915 (0.95%) down-regulated genes (Figure 5.4). Of the twenty most up-regulated

genes (Table E.2), eight were predicted function genes (*A. pisum* hypothetical protein LOC100573940, LOC100574035, LOC100574264 isoform 1, LOC100573859, LOC100574035 and LOC100570532; *A. pisum* maltase 2-like (alpha glucosidase)). Two genes have defined functions: *A. pisum* ACYPI000014 (cathepsin B) and GL24774 (*D. persimilis* N-glycan biosynthesis) and the remaining ten had no functional annotation. Among the ten most down-regulated genes (Table E.2), eighteen had no functional annotation, one was homologous to hypothetical protein of the Gulf Coast tick, *Amblyomma maculatum* Koch and one gene had predicted function of *A. pisum* hypothetical protein LOC100571804.

GO analysis revealed that 1503, 2371 and 2092 DEGs were annotated to 217, 392 and 1436 GO terms of cellular component, molecular function and biological process, respectively (corrected P-value ≤1) (Figure E.6). DEGs were significantly enriched to seventeen cellular components, in which "intrinsic to membrane" was most strongly presented and "cell" the largest category represented with 1113 DEGs (74.1%). Further, DEGs were significantly enriched to ten molecular functions, two of which contained the majority of DEGs: "catalytic activity" (1393 genes, 58.8%) and "binding" (1332, 56.2%). For the GO category, biological process, twenty three significantly enriched terms were reported between Sus F 96 and Carr. "Cellular process" (1498, 71.6%) and "metabolic process" (1242, 59.4%) contained the largest number of represented terms. Interestingly, the terms "cellular response to hormone stimulus" (p-value 0.87749) and "cellular response to endogenous stimulus" (p-value 0.87749) were the most strongly presented. Between strains Sus F 96 vs Carr, 4728 DEGs were assigned to 252 KEGG pathways. DEG enrichment analyses showed that the first four pathways that involved up- or down-regulated genes in response to thiamethoxam stress were "Cardiac muscle contraction" (61 unigenes), "Fatty acid elongation" (29 unigenes), "Neuroactive ligand-receptor interaction" (96 unigenes) and "Glycerophospholipid metabolism" (69 unigenes).

### 5.4.5 Candidate resistance (detoxification) genes

The expression of transcripts encoding potential resistance genes is shown in Figure 5.5. Only strains Glen twn S and Carr contained up-regulated genes relating to known

insecticide detoxification mechanisms, when compared to Sus F 96. Of these, all were contained to the P450 family, with 6 (Unigene15803, CL627, Unigene4712, CL1190, CL1418 and Unigene12819) and 7 (Unigene15803, CL627, CL1190, Unigene4712, Unigene12819, CL1418 and Unigene12511) genes up-regulated in strain Glen twn S and Carr, respectively (Figure 5.5 and Table E.3). Of these, three had predicted similarity to *CYP305A1* (Unigene15803, CL627 and Unigene4712), two to *CYP6K1* (CL1190 and CL1418) and two to *CYP6A13* (Unigene12511 and Unigene12819). Based on predicted matches, all were contained within the CYP2 and CYP3 clans. In contrast, strain F 101 contained no up-regulated genes in the CE, P450 or GST gene families and instead contained the highest number of down-regulated genes with 15, 54 and 30 down-regulated CEs, P450s and GSTs, respectively. It should be noted that contigs CL1190 and CL1418, although not significantly differentially expressed in strain F 101, were significantly up-regulated in strains Glen twn S and Carr when compared to Sus F 96 (Table E.3). No GSTs were found up-regulated in any of the resistant strains compared to Sus F 96. Down regulated GSTs showing similarity to the delta, omega, sigma and theta classes were found in some resistant strains. Finally, no CEs were up-regulated in resistant strains compared to Sus F 96.

Seven nAChR subunit gene sequences, including α1, α2, α3, α4, α7, β1, and β2 were matched against known genes. All nAChR subunit genes were downregulated in resistant strains, compared to Sus F 96 (Table E.4).
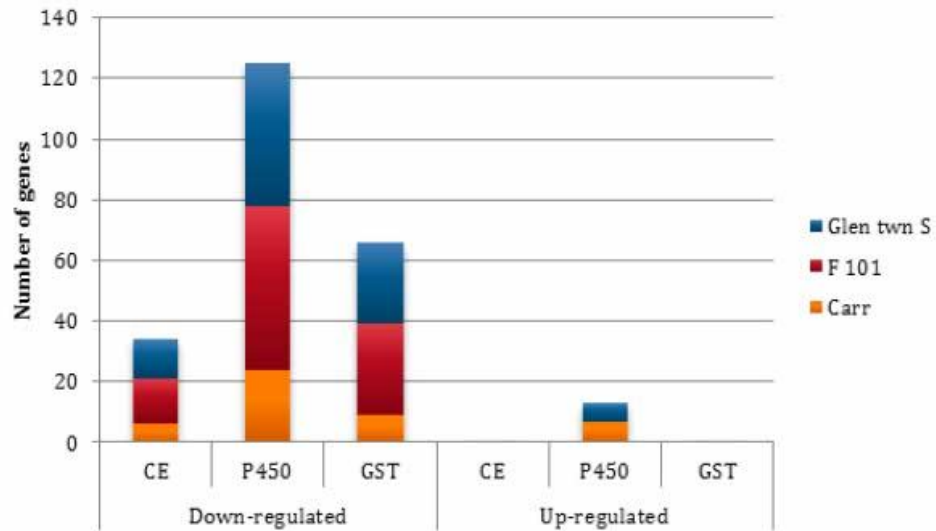
**Figure 5.5** Expression profiles of detoxification-related proteins in RNA-seq analysis of *Aphis gossypii* strains: thiamethoxam resistant strain (F 101, Glen twn S and Carr) compared to a reference susceptible strain (Sus F 96). CE, carboxylesterase; GST, glutathione-S-transferase; P450, cytochrome P450-dependent monoxygenases.

## 5.4.6 Quantitative RT-PCR

Four unigenes, of which two had identified functions relating to detoxification (Contig ID 1190 and 1418) and two matched an RNA virus (RhPV6) of the Bird cherry-oat aphid *Rhopalosiphum padi* (Linnaeus) (Contig ID 10451 and 10452) were selected for further validation. The over-transcription of genes, CL1190 and CL1418, identified from RNA-Seq analysis experiments were confirmed by qRT-PCR in all strain comparisons, although expression ratios obtained from qRT-PCR were frequently higher than those obtained from RNA-Seq analysis (Figure 5.6 and Table E.5). In contrast, expression ratios obtained from qRT-PCR for Unigenes 10451 and 10452, although showing similar trends in up-regulation of expression to RNA-Seq analysis were much smaller in value (Figure 5.6 and Table E.5).
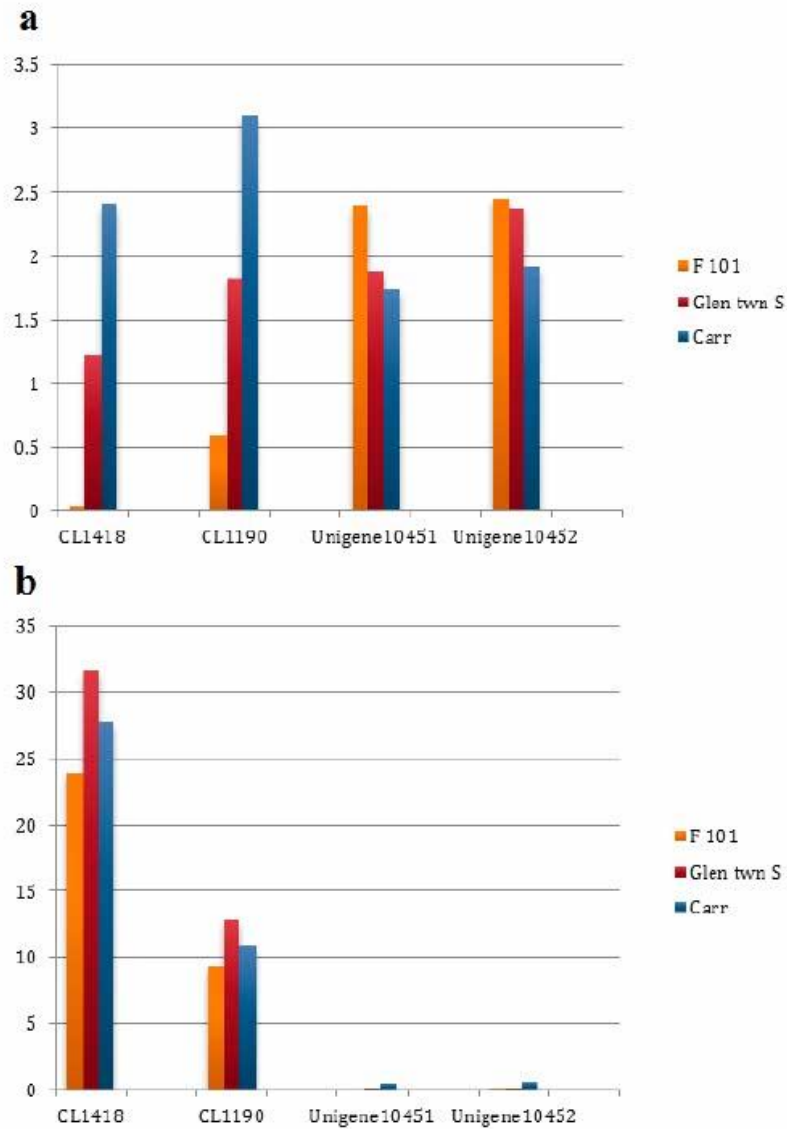
**Figure 5.6** Validation of gene expression of four transcripts selected from RNA-Seq analysis. (a) The fold change (log$_2$Ratio) for genes from RNA-Seq analysis between strain comparisons: F 101/Sus F 96; Glen twn S/Sus F 96; and Carr/Sus F 96 (b) The fold change of each gene was calculated by qRT-PCR using comparative threshold cycle method.

## 5.5 Discussion

The aim of this study chapter was to investigate insecticide resistance mechanisms associated with neonicotinoid resistant *A. gossypii* from Australian cotton. In the present de novo assembly, a total of 132,159,760 clean reads from the pooled transcriptomes of thiamethoxam resistant and susceptible *A. gossypii* strains were generated resulting in a dramatically increased repertoire of resistance-related genes in *A. gossypii* under thiamethoxam stress. Additionally, reads were assembled into 37,167 contigs with an average length of 506 bp and from this 31,042 unigenes were assembled of which 23,372 matched known genes. Therefore, this study has generated a comprehensive transcriptome resource for *A. gossypii* that has characterized the expression of numerous important transcripts encoding proteins involved in insecticide resistance. Consequently, this study will contribute to future research relating to molecular characterization of insecticide resistance mechanisms of *A. gossypii* and other insect pests.

P450s function in insects as enzymatic proteins involved in a vast number of metabolic processes including insecticide detoxification (Li et al. 2006, Schuler 2011). Although metabolic resistance mediated by overexpression of P450s may be triggered by modifications in *cis/trans* regulatory elements or post-translational events (Bass and Field 2011), correlations between gene amplification and overexpression of P450s have been implicated (Puinean et al. 2010, Faucon et al. 2015). According to previous research, the P450 gene families involved in up-regulation and amplification are CYP4, CYP6 and CYP9 (belonging to the CYP3 and CYP4 clans). In the present study, I found seven differentially expressed P450 unigenes (Contig ID 627, 1190, 1418, 4712, 12511, 12819 and 15803) belonging to the CYP2 and CYP3 clans that were up-regulated in strain Carr, and six of these up-regulated in Glen twn S (not contig 12511). In strain F 101, only two transcripts (Contig ID 1190 and 1418) were up-regulated when compared to Sus F 96 (although not significantly differentially expressed). Therefore, my transcriptome analysis centred on the role of contigs 1190 and 1418, despite one of the three strains not being significantly differentially expressed, although still up-regulated. These transcripts were predicted as the P450 gene *CYP6K1*, and when blast searched

against the NCBI database showed 68% and 82% similarity in amino acid sequence to *CYP6K1* of *A. pisum* (XP001948421.1). Contig 1190 matched the amino acid sequence of XP001948421.1 from 1-271 and of the same sequence contig 1418 matched from position 272-514 (Figures E7 and E.8). In the hemipteran insects, *B. tabaci* and *M. persicae*, over-expression of two CYP6 P450 genes (*CYP6CM1* and *CYP6CY3* respectively) has previously been linked to resistance of neonicotinoid insecticides (Karunker et al. 2008, Puinean et al. 2010, Yang et al. 2013). Transcriptional profiles of contigs 1190 and 1418 were validated by qRT-PCR and were found to be highly overexpressed in resistant strains despite significantly lower expression levels obtained from RNA-Seq analysis, especially for strain F 101. This may be explained by the well-known underestimation of expression ratios by RNA-Seq analysis compared with qRT-PCR (Roberts et al. 2011). Discrepancies in the data obtained from RNA-Seq analysis using the Illumina Hi-Seq™ platform and qRT-PCR highlight the importance of qRT-PCR validation of RNA-Seq results.

The expressions of several genes with catalytic/oxidoreductase activity (such as proteins with choline or glucose dehydrogenase activity) were differentially transcribed among resistant and susceptible strains, suggesting a possible relationship between the insecticide resistance phenotype and these physiological processes (Contig ID 273, 324 and 13767). Interestingly, two of these genes, contig 324, annotated as choline dehydrogenase ([EC:1.1.99.1]), and contig 13767, homologous to *A. pisum* gene ACYPI007791 which encodes dehydrogenase/reductase SDR family member 4, are constitutively overexpressed in all three thiamethoxam resistant strains. These genes encode subunits which function in the mitochondria and belong to complexes of the electron transport and respiratory chain. These observations strongly support the hypothesis that mitochondrial energy/redox metabolism are among the mechanisms partially responsible for detoxification of thiamethoxam. Similar trends in up-regulation of mitochondrial genes were recently reported in *A. gambiae* after Plasmodium infection (Kumar et al. 2003).

Among the DEGs, expression levels of Hsp70 family members were dramatically elevated in resistant strain F 101 (Contig ID 2116, 5014, 7780, 10435 and 11682). In insects, Hsps are modulated in response to a variety of chemical and physical stresses

such as heat shock, ultraviolet radiation, chemical pesticides, as well as biotic stresses such as viruses, bacteria and fungi (Parsell and Lindquist 1993, Feder and Hofmann 1999, Sørensen et al. 2003). Their up-regulation is an important part of the cellular stress response induced to maintain stress tolerance and promote cell survival through refolding proteins and preventing their denaturation (Parsell and Lindquist 1993, Feder and Hofmann 1999). Up-regulation of Hsps have been shown to contribute to pesticide tolerance and resistance (Nazir et al. 2001, Feng et al. 2010, Škerl and Gregorc 2010, Chen et al. 2014). For instance, in *D. melanogaster* and *B. mori*, overexpression of Hsp70 was induced by application of the insecticides, chlorpyrifos and pyridalyl, respectively (Nazir et al. 2001, Powell et al. 2011). Similarly, in a spirotetramat resistance strain of *A. gossypii*, up-regulation of five putatively designated Hsp70 unigenes was linked to the resistant phenotype. Therefore, in *A. gossypii*, up-regulation of Hsps may be indicative of an adaptive ability to protect tissues against oxidate stress induced by insecticides (Pournourmahammadi and Abdollahi 2011). Alternatively, as no differentially expressed Hsps were up-regulated in strains Carr and Glen twn S, the dramatically increased expression in strain F 101 may be related to other abiotic stressors, such as temperature (Jones and Zhao 2012). Indeed, the Hsp70 family appears to be the most prominent contributor to temperature tolerance in insects by enabling increased heat tolerance of organisms to protect them from thermal injury and death (Sørensen et al. 2003). This has been evidenced in numerous insect species, e.g. the Oriental fruit moth *Grapholita molesta* (Busck) (Chen et al. 2014); ferment flies: *Drosophila buzzatii* Patterson & Wheeler (Sorensen et al. 1999) and *D. melanogaster* (Nazir et al. 2001); *N. lugens* (Kim et al. 2008); and the Corn earworm, *Helicoverpa zea* (Boddie) (Zhang and Denlinger 2010).

The ABC transporter superfamily is the largest gene family involved in the transport of various substrates across biological membranes, including amino-acids, sugars, lipids, inorganic ions, polysaccharides, metals, peptides, toxic metabolites and drugs (Higgins 1992). Differential expression of certain transporters of B, C and G subfamilies in humans contributes to multidrug resistance of cancer cells against chemotherapeutics (Gottesman et al. 2002): the multidrug resistance proteins (MDR and ABCB subfamily) or P-glycoproteins (Gerlach et al. 1986, Dean et al. 2001); the multidrug resistance associated proteins (MRPs and ABCC subfamily) (Dean et al. 2001); and the breast

cancer resistance protein (BCRP and ABCG2 subfamily) (Doyle and Ross 2003). In insects, physiological functions of ABC transporters include roles in molecule transport, and functions that affect metabolism, development and also insecticide resistance (Dermauw and Van Leeuwen 2014). Some ABC transporters of subfamilies B, C and G have been shown to confer resistance to xenobiotics, including insecticides. For example, in the tobacco hornworm, *Manducta sexta* (Linnaeus), which feeds on nicotine containing tobacco leaves, nicotine is efficiently excreted by P-glycoprotein-like multidrug transporters in the Malpighian tubules (Murray et al. 1994). Also, in the diamondback moth, *P. xylostella*, down-regulation of a novel ABC transporter gene from ABCG subfamily (Pxwhite) is associated with resistance to a Cry toxin, Cry1Ac (Guo et al. 2015). In the present study, down regulation of several transcripts (Contig ID 7980 and 18326) that code for MRPs and include the ABC transporter cassette motif in their structures, are likely to contribute to thiamethoxam resistance in *A. gossypii*.

Genetic changes in genes encoding nAChR subunits are regarded as primary determinants of neonicotinoid resistance in insects (Liu et al. 2006, Bass et al. 2011, Shi et al. 2012, Puinean et al. 2013, Koo et al. 2014). In this study, all nAChR subunits identified through transcriptome analysis were downregulated in each resistant strain compared to Sus F 96. This is consistent with two other studies which have found reduced nAChR subunit expression in neonicotinoid resistant insects, including *A. gossypii*. A transcriptomic survey of thiamethoxam resistant *A. gossypii* by Pan et al. (2015) found downregulation of a nAChR α subunit may contribute to resistance. Elsewhere, in *M. domestica* and *N. lugens*, imidacloprid resistance is correlated with a reduction in expression levels of the nAChR subunits, α2 and Nlα8, respectively (Markussen and Kristensen 2010, Zhang et al. 2015). These results suggest that depletion of the nAChR subunits may relate to thiamethoxam resistance in Australian *A. gossypii*.

In conclusion, this study has contributed a substantial sequence resource for aphids and is likely to accelerate insecticide resistance mechanism research in *A. gossypii* when under thiamethoxam stress. Comparative transcriptome analysis identified a catalogue of candidate genes that might be involved in conferring neonicotinoid resistance in *A. gossypii*. In particular, some genes encoding Hsps, catalytic/oxidoreductase activity

(such as proteins with choline or glucose dehydrogenase activity), ABC transporters, cytochrome P450s and nAChR subunits might play crucial roles in conferring resistance to neonicotinoid compounds. Among the DEGs, up-regulation of cytochrome P450 *CYP6K1* and the role it plays in detoxifying thiamethoxam should be further investigated.

205 of 308

# Chapter 6.   General discussion

The worldwide problem of insecticide resistance has been documented in over 500 arthropod species and results in more frequent applications, increased dosages, decreased yields, and in some cases decreased sensitivity to new, more expensive compounds (Georghiou and Mellon 1983, Soderlund and Bloomquist 1990). Effective insecticide resistance management is crucial to preserving the utility of current and future insecticide chemistries. To prevent or delay the development of resistance in insect and mite pests, it is essential that we understand the mechanisms by which these species develop resistance so that we can implement management strategies to reduce selection on those target sites. During the past decade, with recent advances in high throughput sequencing technology, there have been many studies to uncover the genes, pathways and mechanisms responsible for insecticide resistance in insect pests which lack a fully sequenced genome (Niu et al. 2012, Silva et al. 2012, Zhang et al. 2012, Chen et al. 2014). This information not only dramatically improves our understanding of new mechanisms with regard to insecticide resistance but provides insight to potential tactics to manage pest populations.

For this reason, I completed a study to uncover the genes, pathways and mechanisms responsible for neonicotinoid resistance in *A. gossypii*. This required a multi-faceted approach centred around two main themes. The first was designed to investigate the current status and implications of neonicotinoid resistance in *A. gossypii* collected from Australian cotton using bioassay, molecular genetic based methods as well as a whole plant efficacy trial. The second study theme aimed to elucidate the resistance causing mechanism responsible for neonicotinoid resistance in *A. gossypii* using bioassay with synergist and molecular genetic based methods including state of the art NGS technologies.

Firstly, screening for thiamethoxam and clothianidin resistance using previously established discriminating dose assays detected resistance to both compounds in three strains of *A. gossypii* collected off Australian cotton (Table 2.1). Information on insecticide resistance is important due to the extensive usage of neonicotinoids for controlling *A. gossypii*, with more than 80% of cotton seed planted in Australia treated

with thiamethoxam or other neonicotinoid insecticides (Herron and Wilson 2011). In my study, thiamethoxam was used to produce full log dosage probit lines as it comprises both foliar and seed treatment formulations unlike clothianidin which is only applied foliarly. RFs produced against thiamethoxam were significantly higher than initial detections made during the 2007-08 and 2008-09 cotton seasons (Herron and Wilson 2011) and implied that the selection pressure for resistant genotypes was high in Australian cotton. Indeed, when *A. gossypii* shown resistant via laboratory bioassay were included into a whole plant efficacy trial, resistant aphids were able to survive and reproduce on cotton treated with varying rates of thiamethoxam (Cruiser® and Cruiser Extreme®) (Figures 3.1 and 3.2). Importantly, results of the trial also demonstrated that both rates of thiamethoxam provided adequate protection of susceptible *A. gossypii*. This indicated that if the selection pressure for resistant genotypes could be lowered, the utility of either neonicotinoid pre-germination seed treatment against susceptible *A. gossypii* could be preserved.

One such way to avoid selection over successive generations is the rotation of insecticides between different MoA classes (Mallet 1989). This is particularly true for management of *A. gossypii* because of a very short life cycle (Moran 1992). In practice, rotations of compounds from different MoA classes should provide a sustainable and effective approach to resistance management. Indeed, as resistance is likely more advantageous under insecticidal treatment than it is disadvantageous in the absence of treatment, to be successful, rotation would have to be maintained over successive generations and include many different chemicals (Mallet 1989, Tabashnik 1989). Other well defined resistance management strategies such as the immigration of susceptible types (Tabashnik 1990) are useless in their practicality for *A. gossypii*, as in Australia there is no sexual phase of reproduction and thus no possible dilution of resistance alleles (Wool and Hales 1997).

As all of the seed treatments currently registered for control of *A. gossypii* on cotton belong to the neonicotinoid MoA group 4A, alternative rotation options for growers are very limited (Mass 2014, CottonInfo 2015). At-planting or in-furrow granular insecticides are one possible alternative to seed coated treatments but their use must be carefully considered. For example, when applying at-planting insecticides to the soil or

seed at planting in cooler temperatures seedling emergence can be delayed and in some instances may favour seedling disease (Hake et al. 1996). This is because excessive rates of insecticides may injure seedlings, making them more susceptible to fungal pathogens which thrive at cooler temperatures (Hake et al. 1996). For this reason, at-planting insecticides should never be used unless they are combined with a good fungicide treatment (Hake et al. 1996). The organophosphate at-planting side dressing, phorate (Thimet[®]) belongs to MoA group 1B and is effective at controlling a range of sucking insect pests and mite species present in seedling cotton (Mass 2014, CottonInfo 2015). Previous research has illustrated that phorate offers effective control against neonicotinoid resistant *A. gossypii* and where necessary, may be implemented as a viable alternative to neonicotinoid seed treatments (Herron et al. 2013). However, the use of phorate should be carefully considered as in Australia, resistance to organophosphates has previously been detected in *A. gossypii* across almost all cotton growing regions (Herron and Rophail 2000, Herron et al. 2001). Also listed in the current Cotton Pest Management Guide 2015-16 for control of *A. gossypii* on seedling cotton, is the at-planting insecticide aldicarb (Temik[®]) (CottonInfo 2015). However, when referring to the Australian Pesticides and Veterinary Medicines Authority (APVMA), no current permit exists for use of aldicarb on cotton in Australia (APVMA 2014), leaving phorate as the only viable alternative.

Thus, where use of neonicotinoid seed treatments isn't practical because of confirmed resistance, any chemical control strategy needs to be built around the efficacy of phorate. Therefore, as an adjunct to the first plant efficacy trial, I investigated the suitability of phorate to replace the use of neonicotinoid containing seed treatments. As phorate is an at-planting side dressing its place in any control strategy is fixed i.e. it will always be used first. As resistance management of *A. gossypii* is based on the alternation of chemical groups after each chemical treatment cycle, the first foliar spray can't comprise the IPM friendly carbamate insecticide pirimicarb (Pirimor[®]), as there is cross resistance between carbamate and organophosphate insecticides via the *Ace*1 type mutation (Russell et al. 2004). The first foliar spray needs to be from a different chemical group other than group 1A and 1B. The insecticide diafenthiuron (Pegasus[®]) (group 12A), like pirimicarb, is selective to beneficial insects and predatory mites and is therefore useful in IPM programs. Additionally, sulfoxaflor (group 4C) provides

adequate control against *A. gossypii* and has a low toxicity rating to predators, parasitoids and bees on cotton (Mass 2014, CottonInfo 2015).

Pivotal to prolonging the life of current and future neonicotinoid insecticides is to understand the dynamics of the molecular basis of insecticide resistance. While the R81T point mutation in loop D of the nAChR $\beta$1 subunit gene has been associated with neonicotinoid resistance in numerous strains of *A. gossypii* and *M. persicae* from outside Australia (Bass et al. 2011, Shi et al. 2012, Koo et al. 2014), in my strains the R81T mutation did not confer resistance. Previous studies have shown that the amino acid at this position within loop D is a key determinant of neonicotinoid binding to nAChRs (Shimomura et al. 2006, Yao et al. 2008, Toshima et al. 2009). It is surprising then that in Australian field populations of *A. gossypii*, the R81T mutation was not detected, especially given the high level use of neonicotinoid containing insecticides in Australian cotton systems at the time my strains were collected (Herron and Wilson 2011, APVMA 2013). I consider the way neonicotinoid insecticides are used between countries may be a major contributing factor to specific resistance mechanism selection. Interestingly, specific mechanism selection does not seem to be correlated to the level of resistance in the observed phenotype, as previous populations of *A. gossypii* where the R81T mutation has evolved have exhibited varying levels of resistance to several neonicotinoid compounds (Koo et al. 2014). For example, in a strain of *A. gossypii* (BY-A) from South Korea displaying the R81T mutation, RFs against thiamethoxam and dinotefuran were below <10-fold, while in a second strain (BY-B) exhibiting the R81T mutation, resistance to thiamethoxam was 69-fold (Koo et al. 2014). Importantly, what does seem to be consistent between strains of *A. gossypii* displaying the R81T mutation is that they have all been documented to display >10-fold resistance to imidacloprid (Bass et al. 2011, Shi et al. 2012, Koo et al. 2014, Kim et al. 2015). For example, Koo et al. (2014) surveyed six populations of *A. gossypii* in South Korea and found five strains displayed the R81T mutation and one (strain BY-B) possessed the susceptible type sequence. Strain BY-B was the only strain to display <10-fold resistance to imidacloprid with all other five strains displaying levels >26-fold. Similarly, Shi et al. (2012) documented the R81T mutation in a strain of *A. gossypii* possessing 66-fold resistance and Kim et al. (2015) in a highly imidacloprid resistant (3800-fold) strain of *A. gossypii*. In contrast, in this present study data suggested that

strains studied here were susceptible to imidacloprid. This was demonstrated by transferring samples of each resistant strain to individual cotton plants sprayed with the discriminating dose of imidacloprid but no strain was found surviving after seven days.

Synergist bioassays have repeatedly been used as diagnostic tools to identify metabolic resistance mechanisms in insect species displaying resistance to neonicotinoid compounds (Zewen et al. 2003, Gao et al. 2014, Khan et al. 2015). The mode of action of the majority of synergists is to block the metabolic systems that would otherwise break down insecticide molecules (Oppenoorth 1971). Thus, if the toxicity of an insecticide is increased when applied with a synergist, compared to being applied alone, it may be deduced that detoxifying enzymes are contributing some resistance to that insecticide (Raffa and Priester 1985). Previous studies had indicated that P450s may play a role in conferring resistance to thiamethoxam and as such this metabolic detoxification family was investigated using synergist bioassays (Gao et al. 2014, Khan et al. 2015). The synergist PBO has previously been linked to two major metabolic enzyme systems, P450s and non-specific esterases (Sun and Johnson 1960, Scott 1990). In my study, application of PBO in tandem with thiamethoxam reduced RFs from 7-fold to >1-fold indicating that the proportion of resistant types in the tested population was significantly decreased in the presence of PBO compared to thiamethoxam alone (Table 4.1). Although preliminary, these results suggest that addition of a synergist to thiamethoxam containing treatments may overcome insecticide resistance in the field and reduce the amount of product necessary to control resistant aphids. For example, a recent field trial by Universita Cattolica del Sacro Cuore compared a neonicotinoid-synergist formulation containing just 50% of the registered field dose of insecticide against neonicotinoid-resistant *M. persicae* to a commercial formulation containing the same dose of the same insecticide (Moores 2015). The neonicotinoid-synergist formulation provided 100% control of the resistant populations tested compared to just 5% control with the commercial formulation (Moores 2015). Given the ever increasing difficulty in developing novel insecticide chemistries that target new insecticide targets, the development of mixtures of active compounds and their synergists may provide valuable control strategies of the future. In Australian cotton, the use of microencapsulated pyrethroids and PBO in a tank mix has previously been shown to

give excellent control of highly resistant populations of *H. armigera* and *B. tabaci* in cotton (Gunning et al. 2004).

Before my study, there was little transcriptomic data for *A. gossypii* characterising resistance causing mechanisms. For instance, Pan et al. (2015) conducted a transcriptome study between thiamethoxam susceptible and resistant *A. gossypii* to identify potential resistance causing genes; however, the resistant strain used in that study was artificially selected from a susceptible strain and as such, may not be a reliable indicator of the genetics responsible for resistance in *A. gossypii*. This is because when laboratory-based selection starts with populations of limited size and diversity, extremely rare resistant variants that will eventually lead to field resistance and control failures are usually lacking from the small laboratory populations under selection (Roush and Miller 1986, Roush and McKenzie 1987). As a result, selection of laboratory strains within a continuous phenotypic distribution, favours a polygenic response that is not indicative of resistance found in the field (Georghiou 1972, Roush and McKenzie 1987). Conversely, when selection occurs for phenotypes outside of this distribution, i.e. in a field population where insecticide application is designed to kill every individual it makes contact with, a monogenic response involving a rare variant is typically favoured (McKenzie 1985). Therefore, as strains in my study were initially generated from field populations that received considerable selection pressure from neonicotinoid insecticides preceding their collection (Herron and Wilson 2011) and were heterogeneous in nature when established into laboratory culture, (see Chapter 2), I believe that my strains would more likely contain any potential rare variants that correspond to resistance alleles likely to trigger control failures in the field. Not surprisingly then, when comparing the list of candidate resistance genes generated in my transcriptome study to the study of Pan et al. (2015) clear distinctions are evident. Of most contrasting to my study is their finding that P450 gene expression didn't significantly fluctuate in their resistant strain when compared to the susceptible strain (Pan et al. 2015). When interpreted, their results imply that P450-mediated resistance is not linked to thiamethoxam-resistance adaptation (at least in their strain). The results of my transcriptome study are in complete contrast to the finding of Pan et al. (2015). For instance, the up-regulation of transcripts CL1190 and CL1418, (putatively identified as *CYP6K1* based on alignment to XP001948421.1) from my RNA-Seq analysis and

subsequent quantitative analysis of transcript expression via qRT-PCR, coupled with the synergistic effects of PBO demonstrated in Chapter 4 provide the first direct evidence of metabolic detoxification acting as the primary causal resistance mechanism against thiamethoxam in field strains of *A. gossypii*.

## 6.1 Future work

In Chapter 2 I demonstrated that resistance to thiamethoxam significantly reverted despite routine pressuring. Consequently, instability of the resistance causing allele(s) shown in my study may lead to populations reverting to susceptible in the absence of adequate selection pressure (ffrench-Constant and Roush 1990). This speculation is consistent with the conclusions of Herron and Wilson (2011) that neonicotinoid resistance in laboratory culture may be relatively unstable in *A. gossypii*. Further, the concept is consistent with the theory that resistance genes carry a fitness cost that cause individuals to forego some other attribute or quality which gives susceptible insects an advantage in the absence of insecticide (Crow 1957). Evidence of deleterious pleiotropic effects associated with resistance to neonicotinoid compounds exist for *N. lugens* (Liu and Han 2006) and B-type *B. tabaci* (Feng et al. 2009) and elsewhere has been strongly hypothesized based on documented resistance reversion in the absence of adequate selection pressure (Nauen et al. 2002, Gorman et al. 2007). These effects are generally measured by way of a fitness study to insecticides (reviewed in (Roush and McKenzie 1987)) either by (i) comparing one fitness component e.g. survival, development rate or fecundity between resistant and susceptible strains in the absence of insecticide or by (ii) placing resistant insects in competition with susceptible ones. For that reason I consider it particularly important that any future work should include a fitness study to investigate the potential costs associated with neonicotinoid resistance. Once quantified the fitness data could support improved IPM to better manage thiamethoxam resistant *A. gossypii* in Australian cotton fields.

In Chapter 5 I found two transcripts relating to the same P450 gene (based on alignment to XP001948421.1) putatively identified as *CYP6K1*, and overexpressed in each thiamethoxam resistant strain studied, providing direct evidence that this gene plays a role in resistance. In my study, preliminary sequencing did not detect any allelic

variants in the gene sequence which may have corresponded with the increased level of gene expression observed in RNA-Seq and qRT-PCR analysis. However, to be precise, obtaining the full length gene sequence using genomic DNA would be an essential future study to elucidate any potential SNP(s) which may be conferring the resistant genotype observed. If a link is confirmed, the development of a molecular diagnostic to reliably associate this mechanism with thiamethoxam resistance would provide a rapid and cost effective assay for monitoring of resistant genotypes arising in the field.

One notable advantage of DNA-based diagnostic tests using SNPs as resistance markers is that they are able to effectively distinguish between susceptible (SS), resistant (RR) and heterozygote (RS) genotypes. This is unlike traditional bioassay tests, i.e. discriminating dose tests which are unable to detect individuals heterozygous for a recessive resistance allele (Roush and Miller 1986, ffrench-Constant and Roush 1990). In the early stages of resistance development when allele frequencies are low, resistance alleles are predominantly found as heterozygotes. Thus, use of discriminating dose tests for resistance monitoring may potentially lead to lower detection sensitivity for resistance alleles (Roush and Miller 1986). Implementation of molecular diagnostics to reliably assess the extent and distribution of resistant populations in the field will facilitate design of insecticide resistance management programs that can contain the spread of resistance from its earliest onset. In *M. persicae*, development of a high throughput real-time PCR assay for detection of the R81T mutation has proven invaluable for resistance monitoring of this aphid pest against neonicotinoid compounds (Puinean et al. 2013).

## Appendix A.    Supplementary material referred to in all Chapters

**Table A.1** Discriminating dose data of *Aphis gossypii* thiamethoxam resistant strains (F 101, Glen twn S and Carr) after routine pressuring with varying rates of thiamethoxam (Actara® 250 g/kg).

|  |  | F 101[1] | Glen twn S[1] | Carr[2] |
|---|---|---|---|---|
| **2012** | April | 90 | 89 | 87 |
|  | July | 78 | 62 | 84 |
|  | September | 80 | 72 | 81 |
| **2013** | January | 85 | 84 | 68 |
|  | March | 67 | 79 | 67 |
|  | May | 96 | 87 | 70 |
|  | September | 93 | 88 | 74 |
|  | November | 90 | 90 | 88 |
| **2014** | February | 85 | 88 | 92 |
|  | May | 91 | 91 | 94 |
|  | August | 92 | 90 | 93 |
|  | November | 91 | 92 | 81 |
| **2015** | January | 93 | 89 | 82 |
|  | April | 92 | 90 | 80 |
|  | June | 93 | 89 | 79 |

[1]Percent susceptible of a sample population collected from the stock cage after pressuring with 0.05 g a.i./L of thiamethoxam (Actara® 250 g/kg)
[2]Percent susceptible of a sample population collected from the stock cage after pressuring with 0.1 g a.i./L of thiamethoxam (Actara® 250 g/kg).

# Appendix B.    Primers used in this study

**Table B.1** Primers used in Chapter 2.

| Gene | Designation | Sequence |
|---|---|---|
| Voltage gated sodium channel (VGSC) | KDR_DPI1 Forward | TCTTGGCCCACACTTAATCTTT |
| | KDR_DPI4 Reverse | CTCGCCGTTTGCATCTTATT |
| Acetylcholinesterase | AceF | CAAGCCATCATGGAATCAGG |
| | AceR | TCATCACCATGCATCACACC |

**Table B.2** Primers used in Chapter 4.

| Gene | Designation | Sequence |
|---|---|---|
| nicotinic acetylcholine receptor β1 subunit | Int1_For | CTGTCCAGAACATGACCGAA |
| | Int2_Rev | GTGGTAACCTGAGCACCTGT |

**Table B.3** Primers used in Chapter 5

| Gene | Designation | Sequence |
|---|---|---|
| β-actin | β-actin_F1 | AGCTCTATTCCAACCTTCCTTCT |
| | β-actin_R1 | TGTATGTAGTCTCGTGGATACCG |
| CL1190 | CL1190_F1 | CTGCAGTCATCGTTTTCACG |
| | CL1190_R1 | ACGTCCGTGTTAGCCAAGAG |
| | CL1190_F2 | CGTGATCGGTGAAGTACGAA |
| | CL1190_R2 | CATTGTTTGGCAACGTGTTC |
| | CL1190_F3 | CGTGATCGGTGAAGTACGAA |
| | CL1190_R3 | CATTGTTTGGCAACGTGTTC |
| CL1418 | CL1418_F1 | TGACGGGAATTACGGTTTGT |
| | CL1418_R1 | TATTACCCCGATCCGATGAG |
| | CL1418_F2 | CTCATCGGATCGGGGTAATA |
| | CL1418_R2 | CACAACGGGCAATTAAACAA |
| | CL1418_F3 | ATACTTGCGACCAAGCTCGT |
| | CL1418_R3 | CATGTTCACTGCTGGTTCAGA |
| Unigene10451 | 10451_F1 | GCGCCAAAATTGGAGTTTA |
| | 10451_R1 | CAGACACAAAGCGACGGTTA |
| | 10451_F2 | TGGCGTTATACACCCCTTGT |
| | 10451_R2 | CAGACACAAAGCGACGGTTA |
| | 10451_F3 | TGGCGTTATACACCCCTTGT |
| | 10451_R3 | CAGACACAAAGCGACGGTTA |
| Unigene10452 | 10452_F1 | TGAGTTGGTGTGCATTAGCTG |
| | 10452_R1 | CAAAACCCCAGCGTCTAAAA |
| | 10452_F2 | CGCAATAACGTCGAACTGAA |
| | 10452_R2 | CGTACCTGTTTTGGCAGACA |
| | 10452_F3 | GTGTGTGTGCGAGACTTTCC |
| | 10452_R3 | CCCATCATATTCCTGCGATT |

# Appendix C.　　Supplementary material from Chapter 3

## Efficacy of two thiamethoxam pre-germination seed treatments and a phorate side-dressing against neonicotinoid- and pirimicarb-resistant cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae)

Kate L Marshall,[1]* Damian Collins,[1] Lewis J Wilson[2] and Grant A Herron[1]

[1]New South Wales Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Woodbridge Road, Menangle, NSW 2568, Australia.
[2]The Commonwealth Scientific and Industrial Research Organisation (CSIRO) Agriculture Flagship, Locked Bag 59, Narrabri, NSW 2390, Australia.

**Abstract**　In a glasshouse trial with potted cotton plants grown from thiamethoxam-treated seed, neither 2.76 g a.i./kg seed (Cruiser®) nor 5.52 g a.i./kg seed (Cruiser Extreme®) protected plants from neonicotinoid-resistant *Aphis gossypii* Glover, 1877. Against susceptible *A. gossypii* each treatment was highly effective, providing control of >90% for 42 days. Continued use of either thiamethoxam treatment against resistant *A. gossypii* will select for resistant phenotypes and probably restrict the useful life of the neonicotinoid insecticides against this pest. In a separate trial, side-dressing of cotton seed with phorate 200 g/kg (Thimet®) effectively provided plants with protection from susceptible *A. gossypii*. The insecticidal activity of phorate-treated plants against pirimicarb-resistant *A. gossypii* was not statistically different to untreated plants ($P > 0.05$). To maintain the effectiveness of pirimicarb in the Australian cotton integrated pest management strategy, the use of phorate as an alternative pre-germination treatment to thiamethoxam for aphid control must be managed. We recommend that the first foliar spray applied to cotton treated with phorate at planting should not be pirimicarb or any other insecticide affected by insensitive cholinesterase (ACE1) type resistance.

**Key words**　cotton aphid, Cruiser®, Cruiser Extreme®, resistance management, Thimet®.

## INTRODUCTION

The cotton aphid, *Aphis gossypii*, is a significant pest of cotton, *Gossypium hirsutum* L., and cucurbits both in Australia and worldwide (Blackman & Eastop 2000). In Australian cotton, *A. gossypii* can be found on seedling cotton (October) and typically builds to levels that require control during the mid-late growing season (January–March). *Aphis gossypii* feeding can reduce leaf photosynthesis (Heimoana 2012) and spread plant viruses such as cotton bunchy top virus (CBTV) (Reddall *et al.* 2004; Ellis *et al.* 2013) or cotton leaf roll virus (Corrêa *et al.* 2005) that dramatically reduce the yield potential of affected plants. The excretion of honeydew by aphids (Hequet *et al.* 2000) contaminates the lint of matured fruit (bolls). Damaged lint attracts a lower price and damages the reputation of the region from which it is sourced. Furthermore, it is not economical to clean the lint and contaminated lint binds to machinery during spinning, necessitating shutdown and cleaning.

Economically significant outbreaks of aphids are partially induced by applications of insecticides against other pests (Wilson *et al.* 1999). These pesticides reduce beneficial populations without controlling aphids, which then rapidly increase. Since the advent and widespread uptake of *Bt* cotton, containing the *Cry* proteins to control the primary pests, *Helicoverpa* spp., the use of insecticides has declined dramatically (Wilson *et al.* 2013). However, some species not controlled by the *Cry* proteins have emerged as pests and require targeted control, especially green mirids (*Creontiades dilutus* Stål) (Wilson *et al.* 2013). Insecticides targeted against this pest are generally disruptive to beneficial species but do not control aphids – which consequently have persisted as an important pest. The capacity to control aphids, throughout the crop growing cycle, is consequently important for cotton production.

In Australia, neonicotinoid seed treatments containing thiamethoxam or side-dressings of granular insecticides, such as the organophosphate phorate at planting, are used to control a range of seedling pests such as thrips and wireworms (Elateridae), but also control *A. gossypii* and hence the risk of CBTV transmission. These treatments offer increased selectivity compared with neonicotinoid or organophosphate foliar sprays, which can be highly disruptive to beneficial insect populations (Mass 2013). Cruiser® (thiamethoxam at 2.76 g a.i./kg seed) and Cruiser Extreme® (thiamethoxam at 5.52 g a.i./kg seed) provide early season seedling protection (30–40 days) against *A. gossypii* and several other sucking insect pests

*kate.marshall@dpi.nsw.gov.au

352   K L Marshall et al.

(Maienfisch *et al.* 2001). However, the effectiveness of these products against *A. gossypii* may be threatened because of resistance to neonicotinoid (Herron & Wilson 2011), carbamate and organophosphate insecticides (Herron *et al.* 2001).

Neonicotinoid resistance in *A. gossypii* (Herron & Wilson 2011) was first recorded in the 2007–2008 cotton season and attributed to long-term, widespread use of the neonicotinoid cotton seed treatments. If neonicotinoid-resistant *A. gossypii* are present at the start of the cotton season, the use of neonicotinoid seed treatments may be only partially effective and so could exacerbate resistance. Phorate side-dressing has been suggested as a possible alternative to the neonicotinoid seed treatments, but its suitability as a viable replacement has not been explored, nor has its effectiveness to control carbamate (pirimicarb)-resistant *A. gossypii* been revealed. Similarly, the efficacy of the standard and higher rate thiamethoxam seed treatments against neonicotinoid-resistant aphids *in planta* has not been established. Here we report the results of a glasshouse trial that investigated the effectiveness of these treatments against resistant *A. gossypii*.

## MATERIALS AND METHODS

### Chemicals tested

Cotton seed (variety Sicot 71) treated with thiamethoxam at either 2.76 g a.i./kg seed (Cruiser® Insecticide Seed Treatment) or 5.52 g a.i./kg seed (Cruiser Extreme® Insecticide Seed Treatment) was obtained from Cotton Seed Distributors, Wee Waa, NSW. Phorate 200 g/kg insecticide (Thimet® 200 G Systemic Granular Insecticide) was obtained from Barmac Industries Pty Ltd.

### Aphids

A reference susceptible strain (F 96) was maintained on insecticide-free cotton in an insect-proof cage at 25 ± 4°C under natural light at the Elizabeth Macarthur Agricultural Institute, Camden. Resistant *A. gossypii* strains Glen twn S and Mon P were collected from commercial cotton. Strain Glen twn S was neonicotinoid resistant while strain Mon P was pirimicarb/omethoate resistant (Herron *et al.* 2013). Strain Glen twn S was routinely pressured monthly by exposure to foliar sprays at double the discriminating dose of thiamethoxam (i.e. 0.04 g/L) (Herron & Wilson 2011). Strain Mon P was similarly pressured monthly using a dose 10-fold the discriminating dose of pirimicarb (i.e. 0.1 g/L) (Herron *et al.* 2000). Both strains were pressured a week prior to the initial testing to ensure resistance remained at a high level throughout the trial interval.

### Thiamethoxam trial

Cotton seed treatments were: untreated control (cotton seed variety Sicot 71); 2.76 g a.i./kg seed (Cruiser®) and; 5.52 g

a.i./kg seed (Cruiser Extreme®). On 7 November 2011, 60 seeds of each treatment group were individually planted into plastic pots (11.5 cm diameter) filled with NativeMix™ premium potting mix and held in a room maintained at 28 ± 2°C. Each pot contained only one treatment with all pots planted concurrently (180 treated pots total). At planting and on another three occasions over the following 6 days, 150 mL of water was poured over the soil surface of each pot.

A week after planting the dicotyledons had emerged. At this time (day 0), six pots from each treatment group were transferred onto individual saucers in insect-proof cages maintained at 25 ± 4°C but subject to natural light. Pots were watered by filling their saucers with 200 mL on initial placement into the cages and as necessary during the trial period.

A randomised complete block design was used. Strains were randomised to cages ('whole-plots') and treatments were randomised to three pots within cages ('sub-plots'). Two apterous adult aphids (susceptible or resistant) were placed onto each of the plants within each cage such that three cages contained only susceptible aphids and three contained only resistant aphids. On day 7, all leaves were removed from each plant and final aphid numbers were counted with the aid of a stereo microscope. This process was repeated with new plants at weekly interval until day 49 by which time susceptible aphids could survive on both thiamethoxam treatments.

### Phorate trial

Cotton seed treatments were: untreated control (cotton seed variety Sicot 71) and phorate 200 g/kg (Thimet®) at a dose of 34.4 mg/pot. In a separate trial beginning on 7 July 2013, 60 seeds of each treatment group were planted individually and maintained as above. The dose (34.4 mg/pot) of phorate applied was equivalent to that indicated on the product label for short period protection (3 kg/ha) and assumed a row length equal to the diameter of the pot. Trial design was as above.

### Statistical analysis

Analyses were conducted for each trial using generalised linear mixed models in ASReml (Gilmour *et al.* 2009). The response (number of aphids) was analysed as quasi-Poisson (overdispersed Poisson with log link) for each trial using a mixed model comprising fixed strain, treatment (within strain) and linear day effects and all associated interactions, and random factor day effects and interactions with treatment, strain, strain by treatment, as well as cage, cage by day and position. Wald-type F-tests for fixed terms in the model are reported, as well as contrasts to test for treatment efficacy and interactions between treatment efficacy and (linear) day, for each strain in turn.

The Henderson–Tilton formula (Henderson & Tilton 1955) for treatment control is $100[1 - Ta*Cb/Tb*Ca] = 100[1 - Ta/Ca]$, where Ta and Ca are the number of aphids surviving at the end of the week, and Tb and Cb are the starting number of

aphids used for each pot (2) which cancel out from top and bottom. The ratio Ta/Ca could be estimated, along with an approximate 95% confidence interval, by back-transforming the predicted difference between each treatment and control at each time-point (since a log link was used, and so absolute differences on the log scale correspond to multiplicative effects on the back-transformed scale).

## RESULTS

### Thiamethoxam trial

There were significant ($P < 0.05$) treatment within strain effects and significant interactions of treatment within strain with day (Table 1). The non-zero variance components indicated differences in individual day effects across treatments, and both cage and cage by day effects, as well as residual overdispersion (relative to a Poisson distribution), indicated by a residual variance (3.02) greater than 1 (Table 2). For strain F 96, the interactions of treatment with lin(day) were either non-significant ($P > 0.05$) for the higher rate or just significant ($P < 0.05$) for the lower rate. However, there were statistically highly significant ($P < 0.0001$) treatment within strain effects for both rates of thiamethoxam compared with untreated cotton seed, as expected (Table 1). Both Cruiser® and Cruiser Extreme® provided 100% protection of strain F 96 for 14 days (Table 3). Control of strain F 96 remained very

high (>90%) until day 49 where Cruiser® showed a decrease to 87%. Residual insecticidal activity of Cruiser Extreme® provided greater control at 49 days of 93.3%. Interactions of treatment with day for strain Glen twn S were both significant ($P < 0.05$) for each rate of thiamethoxam when compared with untreated cotton seed, indicating the reduction in treatment efficacy over time. Cruiser Extreme® provided higher initial and residual protection compared with Cruiser® (Table 3 and Fig. 1) but neither treatment adequately controlled resistant *A. gossypii*. From day 28, the effectiveness of Cruiser® against strain Glen twn S was similar to untreated cotton (Table 3).

### Phorate trial

There were statistically highly significant ($P < 0.001$) treatment within strain effects for strain F 96 (and interactions with day), but not for strain Mon P ($P > 0.05$) (Table 4). The non-zero variance components indicated day effects (fac(day)), replicate and replicate by day effects, cage by day effects, treatment, strain by day effects and position effects as well as residual overdispersion (relative to a Poisson distribution), indicated by a residual variance (2.618) greater than 1 (Table 5). Phorate provided robust protection of strain F 96 for the duration of the trial, with control only decreasing below 90% at day 35 (Table 6). From day 42, phorate provided residual control of 80.9%, decreasing to 67.6% control at day 49. Strain Mon P survived well on phorate-treated cotton from day 0 (Fig. 2). Population size of strain Mon P when challenged with phorate showed no statistical significance compared with untreated cotton (Table 4).

## DISCUSSION

The efficacy of two neonicotinoid seed treatments against neonicotinoid susceptible and resistant *A. gossypii* and an organophosphate at-planting treatment against carbamate susceptible and resistant *A. gossypii* were evaluated under simulated field conditions in a glasshouse trial. Raw data produced were transformed via Henderson–Tilton analysis to account for variability seen in *A. gossypii* numbers on untreated controls. We believe that due to the low starting number of two aphids each week, the variability seen in aphid populations from plant to plant was typical. Predicted values were produced for each time-point of the trial which offer a more realistic estimate of the control provided by each treatment.

We have clearly shown that formulated thiamethoxam at either rate (2.76 g a.i./kg seed and 5.52 g a.i./kg seed) is highly effective for protection against neonicotinoid susceptible *A. gossypii* and continues to be a viable option for aphid control. These results are in conformity with previous studies investigating the efficacy of thiamethoxam as a seed treatment against susceptible *A. gossypii*. Maienfisch *et al.* (2001) found that against sucking insect pests of cotton, rates between 105 and 350 g a.i./100 kg seed gave excellent control for 21–45 days. Prasanna *et al.* (2004) also found that thiamethoxam 70WS at a rate of 2.85 g a.i./kg seed effective until 40 days

**Table 1**  Wald-F test statistics for fixed effects of thiamethoxam analysis

|  | DF (num, den) | F-statistic | P-value |
|---|---|---|---|
| strain | 1,53 | 9.164 | 0.0273 |
| lin(day) | 1,16.4 | 9.660 | 0.0066 |
| strain/trt | 4,29.3 | 13.810 | 0.0000 |
| sus:cruiser vs control | 1,61.7 | 31.250 | 0.0000 |
| sus:extreme vs control | 1,73.7 | 34.470 | 0.0000 |
| res:cruiser vs control | 1,11.5 | 4.740 | 0.0512 |
| res:extreme vs control | 1,12.3 | 8.228 | 0.0139 |
| strain × lin(day) | 1,36.7 | 9.840 | 0.0034 |
| strain/trt × lin(day) | 4,32.2 | 3.778 | 0.0125 |
| sus:{cruiser vs control} × lin(day) | 1,80.1 | 4.110 | 0.0460 |
| sus:{extreme vs control} × lin(day) | 1,79.2 | 3.244 | 0.0755 |
| res:{cruiser vs control} × lin(day) | 1,11.4 | 7.003 | 0.0221 |
| res:{extreme vs control} × lin(day) | 1,14.0 | 9.882 | 0.0072 |

lin, linear; res, resistant strain; sus, susceptible strain; trt, treatment.

**Table 2**  Non-zero variance component and standard error (SE) for random terms of thiamethoxam analysis

|  | Component | SE | Z-ratio |
|---|---|---|---|
| cage | 0.0097 | 0.0309 | 0.3143 |
| cage × fac(day) | 0.1266 | 0.0610 | 2.0738 |
| trt × fac(day) | 0.2708 | 0.1430 | 1.8943 |
| Residual | 3.0163 | 0.5196 | 5.8054 |

fac, factor; trt, treatment.

354　K L Marshall et al.

*Table 3* Estimated treatment efficacies (Est) and approximate 95% confidence intervals of two varying rates of formulated thiamethoxam (g a.i./kg seed) against neonicotinoid susceptible and neonicotinoid-resistant *Aphis gossypii*

Susceptible strain F 96

|  |  | Untreated | | 2.76 | | 5.52 | |
|---|---|---|---|---|---|---|---|
|  |  | Est | 95% CI | Est | 95% CI | Est | 95% CI |
| Day 7 | Aphids | 13.5 | (7.8, 23.4) | 0.0 | (0.0, 1.4) | 0.0 | (0.0, 1.0) |
|  | % Control | – | – | 100.0 | (89.3, 100.0) | 100.0 | (92.5, 100.0) |
| Day 14 | Aphids | 16.3 | (10.0, 26.5) | 0.0 | (0.0, 1.1) | 0.0 | (0.0, 1.0) |
|  | % Control | – | – | 100.0 | (93.1, 100.0) | 100.0 | (93.8, 100.0) |
| Day 21 | Aphids | 14.3 | (9.0, 22.6) | 0.0 | (0.0, 1.4) | 0.1 | (0.0, 5.6) |
|  | % Control | – | – | 99.9 | (89.8, 100.0) | 99.5 | (60.9, 100.0) |
| Day 28 | Aphids | 11.2 | (6.8, 18.2) | 0.1 | (0.0, 2.8) | 0.1 | (0.0, 2.0) |
|  | % Control | – | – | 99.4 | (75.0, 100.0) | 99.5 | (82.1, 100.0) |
| Day 35 | Aphids | 11.9 | (7.2, 19.7) | 0.2 | (0.0, 2.4) | 0.2 | (0.0, 1.7) |
|  | % Control | – | – | 98.3 | (79.0, 99.9) | 98.3 | (85.1, 99.8) |
| Day 42 | Aphids | 21.7 | (13.7, 34.5) | 1.2 | (0.3, 4.2) | 0.9 | (0.3, 3.2) |
|  | % Control | – | – | 94.6 | (80.5, 98.5) | 95.8 | (85.2, 98.8) |
| Day 49 | Aphids | 35.3 | (21.9, 56.8) | 4.6 | (1.7, 12.6) | 2.4 | (0.7, 8.7) |
|  | % Control | – | – | 87.0 | (65.2, 95.2) | 93.3 | (75.7, 98.1) |

Resistant strain Glen two S

|  |  | Est | 95% CI | Est | 95% CI | Est | 95% CI |
|---|---|---|---|---|---|---|---|
| Day 7 | Aphids | 38.2 | (24.0, 60.8) | 5.6 | (2.5, 12.6) | 1.5 | (0.5, 4.9) |
|  | % Control | – | – | 85.3 | (67.6, 93.3) | 96.1 | (87.1, 98.8) |
| Day 14 | Aphids | 36.3 | (23.3, 56.6) | 4.9 | (2.2, 11.1) | 2.2 | (0.8, 6.1) |
|  | % Control | – | – | 86.5 | (69.3, 94.1) | 94.0 | (82.9, 97.9) |
| Day 21 | Aphids | 25.1 | (16.2, 38.9) | 7.1 | (3.6, 13.9) | 18.3 | (10.7, 31.2) |
|  | % Control | – | – | 71.9 | (43.8, 85.9) | 27.2 | (−23.8, 57.2) |
| Day 28 | Aphids | 15.5 | (9.6, 25.0) | 15.1 | (8.6, 26.4) | 8.8 | (4.6, 16.7) |
|  | % Control | – | – | 2.6 | (−79.8, 47.2) | 43.4 | (−14.0, 71.9) |
| Day 35 | Aphids | 13.0 | (7.8, 21.5) | 14.1 | (7.9, 25.3) | 9.8 | (5.2, 18.7) |
|  | % Control | – | – | −8.9 | (−110.3, 43.6) | 24.3 | (−54.6, 62.9) |
| Day 42 | Aphids | 18.8 | (11.6, 30.3) | 22.8 | (13.5, 38.3) | 18.5 | (10.7, 32.0) |
|  | % Control | – | – | −21.3 | (−109.1, 29.7) | 1.4 | (−74.5, 44.3) |
| Day 49 | Aphids | 24.0 | (14.7, 39.3) | 24.7 | (14.6, 41.7) | 20.8 | (12.1, 35.9) |
|  | % Control | – | – | −2.8 | (−72.0, 38.6) | 13.4 | (−48.3, 49.4) |

post seedling emergence, while the higher rate of 4.28 g a.i./kg seed still provided superior control of *A. gossypii* when compared with untreated plants at 60 days, although not statistically significant. Zidan (2012) also found that thiamethoxam 70WS at the recommended rate of 4.9 g a.i./kg seed provided effective control of *A. gossypii*, although when compared with our results provided significantly reduced residual protection.

In contrast to neonicotinoid susceptible *A. gossypii*, our results have revealed that neither rate of thiamethoxam gives adequate control against neonicotinoid-resistant *A. gossypii*. It is likely that ongoing widespread reliance on neonicotinoid seed treatments, at either rate, will continue to select for resistant genotypes. Cross-resistance between members of the neonicotinoid group 4A mode of action (MoA) in *A. gossypii* has been reported elsewhere (Wang *et al.* 2007; Shi *et al.* 2011) and suggests that control of resistant populations is likely to be lost if neonicotinoid use is not managed better. The Insecticide Resistance Management Strategy (IRMS) for control of sucking insect pests of cotton recommends chemical rotation as the primary strategy for control of resistant *A. gossypii* (Mass 2013). Other well-defined

strategies in the IRMS such as use of refugia for control of *Helicoverpa* spp. are limited in their practicality for *A. gossypii* due to a short life cycle and there being no sexual phase of reproduction occurring in Australia (Smith *et al.* 2006). If chemical rotation is maintained over successive generations, then in the absence of selection the resistant population should return to susceptibility. It should be mentioned that this strategy relies on their being an associated fitness cost to the observed resistance. Fortunately, reversion to susceptibility in the absence of insecticide pressure has been noted to occur in laboratory strains of neonicotinoid-resistant *A. gossypii* (KL Marshall unpubl. data 2013). This would suggest that at least in some populations of *A. gossypii*, genes conferring neonicotinoid resistance do not appear to be fixed. Neonicotinoid seed dressings are primarily targeted against other pests where they continue to provide cost-effective control (Mass 2013), so restriction in their use without a viable alternative is impractical. Phorate is registered for the control of *A. gossypii* at planting and has previously been shown to control neonicotinoid-resistant *A. gossypii* as it possesses a distinct MoA to neonicotinoids (Herron *et al.* 2013). However, established cross-resistance

Chemical efficacy against *Aphis gossypii*   355



**Fig. 1.** Fitted trend for the thiamethoxam analysis, for each strain by treatment combination (thiamethoxam at 5.52 g a.i./kg seed, Cruiser Extreme® Insecticide Seed Treatment; thiamethoxam at 2.76 g a.i./kg seed, Cruiser® Insecticide Seed Treatment; untreated cotton seed, variety Sicot 71). The solid line represents the fitted trend, with dotted lines representing the 95% confidence interval. The raw data for each replicate is numbered 1 to 3 in each panel (with replicates 1 and 3 shifted slightly left and right, respectively, to avoid overlap).

**Table 4** Wald-F test statistics of fixed effects for phorate analysis

|  | DF (num, den) | F-statistic | P-value |
|---|---|---|---|
| strain | 1,5.0 | 3.4440 | 0.1223 |
| lin(day) | 1,4.8 | 0.5352 | 0.4987 |
| strain/trt | 2,5.9 | 10.4000 | 0.0115 |
| sus {phorate vs control} | 1,23.0 | 18.8700 | 0.0002 |
| res {phorate vs control} | 1,3.6 | 0.1018 | 0.7675 |
| strain × lin(day) | 1,4.8 | 0.0548 | 0.8245 |
| strain/trt × lin(day) | 2,8 | 3.7490 | 0.0707 |
| sus {phorate vs control} × lin(day) | 1,40.5 | 7.8890 | 0.0076 |
| res {phorate vs control} × lin(day) | 1,3.7 | 1.0180 | 0.3746 |

lin, linear; res, resistant strain; sus, susceptible strain; trt, treatment.

**Table 5** Non-zero variance component and standard error (SE) for random terms of phorate analysis

|  | Component | SE | z.ratio |
|---|---|---|---|
| fac(day) | 0.3424 | 0.4020 | 0.8475 |
| rep | 0.1070 | 0.1239 | 0.8388 |
| rep × fac(day) | 0.0122 | 0.0553 | 0.2181 |
| cage × fac(day) | 0.0823 | 0.0764 | 1.1067 |
| trt × fac(day) | 0.0806 | 0.0931 | 0.8854 |
| strain × fac(day) | 0.3051 | 0.2512 | 1.213 |
| position | 0.0043 | 0.0227 | 0.1907 |
| Residual | 2.6847 | 0.6312 | 4.0102 |

fac, factor; rep, replicate; trt, treatment.

221 of 308

356　K L Marshall et al.

*Table 6* Estimated treatment efficacies (Est) and approximate 95% confidence intervals of phorate as a side-dressing against pirimicarb susceptible and pirimicarb-resistant *Aphis gossypii*

Susceptible strain F 96

| | | Untreated | | 3 kg/ha | |
|---|---|---|---|---|---|
| | | Est | 95% CI | Est | 95% CI |
| Day 7 | Aphids | 11.6 | (5.6, 23.9) | 0.0 | (0.0, 0.7) |
| | % Control | – | – | 99.7 | (94.2, 100.0) |
| Day 14 | Aphids | 17.0 | (8.7, 33.0) | 0.2 | (0.0, 1.8) |
| | % Control | – | – | 99.0 | (89.8, 99.9) |
| Day 21 | Aphids | 12.8 | (6.3, 25.7) | 0.3 | (0.0, 2.0) |
| | % Control | – | – | 97.6 | (85.7, 99.6) |
| Day 28 | Aphids | 5.3 | (2.3, 12.5) | 0.4 | (0.1, 1.9) |
| | % Control | – | – | 92.2 | (68.6, 98.0) |
| Day 35 | Aphids | 4.2 | (1.7, 10.4) | 0.4 | (0.1, 1.7) |
| | % Control | – | – | 89.3 | (67.8, 96.5) |
| Day 42 | Aphids | 11.5 | (5.7, 23.3) | 2.2 | (0.0, 5.5) |
| | % Control | – | – | 80.9 | (60.9, 90.7) |
| Day 49 | Aphids | 27.0 | (14.6, 50.0) | 8.8 | (4.0, 19.1) |
| | % Control | – | – | 67.6 | (36.5, 83.4) |

Resistant strain Mon P

| | | Est | 95% CI | Est | 95% CI |
|---|---|---|---|---|---|
| Day 7 | Aphids | 7.1 | (3.2, 15.8) | 3.8 | (1.5, 9.6) |
| | % Control | – | – | 46.0 | (−26.0, 76.9) |
| Day 14 | Aphids | 30.9 | (17.0, 56.4) | 26.6 | (14.4, 49.1) |
| | % Control | – | – | 14.0 | (−33.1, 44.5) |
| Day 21 | Aphids | 26.8 | (14.6, 49.2) | 27.1 | (14.7, 50.0) |
| | % Control | – | – | −1.3 | (−59.1, 35.5) |
| Day 28 | Aphids | 10.7 | (5.3, 21.4) | 18.9 | (9.9, 35.9) |
| | % Control | – | – | −76.8 | (−208.6, −1.3) |
| Day 35 | Aphids | 4.3 | (1.9, 10.1) | 5.5 | (2.4, 12.5) |
| | % Control | – | – | −26.3 | (−160.1, 38.7) |
| Day 42 | Aphids | 45.0 | (25.3, 80.1) | 53.3 | (30.2, 94.1) |
| | % Control | – | – | −18.4 | (−68.8, 17.0) |
| Day 49 | Aphids | 22.8 | (12.1, 43.0) | 24.0 | (12.8, 45.1) |
| | % Control | – | – | −5.3 | (−71.9, 35.5) |

between the organophosphate and carbamate chemical classes via insensitive cholinesterase type resistance (*ACE1*) will select for high level resistance in *A. gossypii* pest populations if used sequentially and may lead to control failures as previously seen (Herron *et al.* 2001; Andrews *et al.* 2004; Benting & Nauen 2004). The IRMS lists the carbamate (pirimicarb) as a favourable first foliar spray for use against *A. gossypii* due to its softness on beneficial insect species (Mass 2013). Herron *et al.* (2013) suggested that pirimicarb-resistant *A. gossypii* would not be controlled by phorate. The results of our glasshouse trial confirm those laboratory findings. If phorate is to successfully substitute for a neonicotinoid seed dressing, the interaction with pirimicarb must be carefully considered. If phorate is used to control neonicotinoid-resistant *A. gossypii*, then pirimicarb, or any other chemical associated with *ACE1*-type resistance, should not immediately follow as the first foliar spray.

## ACKNOWLEDGEMENT

## REFERENCES

Andrews MC, Callaghan A, Field LM, Williamson MS & Moores GD. 2004. Identification of mutations conferring insecticide-insensitive



*Fig. 2.* Fitted trends for the phorate analysis, for each strain by treatment combination (phorate equivalent to 3 kg/ha, Thimet® 200 G Systemic Granular Insecticide; untreated cotton seed, variety Sicot 71). The solid line represents the fitted trend, with dotted lines representing the 95% confidence interval. The raw data for each replicate is numbered 1 to 3 in each panel (with replicates 1 and 3 shifted slightly left and right, respectively, to avoid overlap).

AChE in the cotton-melon aphid, *Aphis gossypii* Glover. *Insect Molecular Biology* 13, 555-561.

Benting J & Nauen R. 2004. Biochemical evidence that an S431F mutation in acetylcholinesterase-1 of *Aphis gossypii* mediates resistance to pirimicarb and omethoate. *Pest Management Science* 60, 1051-1055.

Blackman RL & Eastop VF. 2000. *Aphids on the World's Crops*, 2nd edn., John Wiley and Sons, Chichester, UK.

Corrêa RL, Silva TF, Simões-Araújo JL, Barroso PAV, Vidal MS & Vaslin MFS. 2005. Molecular characterization of a virus from the family Luteoviridae associated with cotton blue disease. *Archives of Virology* 150, 1357-1367.

Ellis MH, Silva TF, Stiller WN *et al.* 2013. Identification of a new Polerovirus (family Luteoviridae) associated with cotton bunchy top disease in Australia. *Australasian Plant Pathology* 42, 261-269.

Gilmour AR, Gogel BJ, Cullis BR & Thompson R. 2009. *ASReml User Guide Release 3.0.* VSN International Ltd, Hemel Hempstead, HP1 1ES, UK.

Heimoana SC. 2012. The effects of aphids (*Aphis gossypii*) Glover on photosynthesis in cotton (*Gossypium hirsutum*). The University of Queensland.

Henderson CF & Tilton EW. 1955. Tests with acaricides against the brown wheat mite. *Journal of Economic Entomology* 48, 157-161.

Hequet E, Ethridge D, Cole B & Wyatt B. 2000. How cotton stickiness measurements relate to spinning efficiency. In: Proc. Engineered Fiber Selection System Conference (EFS).

Herron G, Powis K & Rophail J. 2000. Baseline studies and preliminary resistance survey of Australian populations of cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae). *Australian Journal of Entomology* 29, 33-38.

Herron GA & Wilson LJ. 2011. Neonicotinoid resistance in *Aphis gossypii* Glover (Aphididae: Hemiptera) from Australian cotton. *Australian Journal of Entomology* 50, 93-98.

Herron GA, Powis K & Rophail J. 2001. Insecticide resistance in *Aphis gossypii* Glover (Hemiptera: Aphididae), a serious threat to Australian cotton. *Australian Journal of Entomology* 40, 85-91.

Herron GA, Langfield BJ, Bogema DR & Chen Y. 2013. Baseline susceptibility and cross-resistance in *Aphis gossypii* Glover (Aphididae:

Hemiptera) to phorate and sulfoxaflor. *Australian Journal of Entomology* 53, 32-35.

Maienfisch P, Angst M, Brandl F *et al.* 2001. Chemistry and biology of thiamethoxam: a second generation neonicotinoid. *Pest Management Science* 57, 906-913.

Maas S. 2013. *Cotton Pest Management Guide 2013-14.* Greenmount Press, Toowoomba, Australia.

Prasanna AR, Bheemanna M & Patil BV. 2004. Evaluation of Thiamethoxam 70WS as seed treatment against leaf miner and early sucking pests on hybrid cotton. *Karnataka Journal of Agricultural Science* 17, 238-241.

Reddall A, Ali A, Able JA *et al.* 2004. Cotton bunchy top: an aphid and graft transmitted cotton disease. *Australasian Plant Pathology* 33, 197-202.

Shi X-B, Shi X-G, Wang H-Y, Xia X-M & Wang K-Y. 2011. Cross-resistance of the imidacloprid-resistant population of *Aphis gossypii* Glover (Homoptera: Aphididae) to other neonicotinoid insecticides and changes in activities of related enzymes. *Acta Entomologica Sinica* 54, 1027-1033.

Smith T, Wilson L, Heimoana S, Herron G & Franzmann B. 2006. Over-winter host plants of cotton aphid (*Aphis gossypii*) and implications for managing abundance and resistance. In: Proceedings 13th Australian Cotton Growers Conference. Gold Coast, 8-10 August 2006.

Wang KI, Guo QL, Xia XM, Wang HY & Lin TX. 2007. Resistance of *Aphis gossypii* (Homoptera: Aphididae) to selected insecticides on cotton from five cotton production regions in Shandong, China. *Journal of Pesticide Science* 32, 372-378.

Wilson L, Downes S, Khan M *et al.* 2013. IPM in the transgenic era: a review of the challenges from emerging pests in Australian cotton systems. *Crop and Pasture Science* 64, 737-749.

Wilson LJ, Bauer LR & Lally DA. 1999. Insecticide-induced increases in aphid abundance in cotton. *Australian Journal of Entomology* 38, 242-243.

Zidan LTM. 2012. Bio-efficacy of three new neonicotinoid insecticides as seed treatment against four early sucking pests of cotton. *American-Eurasian Journal Agricultural and Environmental Science* 12, 535-540.

*Accepted for publication 29 October 2014.*

223 of 308

## Appendix D.   Supplementary material from Chapter 4

# NEONICOTINOID RESISTANCE IN COTTON APHID FROM AUSTRALIA

**AUTHORS** Kate.L.Marshall[1] Yizhou Chen[1] Grant. A. Herron[1]
**ORGANISATION** [1] New South Wales Department of Primary Industries,
Elizabeth Macarthur Agricultural Institute, Woodbridge Road, Menangle
2568, NSW, Australia

## 17th australian COTTON conference
Our Fibre. Our Focus. Our Future

### Summary

We have shown that target site
insensitivity in Australian *Aphis
gossypii* via the R81T mutation
is not the causal mechanism of
neonicotinoid resistance despite
overseas studies implicating such.
Instead we propose metabolic
detoxification as the likely causal
mechanism for resistance in
Australian A. gossypii and we are
currently trying to validate that
hypothesis via transcriptome
analysis. It is not clear why the
R81T mutation is absent but the
difference may relate to limited
imidacloprid use in Australian
cotton and the progressive nature
of the cotton industry itself.

### Introduction

In Australia, cotton aphid, *Aphis gossypii*
Glover is a destructive pest of cotton and
cucurbits and is frequently targeted with
chemical sprays for its control. Resistance
to the organophosphates, carbamates,
pyrethroids and more recently the
neonicotinoids has been detected in A.
gossypii in Australian cotton (Herron et
al. 2001; Herron & Wilson 2011; Marshall
et al. 2012). Insecticide resistance in
A. gossypii has two primary routes;
target site insensitivity and metabolic
detoxification. Target site insensitivity
is caused from modification/s in the
gene of the target site which prevents
binding of the insecticide and renders
the chemical ineffective. In metabolic
detoxification, enzymes which metabolize
the insecticide may be over produced
(gene amplification) or up-regulated (gene
expression), in each case allowing the
insect to metabolize the toxin to a level
suitable for survival. Alternatively, enzymes
may have a greater affinity for binding to
the insecticide, allowing it to be slowly
sequestered over time. Detoxification
and/or sequestration are not mutually
exclusive and often occur together in
insects whereby metabolic detoxification
is the primary mechanism of resistance.

For the three chemical classes;
organophosphates, carbamates and
pyrethroids, the mechanisms by which
A. gossypii confers resistance have been
elucidated as either target site insensitivity
and/or metabolic detoxification. Against
the more recent chemical class, the
neonicotinoids, the causal mechanism
of resistance has not yet been revealed.

Overseas, imidacloprid (a neonicotinoid)
resistance in A. gossypii has been
linked to target site insensitivity via a
modification in the predicted binding
site of neonicotinoid insecticides in the
nicotinic acetylcholine receptor (nAChR)
(Koo et al. 2014; Shi et al. 2012). This
mutation, termed R81T results in an
arginine (AGA) to threonine (ACA) base
substitution at amino acid position 81 in
the loop D region of the β1 subunit of the
nAChR.

To ascertain whether target site
insensitivity was responsible for the
confirmed resistance in Australian A.
gossypii, we amplified the mutation site
within the loop D region of the β1 subunit
through PCR and compared the DNA
sequence of a thiamethoxam resistant A.
gossypii strain (Carrington) from Australia
against a reference imidacloprid resistant
A. gossypii strain (GenBank Accession
number: JQ627836) from China (Shi et al.
2012). Additionally, the cDNA sequences
of a neonicotinoid susceptible strain (F
96) and an additional thiamethoxam
resistant strain (Glentown) from Australia
were included for sequence analysis.

### Methods

#### 1. Bioassay

Insecticide susceptible (strain F 96)
and thiamethoxam resistant (strains
Carrington and Glentown, both collected
off commercial cotton) were bioassayed
against the neonicotinoid insecticide
thiamethoxam (Actara®). Briefly, aphids
in batches of thirty were placed onto an
excised cotton leaf discs fixed in agar in

## NEONICOTINOID RESISTANCE IN COTTON APHID FROM AUSTRALIA

a petri dish and sprayed using a Potter spray tower with serial dilutions of the insecticide prepared with distilled water (Herron et al. 2001). Each strain was tested against five serial concentrations, selected to achieve 0 < x < 100% mortality. After spraying, each petri dish was covered with cling wrap with tiny perforations to reduce condensation and placed in an incubator at 25°C for 24 hours. After this period aphids were assessed as dead or alive with the aid of a stereo microscope.

### 2. Data Analysis

Bioassay data was analysed using a stand-alone probit program developed by Barchie (2001), which ensures that variability between replicates is taken into account. Dose response probit regressions were corrected for control mortality (Abbott 1925) and the $LC_{50}$ and $LC_{99.9}$ plus their 95% fiducial-limits were calculated by applying the method of Finney (1971). Resistance factors were calculated by dividing the $LC_{50}$ of the field-collected population by the value of the susceptible strain.

### 3. PCR Amplification

DNA was extracted from a pooled sample of 200 aphids of strain Carrington and used as a template in a polymerase chain reaction (PCR) protocol using primers (Forward primer: CTGTCCAGAACATGACCGAA and Reverse primer: GTGGTAACCTGAGCACCTGT) designed to amplify the mutation site within the loop D region of the β1 subunit of the nAChR. The amplified DNA was purified and sequenced by the Australian Genomic Research Facility (AGRF). Using the sequencing software program CodonCode Aligner® the sequencing data of strain Carrington was aligned to the reference imidacloprid resistant *A. gossypii* strain (GenBank accession number: JQ627836) for comparison.

Additionally, cDNA sequences were produced for susceptible strain F 96 and thiamethoxam resistant strains Carrington, and Glentown for further analysis.

### Results

#### Bioassay Results

| Strain | $LC_{50}$ (95% FL[a]) (g/L) | Slope± SE[b] | RF[c] (95% CI[d]) |
|---|---|---|---|
| Susceptible | 0.00038(0.00031-0.00046) | 2.4±0.24 | - |
| Carrington | 0.03(0.027-0.039) | 2.2±0.19 | 85.00(65.29-110.66) |
| Glentown | 0.02(0.01-0.03) | 1.2±0.20 | 51.3(30.5-86.2) |

[a]fiducial limits; [b]standard error; [c]resistance factor; [d]confidence interval.

TABLE 1. Full log dose probit regression summary of neonicotinoid susceptible strain F 96 and thiamethoxam resistant strains Carrington and Glentown against thiamethoxam

#### Sequencing Results

Sequence alignment between susceptible strain F 96, thiamethoxam resistant strains Carrington and Glentown and the reference imidacloprid resistant *A. gossypii* strain (Genbank accession number: JQ627836) confirmed that the region amplified were the loop D region of the β1 subunit. Comparative sequence analysis identified that all strains sequenced from Australia possessed a nucleotide G at base position 242 in the consensus region of DNA (AGA), whilst the reference imidacloprid resistant *A. gossypii* strain (Genbank accession number: JQ627836) possessed the nucleotide C (ACA), the later resulting in a corresponding codon change at position 81 from arginine to threonine (R81T) (Fig.1).



FIGURE 1. Comparative sequence analysis of Aphis gossypii strains susceptible F 96, Carrington (cDNA and gDNA), Glentown and imidacloprid resistant (Genbank accession number JQ627836). (Note: mutation site R81T boxed in red)

225 of 308

## NEONICOTINOID RESISTANCE IN COTTON APHID FROM AUSTRALIA

### Discussion

Through comparative sequence analysis, our results have illustrated that in Australian *A. gossypii* the causal mechanism of neonicotinoid resistance is not a target site insensitivity via the *R81T* mutation (Fig. 1). Studies overseas which have found the *R81T* mutation in *A. gossypii*, have described it firstly as the putative mechanism for imidacloprid resistance, and secondly as a cross resistance mechanism to other neonicotinoid insecticides (Koo et al. 2014; Shi et al. 2012). Interestingly, our strains display resistance to thiamethoxam (Table 1) but recent bioassay data has shown they are susceptible to imidacloprid (unpubl. data). Although there is confirmed cross resistance between members of the neonicotinoid mode of action group 4A (Shi et al. 2011; Wang et al. 2007) the spectrum of resistance displayed may be dependent on exposure to each chemical. Infrequent use of imidacloprid in Australian cotton may have increased the susceptibility of *A. gossypii* to this chemical. Additionally, in Australia the majority of cotton seed planted is coated with thiamethoxam as a pre germination seed treatment for the control of all early season insect pests. If the *R81T* mutation develops in response to imidacloprid, *A. gossypii* in Australian cotton may not have had enough exposure to develop the *R81T* mutation. Alternatively, we consider in the absence of imidacloprid a metabolic resistance developed via detoxification to the secondary analogs of imidacloprid and in particular to thiamethoxam. Research to validate a metabolic detoxification theory as the primary mechanism of neonicotinoid resistance in Australian *A. gossypii* from cotton is underway.

### Conclusion

The mutation responsible for imidacloprid resistance (*R81T*) in *A. gossypii* strains overseas is not present in Australia. Thiamethoxam is used widely in Australian cotton as a pre germination seed treatment whilst imidacloprid use is limited. This could explain the reason

why the causal mechanism of resistance to neonicotinoids in Australia, in particular to thiamethoxam may develop from a different origin.

### References

Abbott, WS. 1925 A method for computing the effectiveness of an insecticide. *Journal of Economic Entomology* 18, 265-267.

Finney, DJ. 1971. *Probit Analysis*, Cambridge University Press, Cambridge, UK.

Herron, GA, Powis, K & Rophail, J. 2001 Insecticide resistance in *Aphis gossypii* Glover (Hemiptera: Aphididae), a serious threat to Australian cotton. *Australian Journal of Entomology* 40, 85–91.

Herron, GA & Wilson, LJ. 2011 Neonicotinoid resistance in *Aphis gossypii* Glover (Aphididae: Hemiptera) from Australian cotton. *Australian Journal of Entomology* 50, 93-98.

Koo, H-N, An, J-J, Park, S-E, Kim, J-I & Kim, G-H. 2014 Regional susceptibilities to 12 insecticides of melon and cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae) and a point mutation associated with imidacloprid resistance. *Crop Protection* 55, 91-97.

Marshall, KL, Moran, C, Chen, Y & Herron, GA. 2012 Detection of kdr pyrethroid resistance in the cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae), using a PCR-RFLP assay. *Journal of Pesticide Science* 37, 169-172.

Shi, X-B, Shi, X-G, Wang, H-Y, Xia, X-M & Wang, K-Y. 2011 Cross-resistance of the imidacloprid-resistant population of *Aphis gossypii* Glover (Homoptera: Aphididae) to other neonicotinoid insecticides and changes in activities of related enzymes. *ACTA ENTOMOLOGICA SINICA* 54, 1027-1033.

Shi, X, Zhu, Y, Xia, X, Qiao, K, Wang, H & Wang, K. 2012 The mutation in nicotinic acetylcholine receptor β1 subunit may

confer resistance to imidacloprid in *Aphis gossypii* (Glover). *Journal of Food, Agriculture and Environment* 10, 1227-1230.

Wang, KI, Guo, QL, Xia, XM, Wang, HY & Liu, TX. 2007 Resistance of *Aphis gossypii* (Homoptera: Aphididae) to selected insecticides on cotton from five cotton production regions in Shandong, China. *Journal of Pesticide Science* 32, 372-378.

### Correspondence should be addressed to:

kate.marshall@dpi.nsw.gov.au

Australian Government
Cotton Research and
Development Corporation

NSW | Department of Primary Industries

UNIVERSITY OF TECHNOLOGY SYDNEY

Primer-BLAST Primer-Blast results
NCBI/ Primer-BLAST : results: Job id=JSID_01_134596_130.14.22.10_9002 more...

Input PCR template
JQ627836.1 Aphis gossypii nicotinic acetylcholine receptor beta 1 subunit mRNA, complete cds
Range
60 - 240
Specificity of primers
primer specificity was not determined as specificity checking option was not selected.
Other reports
Search Summary
Search parameters and other details

| Search parameter name | Search parameter value |
|---|---|
| Number of Blast hits analyzed | 0 |
| Entrez query | |
| Min total mismatches | 2 |
| Min 3' end mismatches | 2 |
| Defined 3' end region length | 5 |
| Mismatch threshold to ignore targets | 6 |
| Misprimed product size deviation | 4000 |
| Max number of Blast target sequences | 50000 |
| Blast E value | 30000 |
| Blast word size | 7 |
| Max candidate primer pairs | 1000 |
| Min PCR product size | 50 |
| Max PCR product size | 100 |
| Min Primer size | 15 |
| Opt Primer size | 20 |
| Max Primer size | 25 |
| Min Tm | 57 |
| Opt Tm | 60 |
| Max Tm | 63 |
| Max Tm difference | 3 |
| Repeat filter | AUTO |
| Low complexity filter | Yes |

Summary of primer pairs



Detailed primer reports
Primer pair 1

| | Sequence (5'->3') | Template strand | Length | Start | Stop | Tm | GC% | Self complementarity | Self 3' complementarity |
|---|---|---|---|---|---|---|---|---|---|
| Forward primer | CTGTCCAGAACATGACCGAA | Plus | 20 | 134 | 153 | 57.54 | 50.00 | 5.00 | 0.00 |
| Reverse primer | ACGTTCGATTTCATTATTTGACTCT | Minus | 25 | 233 | 209 | 57.40 | 32.00 | 4.00 | 1.00 |
| Internal oligo | | Plus | | | | | | | |
| Product length | 100 | | | | | | | | |
| Product Tm | | | | | | | | | |

1 of 3                                      31/07/2012 11:32 AM

**Figure D.1** Primer-BLAST results based on the nicotinic receptor β1 subunit of *Aphis gossypii* (GenBank accession number JQ627836.1) used to design primers in Table B.2.

Product
Tm -
min(OLIGO
Tm)
Exon
junction
**Total intron**
**size**
Products on intended target

Products on allowed transcript variants

Products on potentially unintended templates

Products on target templates

**Primer pair 2**

| | Sequence (5'->3') | Template strand | Length | Start | Stop | Tm | GC% | Self complementarity | Self 3' complementarity |
|---|---|---|---|---|---|---|---|---|---|
| Forward primer | AGCATTCGTTCAACTCATCAACG | Plus | 23 | 177 | 199 | 60.12 | 43.48 | 5.00 | 3.00 |
| Reverse primer | ACCAAACGTTCGATTTCATTATTTG | Minus | 25 | 238 | 214 | 57.44 | 32.00 | 6.00 | 2.00 |
| Internal oligo | | Plus | | | | | | | |
| Product length | 62 | | | | | | | | |

Product
Tm

Product
Tm -
min(OLIGO
Tm)
Exon
junction
**Total intron**
**size**
Products on intended target

Products on allowed transcript variants

Products on potentially unintended templates

Products on target templates

**Primer pair 3**

| | Sequence (5'->3') | Template strand | Length | Start | Stop | Tm | GC% | Self complementarity | Self 3' complementarity |
|---|---|---|---|---|---|---|---|---|---|
| Forward primer | GCATTCGTTCAACTCATCAACG | Plus | 22 | 178 | 199 | 58.84 | 45.45 | 5.00 | 3.00 |
| Reverse primer | CAACCAAACGTTCGATTTCATTATT | Minus | 25 | 240 | 216 | 57.44 | 32.00 | 8.00 | 2.00 |
| Internal oligo | | Plus | | | | | | | |
| Product length | 63 | | | | | | | | |

Product
Tm

Product
Tm -
min(OLIGO
Tm)
Exon
junction

**Figure D.1 (cont'd)** Primer-BLAST results based on the nicotinic receptor β1 subunit of *Aphis gossypii* (GenBank accession number JQ627836.1) used to design primers in Table B.2.

**Total intron size**

Products on intended target

Products on allowed transcript variants

Products on potentially unintended templates

Products on target templates

**Primer pair 4**

| | Sequence (5'->3') | Template strand | Length | Start | Stop | Tm | GC% | Self complementarity | Self 3' complementarity |
|---|---|---|---|---|---|---|---|---|---|
| Forward primer | CCAGAACATGACCGAAAAAGTCA | Plus | 23 | 138 | 160 | 59.44 | 43.48 | 4.00 | 1.00 |
| Reverse primer | CCAAACGTTCGATTTCATTATTTGA | Minus | 25 | 237 | 213 | 57.21 | 32.00 | 6.00 | 2.00 |
| Internal oligo | | Plus | | | | | | | |
| Product length | 100 | | | | | | | | |
| Product Tm | | | | | | | | | |
| Product Tm - min(OLIGO Tm) | | | | | | | | | |
| Exon junction | | | | | | | | | |
| Total intron size | | | | | | | | | |

Products on intended target

Products on allowed transcript variants

Products on potentially unintended templates

Products on target templates

**Figure D.1 (cont'd)** Primer-BLAST results based on the nicotinic receptor β1 subunit of *Aphis gossypii* (GenBank accession number JQ627836.1) used to design primers in Table B.2.

## Appendix E. Supplementary material from Chapter 5

Total RNA

Eucaryote → Enrich mRNA by Oligo(dT)

Prokaryote → Remove rRNA

RNA fragment

Random hexamer primed cDNA synthesis

Size selection and PCR amplification

Illumina sequencing
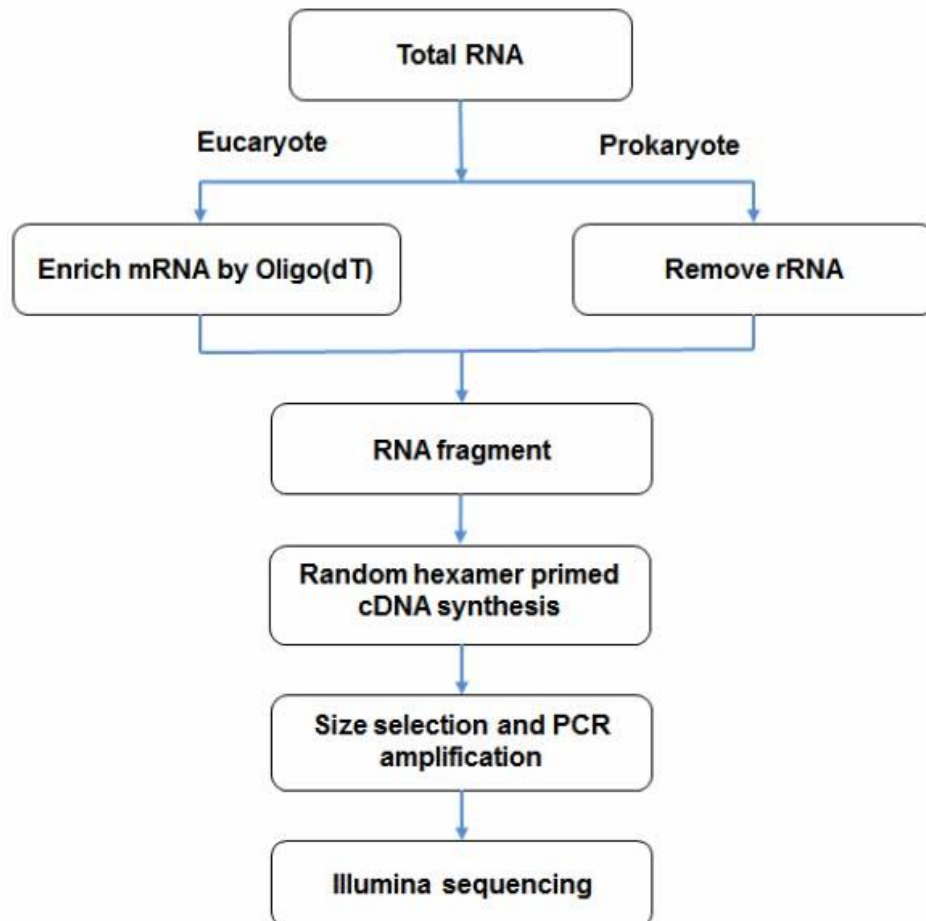
**Figure E.1** Schematic diagram illustrating the experimental pipeline of transcriptome assembly used in this study.
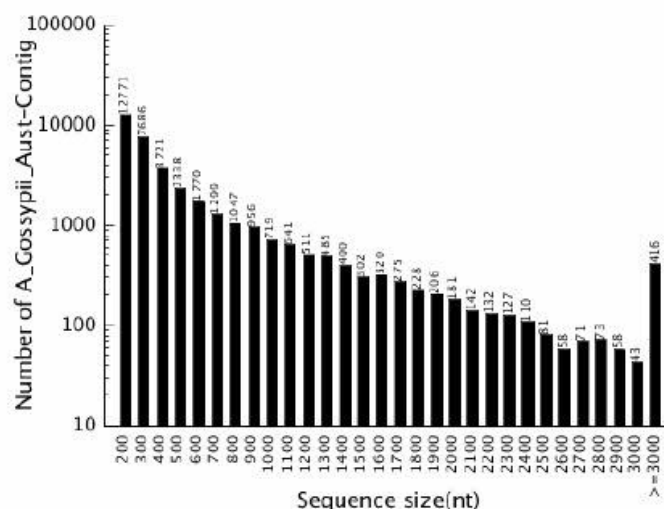
**Figure E.2** Length distribution of contigs. 'A_Gossypii_Aust-Contig' indicates that the contigs were those assembled from reads from the pooled transcriptome of four *Aphis gossypii* strains (including the reference susceptible (Sus F 96) and thiamethoxam resistant (F 101, Glen twn S and Carr)).
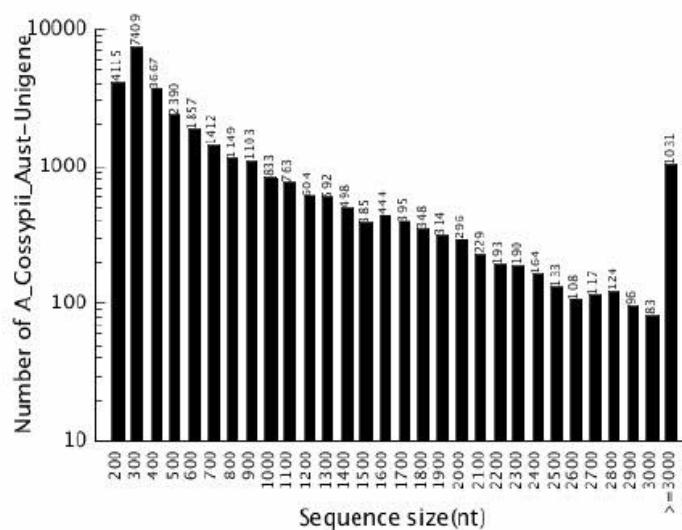


**Figure E.3** Length distribution of unigenes. 'A_Gossypii_Aust_Unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptomes of four *Aphis gossypii* strains (including the reference susceptible (Sus F 96) and thiamethoxam resistant (F 101, Glen twn S and Carr)).
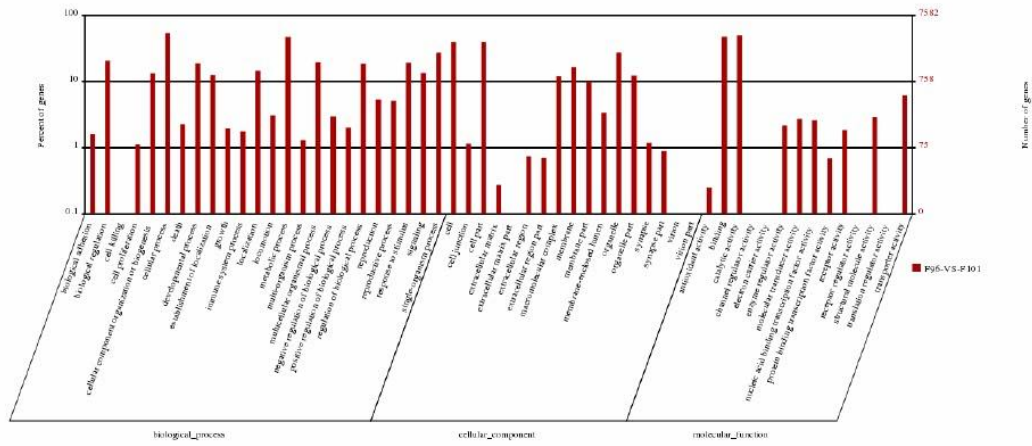
**Figure E.4** Histogram presentation of the gene ontology classification. GO categories, shown in the x-axis, are grouped into three main ontologies: biological process, cellular component and molecular function. The right y-axis indicates the number of genes in each category, while the left y-axis indicates the percentage of total genes in that category. The 'F96-VS-F 101' indicates that the unigenes were those assembled from reads from the comparison of a reference susceptible (Sus F 96) and thiamethoxam resistant (F 101) *Aphis gossypii* strains.
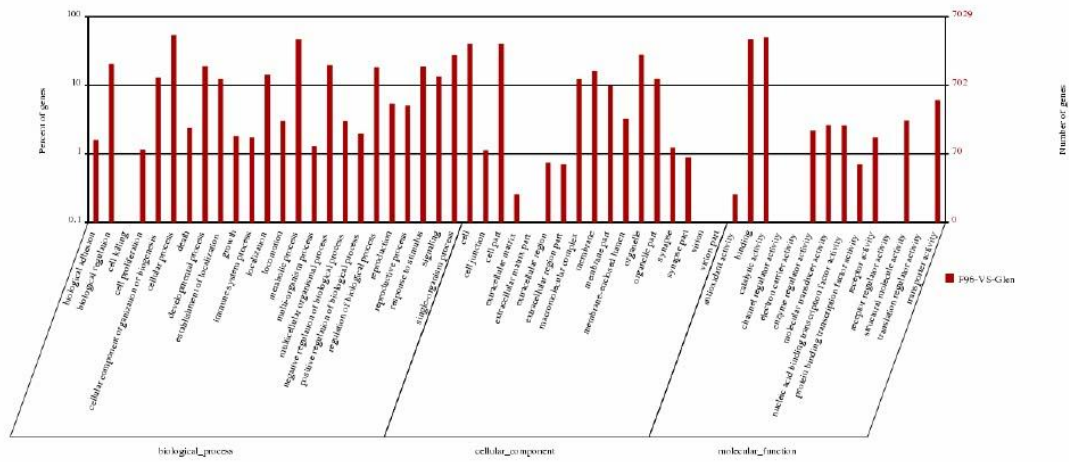
**Figure E.5** Histogram presentation of the gene ontology classification. GO categories, shown in the x-axis, are grouped into three main ontologies: biological process, cellular component and molecular function. The right y-axis indicates the number of genes in each category, while the left y-axis indicates the percentage of total genes in that category. The 'F96-VS-Glen' indicates that the unigenes were those assembled from reads from the comparison of a reference susceptible (Sus F 96) and thiamethoxam resistant (Glen twn S) *Aphis gossypii* strains.
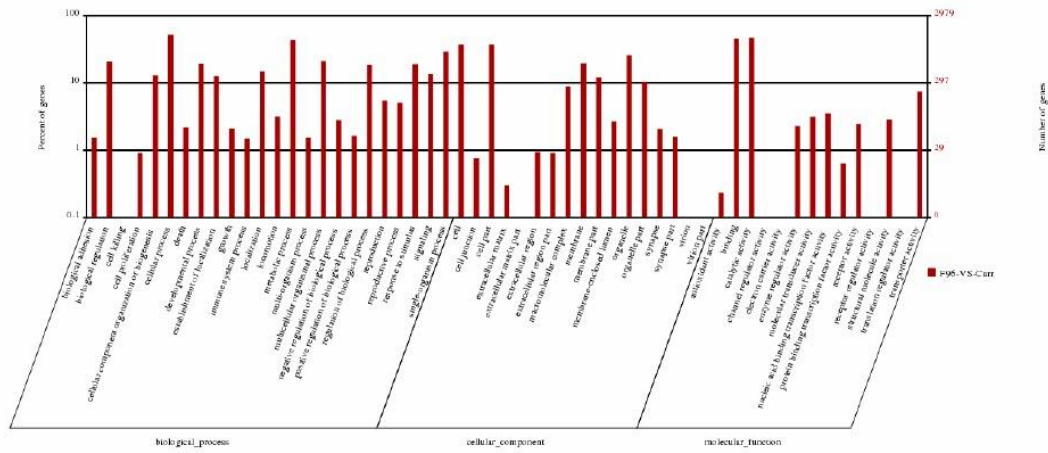
**Figure E.6** Histogram presentation of the gene ontology classification. GO categories, shown in the x-axis, are grouped into three main ontologies: biological process, cellular component and molecular function. The right y-axis indicates the number of genes in each category, while the left y-axis indicates the percentage of total genes in that category. The 'F96-VS-Carr' indicates that the unigenes were those assembled from reads from the comparison of a reference susceptible (Sus F 96) and thiamethoxam resistant (Carr) *Aphis gossypii* strains.

## Results colour-coded for amino acid conservation

The current colourscheme of the alignment is for **amino acid conservation**.

The conservation scoring is performed by PRALINE. The scoring scheme works from 0 for the least conserved alignment position, up to 10 for the most conserved alignment position. The colour assignments are:

Unconserved 0 1 2 3 4 5 6 7 8 9 10 Conserved



**Figure E.7** PRALINE alignment of the predicted cytochrome P450 gene 6k1-like of the pea aphid *Acyrthosiphon pisum* (Accession number: XP001948421.1) and *Aphis gossypii* sequence Contig 1190 (firstly translated using ExPASy (Gasteiger et al. 2003)).

## Results colour-coded for amino acid conservation

The current colourscheme of the alignment is for **amino acid conservation**.

The conservation scoring is performed by PRALINE. The scoring scheme works from 0 for the least conserved alignment position, up to 10 for the most conserved alignment position. The colour assignments are:

Unconserved `0 1 2 3 4 5 6 7 8 9 10` Conserved



**Figure E.8** PRALINE alignment of the predicted cytochrome P450 gene 6k1-like of the pea aphid *Acyrthosiphon pisum* (Accession number: XP001948421.1) and *Aphis gossypii* sequence Contig 1418 (firstly translated using ExPASy (Gasteiger et al. 2003)).

**Table E.1** Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'. The 'A_Gossypii_Aust_unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptome of four *Aphis gossypii* strains (including a reference susceptible (Sus F 96) and three thiamethoxam resistant (F 101, Glen twn S and Carr)).

| # | Pathway | Count (15460) | Pathway ID |
|---|---------|---------------|------------|
| 1 | Metabolic pathways | 2109 | ko01100 |
| 2 | RNA transport | 549 | ko03013 |
| 3 | Focal adhesion | 516 | ko04510 |
| 4 | Regulation of actin cytoskeleton | 491 | ko04810 |
| 5 | Pathways in cancer | 484 | ko05200 |
| 6 | Purine metabolism | 448 | ko00230 |
| 7 | HTLV-I infection | 430 | ko05166 |
| 8 | Epstein-Barr virus infection | 426 | ko05169 |
| 9 | MAPK signaling pathway | 370 | ko04010 |
| 10 | Spliceosome | 364 | ko03040 |
| 11 | Vascular smooth muscle contraction | 358 | ko04270 |
| 12 | Endocytosis | 353 | ko04144 |
| 13 | Pyrimidine metabolism | 342 | ko00240 |
| 14 | Huntington's disease | 338 | ko05016 |
| 15 | Transcriptional misregulation in cancer | 328 | ko05202 |
| 16 | Ubiquitin mediated proteolysis | 327 | ko04120 |
| 17 | Tight junction | 322 | ko04530 |
| 18 | mRNA surveillance pathway | 312 | ko03015 |
| 19 | Bile secretion | 308 | ko04976 |
| 20 | Amoebiasis | 306 | ko05146 |
| 21 | Insulin signaling pathway | 298 | ko04910 |
| 22 | Protein processing in endoplasmic reticulum | 290 | ko04141 |
| 23 | Lysosome | 288 | ko04142 |

(Continued)

**Table E.1 (cont'd)** Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'. The 'A_Gossypii_Aust_unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptome of four *Aphis gossypii* strains (including a reference susceptible (Sus F 96) and three thiamethoxam resistant (F 101, Glen twn S and Carr)).

| # | Pathway | Count (15460) | Pathway ID |
|---|---------|---------------|------------|
| 24 | Influenza A | 283 | ko05164 |
| 25 | Dilated cardiomyopathy | 278 | ko05414 |
| 26 | Calcium signaling pathway | 273 | ko04020 |
| 27 | Vibrio cholerae infection | 267 | ko05110 |
| 28 | Alzheimer's disease | 266 | ko05010 |
| 29 | Hypertrophic cardiomyopathy (HCM) | 261 | ko05410 |
| 30 | Cell cycle | 260 | ko04110 |
| 31 | Phagosome | 249 | ko04145 |
| 32 | Herpes simplex infection | 249 | ko05168 |
| 33 | Chemokine signaling pathway | 240 | ko04062 |
| 34 | Ribosome biogenesis in eukaryotes | 239 | ko03008 |
| 35 | Oocyte meiosis | 229 | ko04114 |
| 36 | RNA degradation | 229 | ko03018 |
| 37 | Neuroactive ligand-receptor interaction | 227 | ko04080 |
| 38 | Leukocyte transendothelial migration | 222 | ko04670 |
| 39 | Salmonella infection | 219 | ko05132 |
| 40 | Wnt signaling pathway | 216 | ko04310 |
| 41 | Dopaminergic synapse | 215 | ko04728 |
| 42 | Pancreatic secretion | 210 | ko04972 |
| 43 | Starch and sucrose metabolism | 206 | ko00500 |
| 44 | Adherens junction | 205 | ko04520 |
| 45 | Axon guidance | 202 | ko04360 |

(Continued)

**Table E.1 (cont'd)** Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'. The 'A_Gossypii_Aust_unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptome of four Aphis gossypii strains (including a reference susceptible (Sus F 96) and three thiamethoxam resistant (F 101, Glen twn S and Carr)).

| # | Pathway | Count (15460) | Pathway ID |
|---|---------|---------------|------------|
| 46 | Tuberculosis | 196 | ko05152 |
| 47 | ABC transporters | 196 | ko02010 |
| 48 | Lysine degradation | 195 | ko00310 |
| 49 | Alcoholism | 193 | ko05034 |
| 50 | Phosphatidylinositol signaling system | 189 | ko04070 |
| 51 | Neurotrophin signaling pathway | 188 | ko04722 |
| 52 | Gastric acid secretion | 183 | ko04971 |
| 53 | Salivary secretion | 183 | ko04970 |
| 54 | ECM-receptor interaction | 180 | ko04512 |
| 55 | Progesterone-mediated oocyte maturation | 179 | ko04914 |
| 56 | Protein digestion and absorption | 178 | ko04974 |
| 57 | Pathogenic Escherichia coli infection | 177 | ko05130 |
| 58 | RNA polymerase | 174 | ko03020 |
| 59 | Bacterial invasion of epithelial cells | 173 | ko05100 |
| 60 | Parkinson's disease | 163 | ko05012 |
| 61 | Peroxisome | 161 | ko04146 |
| 62 | Toxoplasmosis | 161 | ko05145 |
| 63 | Oxidative phosphorylation | 159 | ko00190 |
| 64 | Amphetamine addiction | 152 | ko05031 |
| 65 | Viral myocarditis | 150 | ko05416 |
| 66 | Fc gamma R-mediated phagocytosis | 148 | ko04666 |
| 67 | Cardiac muscle contraction | 147 | ko04260 |

(Continued)

**Table E.1 (cont'd)** Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'. The 'A_Gossypii_Aust_unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptome of four Aphis gossypii strains (including a reference susceptible (Sus F 96) and three thiamethoxam resistant (F 101, Glen twn S and Carr)).

| # | Pathway | Count (15460) | Pathway ID |
|---|---------|---------------|------------|
| 68 | Glycerophospholipid metabolism | 144 | ko00564 |
| 69 | GnRH signaling pathway | 144 | ko04912 |
| 70 | Dorso-ventral axis formation | 143 | ko04320 |
| 71 | Inositol phosphate metabolism | 143 | ko00562 |
| 72 | Drug metabolism - other enzymes | 142 | ko00983 |
| 73 | Melanogenesis | 141 | ko04916 |
| 74 | Measles | 140 | ko05162 |
| 75 | Aminoacyl-tRNA biosynthesis | 140 | ko00970 |
| 76 | Hepatitis C | 139 | ko05160 |
| 77 | Gap junction | 138 | ko04540 |
| 78 | Glutamatergic synapse | 137 | ko04724 |
| 79 | ErbB signaling pathway | 136 | ko04012 |
| 80 | Long-term potentiation | 136 | ko04720 |
| 81 | T cell receptor signaling pathway | 135 | ko04660 |
| 82 | Cholinergic synapse | 134 | ko04725 |
| 83 | Cell adhesion molecules (CAMs) | 133 | ko04514 |
| 84 | Vitamin digestion and absorption | 132 | ko04977 |
| 85 | Retinol metabolism | 132 | ko00830 |
| 86 | Other types of O-glycan biosynthesis | 132 | ko00514 |
| 87 | Small cell lung cancer | 131 | ko05222 |
| 88 | Galactose metabolism | 130 | ko00052 |

(Continued)

**Table E.1 (cont'd)** Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'. The 'A_Gossypii_Aust_unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptome of four Aphis gossypii strains (including a reference susceptible (Sus F 96) and three thiamethoxam resistant (F 101, Glen twn S and Carr)).

| # | Pathway | Count (15460) | Pathway ID |
|---|---------|---------------|------------|
| 89 | Pentose and glucuronate interconversions | 129 | ko00040 |
| 90 | Prostate cancer | 128 | ko05215 |
| 91 | Glycerolipid metabolism | 127 | ko00561 |
| 92 | Drug metabolism - cytochrome P450 | 123 | ko00982 |
| 93 | Jak-STAT signaling pathway | 123 | ko04630 |
| 94 | Metabolism of xenobiotics by cytochrome P450 | 121 | ko00980 |
| 95 | Porphyrin and chlorophyll metabolism | 118 | ko00860 |
| 96 | Fanconi anemia pathway | 118 | ko03460 |
| 97 | Synaptic vesicle cycle | 118 | ko04721 |
| 98 | Shigellosis | 117 | ko05131 |
| 99 | NF-kappa B signaling pathway | 116 | ko04064 |
| 100 | Morphine addiction | 114 | ko05032 |
| 101 | Steroid hormone biosynthesis | 113 | ko00140 |
| 102 | Amino sugar and nucleotide sugar metabolism | 111 | ko00520 |
| 103 | Cocaine addiction | 110 | ko05030 |
| 104 | Ribosome | 110 | ko03010 |
| 105 | GABAergic synapse | 110 | ko04727 |
| 106 | Retrograde endocannabinoid signaling | 110 | ko04723 |
| 107 | Cytosolic DNA-sensing pathway | 109 | ko04623 |
| 108 | PPAR signaling pathway | 108 | ko03320 |
| 109 | Glycine, serine and threonine metabolism | 108 | ko00260 |
| 110 | VEGF signaling pathway | 107 | ko04370 |

(Continued)

**Table E.1 (cont'd)** Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'. The 'A_Gossypii_Aust_unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptome of four Aphis gossypii strains (including a reference susceptible (Sus F 96) and three thiamethoxam resistant (F 101, Glen twn S and Carr)).

| # | Pathway | Count (15460) | Pathway ID |
|---|---------|---------------|------------|
| 111 | Phototransduction - fly | 107 | ko04745 |
| 112 | Renal cell carcinoma | 106 | ko05211 |
| 113 | Antigen processing and presentation | 105 | ko04612 |
| 114 | Nucleotide excision repair | 104 | ko03420 |
| 115 | Ascorbate and aldarate metabolism | 104 | ko00053 |
| 116 | Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 104 | ko05412 |
| 117 | TGF-beta signaling pathway | 104 | ko04350 |
| 118 | p53 signaling pathway | 101 | ko04115 |
| 119 | Glioma | 101 | ko05214 |
| 120 | mTOR signaling pathway | 99 | ko04150 |
| 121 | DNA replication | 98 | ko03030 |
| 122 | Serotonergic synapse | 97 | ko04726 |
| 123 | Notch signaling pathway | 97 | ko04330 |
| 124 | Prion diseases | 97 | ko05020 |
| 125 | Fc epsilon RI signaling pathway | 93 | ko04664 |
| 126 | Tyrosine metabolism | 93 | ko00350 |
| 127 | Basal transcription factors | 92 | ko03022 |
| 128 | Vasopressin-regulated water reabsorption | 92 | ko04962 |
| 129 | Glutathione metabolism | 91 | ko00480 |
| 130 | Colorectal cancer | 91 | ko05210 |
| 131 | Amyotrophic lateral sclerosis (ALS) | 90 | ko05014 |
| 132 | Adipocytokine signaling pathway | 89 | ko04920 |

(Continued)

**Table E.1 (cont'd)** Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'. The 'A_Gossypii_Aust_unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptome of four Aphis gossypii strains (including a reference susceptible (Sus F 96) and three thiamethoxam resistant (F 101, Glen twn S and Carr)).

| # | Pathway | Count (15460) | Pathway ID |
|---|---|---|---|
| 133 | Fat digestion and absorption | 88 | ko04975 |
| 134 | Epithelial cell signaling in Helicobacter pylori infection | 87 | ko05120 |
| 135 | Arginine and proline metabolism | 87 | ko00330 |
| 136 | Mineral absorption | 84 | ko04978 |
| 137 | Glycolysis / Gluconeogenesis | 84 | ko00010 |
| 138 | Basal cell carcinoma | 84 | ko05217 |
| 139 | N-Glycan biosynthesis | 83 | ko00510 |
| 140 | Hematopoietic cell lineage | 83 | ko04640 |
| 141 | Legionellosis | 82 | ko05134 |
| 142 | Base excision repair | 81 | ko03410 |
| 143 | Carbohydrate digestion and absorption | 81 | ko04973 |
| 144 | B cell receptor signaling pathway | 80 | ko04662 |
| 145 | Endometrial cancer | 80 | ko05213 |
| 146 | Chronic myeloid leukemia | 80 | ko05220 |
| 147 | Type II diabetes mellitus | 79 | ko04930 |
| 148 | Pyruvate metabolism | 76 | ko00620 |
| 149 | Fructose and mannose metabolism | 74 | ko00051 |
| 150 | Olfactory transduction | 74 | ko04740 |
| 151 | Cytokine-cytokine receptor interaction | 74 | ko04060 |
| 152 | Homologous recombination | 72 | ko03440 |
| 153 | Osteoclast differentiation | 71 | ko04380 |
| 154 | Chagas disease (American trypanosomiasis) | 71 | ko05142 |

(Continued)

**Table E.1 (cont'd)** Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'. The 'A_Gossypii_Aust_unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptome of four Aphis gossypii strains (including a reference susceptible (Sus F 96) and three thiamethoxam resistant (F 101, Glen twn S and Carr)).

| # | Pathway | Count (15460) | Pathway ID |
|---|---------|---------------|------------|
| 155 | Cysteine and methionine metabolism | 71 | ko00270 |
| 156 | Pentose phosphate pathway | 71 | ko00030 |
| 157 | Apoptosis | 71 | ko04210 |
| 158 | Hedgehog signaling pathway | 70 | ko04340 |
| 159 | Glycosphingolipid biosynthesis - ganglio series | 68 | ko00604 |
| 160 | Valine, leucine and isoleucine degradation | 68 | ko00280 |
| 161 | Alanine, aspartate and glutamate metabolism | 68 | ko00250 |
| 162 | Circadian rhythm - fly | 67 | ko04711 |
| 163 | Natural killer cell mediated cytotoxicity | 66 | ko04650 |
| 164 | Acute myeloid leukemia | 66 | ko05221 |
| 165 | Toll-like receptor signaling pathway | 65 | ko04620 |
| 166 | Tryptophan metabolism | 65 | ko00380 |
| 167 | Fatty acid biosynthesis | 64 | ko00061 |
| 168 | Thyroid cancer | 64 | ko05216 |
| 169 | Complement and coagulation cascades | 64 | ko04610 |
| 170 | Pancreatic cancer | 64 | ko05212 |
| 171 | Glycosylphosphatidylinositol(GPI)-anchor biosynthesis | 63 | ko00563 |
| 172 | Long-term depression | 63 | ko04730 |
| 173 | Mismatch repair | 62 | ko03430 |
| 174 | MAPK signaling pathway - fly | 62 | ko04013 |
| 175 | Endocrine and other factor-regulated calcium reabsorption | 61 | ko04961 |
| 176 | Sphingolipid metabolism | 61 | ko00600 |

(Continued)

**Table E.1 (cont'd)** Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'. The 'A_Gossypii_Aust_unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptome of four *Aphis gossypii* strains (including a reference susceptible (Sus F 96) and three thiamethoxam resistant (F 101, Glen twn S and Carr)).

| # | Pathway | Count (15460) | Pathway ID |
|---|---|---|---|
| 177 | Fatty acid metabolism | 61 | ko00071 |
| 178 | Non-small cell lung cancer | 60 | ko05223 |
| 179 | NOD-like receptor signaling pathway | 56 | ko04621 |
| 180 | Proteasome | 55 | ko03050 |
| 181 | Malaria | 53 | ko05144 |
| 182 | Nicotine addiction | 53 | ko05033 |
| 183 | Citrate cycle (TCA cycle) | 52 | ko00020 |
| 184 | Rheumatoid arthritis | 52 | ko05323 |
| 185 | Insect hormone biosynthesis | 51 | ko00981 |
| 186 | Pertussis | 51 | ko05133 |
| 187 | Butanoate metabolism | 50 | ko00650 |
| 188 | Type I diabetes mellitus | 50 | ko04940 |
| 189 | Systemic lupus erythematosus | 49 | ko05322 |
| 190 | Circadian rhythm - mammal | 49 | ko04710 |
| 191 | Glycosaminoglycan biosynthesis - heparan sulfate | 48 | ko00534 |
| 192 | Phenylalanine metabolism | 48 | ko00360 |
| 193 | Melanoma | 47 | ko05218 |
| 194 | Fatty acid elongation | 47 | ko00062 |
| 195 | Leishmaniasis | 46 | ko05140 |
| 196 | beta-Alanine metabolism | 45 | ko00410 |
| 197 | SNARE interactions in vesicular transport | 45 | ko04130 |
| 198 | Phototransduction | 44 | ko04744 |

(Continued)

**Table E.1 (cont'd)** Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'. The 'A_Gossypii_Aust_unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptome of four Aphis gossypii strains (including a reference susceptible (Sus F 96) and three thiamethoxam resistant (F 101, Glen twn S and Carr)).

| #   | Pathway                                   | Count (15460) | Pathway ID |
|-----|-------------------------------------------|---------------|------------|
| 199 | Biosynthesis of unsaturated fatty acids   | 43            | ko01040    |
| 200 | Renin-angiotensin system                  | 42            | ko04614    |
| 201 | Glycosaminoglycan degradation             | 41            | ko00531    |
| 202 | Propanoate metabolism                     | 41            | ko00640    |
| 203 | Collecting duct acid secretion            | 41            | ko04966    |
| 204 | Nicotinate and nicotinamide metabolism    | 41            | ko00760    |
| 205 | Other glycan degradation                  | 40            | ko00511    |
| 206 | Aldosterone-regulated sodium reabsorption | 39            | ko04960    |
| 207 | Bladder cancer                            | 39            | ko05219    |
| 208 | alpha-Linolenic acid metabolism           | 38            | ko00592    |
| 209 | Autoimmune thyroid disease                | 37            | ko05320    |
| 210 | Terpenoid backbone biosynthesis           | 37            | ko00900    |
| 211 | Staphylococcus aureus infection           | 37            | ko05150    |
| 212 | Regulation of autophagy                   | 36            | ko04140    |
| 213 | Arachidonic acid metabolism               | 35            | ko00590    |
| 214 | Ether lipid metabolism                    | 35            | ko00565    |
| 215 | Protein export                            | 33            | ko03060    |
| 216 | Maturity onset diabetes of the young      | 32            | ko04950    |
| 217 | One carbon pool by folate                 | 31            | ko00670    |
| 218 | Taste transduction                        | 31            | ko04742    |
| 219 | Histidine metabolism                      | 30            | ko00340    |
| 220 | RIG-I-like receptor signaling pathway     | 30            | ko04622    |

(Continued)

**Table E.1 (cont'd)** Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'. The 'A_Gossypii_Aust_unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptome of four Aphis gossypii strains (including a reference susceptible (Sus F 96) and three thiamethoxam resistant (F 101, Glen twn S and Carr)).

| # | Pathway | Count (15460) | Pathway ID |
|---|---------|---------------|------------|
| 221 | Glyoxylate and dicarboxylate metabolism | 30 | ko00630 |
| 222 | Glycosaminoglycan biosynthesis - chondroitin sulfate | 30 | ko00532 |
| 223 | Steroid biosynthesis | 29 | ko00100 |
| 224 | Non-homologous end-joining | 29 | ko03450 |
| 225 | Riboflavin metabolism | 28 | ko00740 |
| 226 | Folate biosynthesis | 28 | ko00790 |
| 227 | Linoleic acid metabolism | 27 | ko00591 |
| 228 | Pantothenate and CoA biosynthesis | 23 | ko00770 |
| 229 | Proximal tubule bicarbonate reclamation | 23 | ko04964 |
| 230 | Cyanoamino acid metabolism | 21 | ko00460 |
| 231 | Selenocompound metabolism | 20 | ko00450 |
| 232 | Mucin type O-Glycan biosynthesis | 20 | ko00512 |
| 233 | Sulfur relay system | 17 | ko04122 |
| 234 | African trypanosomiasis | 17 | ko05143 |
| 235 | Taurine and hypotaurine metabolism | 16 | ko00430 |
| 236 | Glycosaminoglycan biosynthesis - keratan sulfate | 14 | ko00533 |
| 237 | Synthesis and degradation of ketone bodies | 14 | ko00072 |
| 238 | Ubiquinone and other terpenoid-quinone biosynthesis | 14 | ko00130 |
| 239 | Sulfur metabolism | 13 | ko00920 |
| 240 | Glycosphingolipid biosynthesis - lacto and neolacto series | 13 | ko00601 |
| 241 | Valine, leucine and isoleucine biosynthesis | 12 | ko00290 |
| 242 | Glycosphingolipid biosynthesis - globo series | 11 | ko00603 |

(Continued)

**Table E.1 (cont'd)** Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'. The 'A_Gossypii_Aust_unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptome of four Aphis gossypii strains (including a reference susceptible (Sus F 96) and three thiamethoxam resistant (F 101, Glen twn S and Carr)).

| # | Pathway | Count (15460) | Pathway ID |
|---|---------|---------------|------------|
| 243 | Asthma | 11 | ko05310 |
| 244 | Biotin metabolism | 11 | ko00780 |
| 245 | Primary bile acid biosynthesis | 10 | ko00120 |
| 246 | Primary immunodeficiency | 10 | ko05340 |
| 247 | Butirosin and neomycin biosynthesis | 10 | ko00524 |
| 248 | Phenylalanine, tyrosine and tryptophan biosynthesis | 9 | ko00400 |
| 249 | Vitamin B6 metabolism | 8 | ko00750 |
| 250 | D-Arginine and D-ornithine metabolism | 6 | ko00472 |
| 251 | Lipoic acid metabolism | 5 | ko00785 |
| 252 | Thiamine metabolism | 5 | ko00730 |
| 253 | Lysine biosynthesis | 4 | ko00300 |
| 254 | D-Glutamine and D-glutamate metabolism | 2 | ko00471 |
| 255 | Caffeine metabolism | 1 | ko00232 |

**Table E.2** Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, $\log_2$ Ratio, False discovery rate (FDR) and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.001 and the absolute value of the $\log_2$ Ratio≥1 were thresholds for determining the significance of gene expression differences.

| GeneID | $\log_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Sus F 96 vs F 101 | | | |
| Unigene6303[a] | 8.66 | 1.54E-08 | - |
| Unigene19540[a] | 8.23 | 2.15E-05 | - |
| Unigene19346[a] | 8.10 | 1.93E-06 | - |
| Unigene16958[a] | 7.96 | 0.000237 | - |
| Unigene8236[a] | 2.72 | 2.48E-24 | EFZ13460.1|hypothetical protein SINV_12007 [Solenopsis invicta] |
| Unigene21349[a] | 2.54 | 1.77E-05 | EFN72115.1|hypothetical protein EAG_00326 [Camponotus floridanus] |
| Unigene10453[a] | 2.52 | 0 | ADY39838.1|Replicase polyprotein [Ascaris suum] |
| Unigene10452[a] | 2.46 | 0 | ACY69873.1|polyprotein-like protein [Glossina morsitans morsitans] |
| Unigene10451[a] | 2.39 | 0 | - |

**(Continued)**

**Table E.2 (cont'd)** Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, log$_2$ Ratio, False discovery rate (FDR) and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.001 and the absolute value of the log$_2$ Ratio≥1 were thresholds for determining the significance of gene expression differences.

| GeneID | log$_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| CL2116.Contig3[a] | 2.37 | 2.93E-131 | XP_001951915.1|/PREDICTED: heat shock protein 70 B2-like [Acyrthosiphon pisum] |
| Unigene17291[a] | 2.25 | 4.37E-28 | XP_001807263.1|PREDICTED: similar to gag-pol polyprotein [Tribolium castaneum] |
| Unigene16335[a] | 2.15 | 1.14E-07 | - |
| Unigene22183[a] | 2.15 | 0.000216 | XP_001807263.1|PREDICTED: similar to gag-pol polyprotein [Tribolium castaneum] |
| Unigene21691[a] | 2.12 | 1.77E-07 | XP_001807662.1|PREDICTED: similar to putative gag-pol protein [Tribolium castaneum] |
| Unigene5014[a] | 2.12 | 9.45E-14 | XP_001951915.1|PREDICTED: heat shock protein 70 B2-like [Acyrthosiphon pisum] |
| CL2116.Contig5[a] | 2.06 | 1.56E-22 | XP_003248918.1|PREDICTED: heat shock protein 68-like, partial [Acyrthosiphon pisum] |
| Unigene7780[a] | 2.04 | 3.91E-25 | XP_003248918.1|PREDICTED: heat shock protein 68-like, partial [Acyrthosiphon pisum] |
| Unigene11682[a] | 1.97 | 1.60E-11 | XP_003248918.1|PREDICTED: heat shock protein 68-like, partial [Acyrthosiphon pisum] |
| Unigene10435[a] | 1.90 | 3.05E-13 | XP_003248918.1|PREDICTED: heat shock protein 68-like, partial [Acyrthosiphon pisum] |
| CL2116.Contig6[a] | 1.89 | 7.44E-26 | XP_003248918.1|PREDICTED: heat shock protein 68-like, partial [Acyrthosiphon pisum] |

**(Continued)**

**Table E.2 (cont'd)** Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, $\log_2$ Ratio, False discovery rate (FDR) and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.001 and the absolute value of the $\log_2$ Ratio≥1 were thresholds for determining the significance of gene expression differences.

| GeneID | $\log_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Unigene15451[b] | -10.60 | 1.63E-31 | XP_001945759.1|PREDICTED: oligopeptidase A-like [Acyrthosiphon pisum] |
| Unigene17197[b] | -10.33 | 7.25E-19 | - |
| Unigene5422[b] | -10.32 | 7.36E-21 | XP_003245146.1|PREDICTED: protein msta, isoform A-like [Acyrthosiphon pisum] |
| Unigene19845[b] | -10.28 | 3.34E-18 | - |
| Unigene18246[b] | -10.21 | 3.38E-19 | - |
| Unigene1782[b] | -10.12 | 3.46E-23 | - |
| Unigene1872[b] | -10.07 | 1.54E-17 | - |
| Unigene17759[b] | -10.05 | 1.50E-15 | - |
| Unigene18414[b] | -10.03 | 7.58E-29 | - |
| Unigene15063[b] | -10.03 | 3.12E-13 | - |
| Unigene19824[b] | -10.00 | 1.52E-16 | NP_001155946.1|exoribonuclease 1 [Acyrthosiphon pisum] |

**(Continued)**

**Table E.2 (cont'd)** Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, log$_2$ Ratio, False discovery rate (FDR) and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.001 and the absolute value of the log$_2$ Ratio≥1 were thresholds for determining the significance of gene expression differences.

| GeneID | log$_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Unigene7980[b] | -9.95 | 1.62E-25 | XP_001948961.2|PREDICTED: probable multidrug resistance-associated protein lethal(2)03659-like [Acyrthosiphon pisum] |
| Unigene18326[b] | -9.94 | 7.48E-24 | XP_001948736.2|PREDICTED: probable multidrug resistance-associated protein lethal(2)03659-like [Acyrthosiphon pisum] |
| Unigene884[b] | -9.93 | 6.89E-15 | - |
| Unigene19195[b] | -9.92 | 7.00E-16 | XP_001943554.1|PREDICTED: hypothetical protein LOC100159424 [Acyrthosiphon pisum] |
| Unigene17685[b] | -9.92 | 1.60E-22 | - |
| Unigene18285[b] | -9.85 | 1.48E-14 | XP_003492803.1|PREDICTED: ATP synthase subunit alpha-like [Bombus impatiens] |
| Unigene18341[b] | -9.84 | 1.50E-15 | XP_003243866.1|PREDICTED: protein toll-like [Acyrthosiphon pisum] |
| Unigene18832[b] | -9.79 | 3.26E-16 | - |
| Unigene19121[b] | -9.76 | 3.22E-15 | - |

(Continued)

**Table E.2 (cont'd)** Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, log$_2$ Ratio, False discovery rate (FDR) and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.001 and the absolute value of the log$_2$ Ratio≥1 were thresholds for determining the significance of gene expression differences.

| GeneID | log$_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Sus F 96 vs Glen twn S | | | |
| Unigene6303[a] | 9.45 | 5.14E-14 | - |
| Unigene5141[a] | 8.90 | 1.54E-09 | - |
| Unigene17389[a] | 8.80 | 2.93E-09 | - |
| Unigene15492[a] | 8.61 | 1.75E-06 | XP_001948285.2|PREDICTED: maltase 2-like [Acyrthosiphon pisum] |
| CL1027.Contig2[a] | 8.58 | 7.98E-05 | - |
| Unigene14552[a] | 8.48 | 3.80E-24 | XP_002027279.1|GL24774 [Drosophila persimilis] |
| Unigene15706[a] | 8.44 | 7.98E-05 | - |
| Unigene1875[a] | 8.16 | 6.29E-06 | XP_003241394.1|PREDICTED: hypothetical protein LOC100575926 [Acyrthosiphon pisum] |
| Unigene19078[a] | 8.12 | 7.98E-05 | XP_003245035.1|PREDICTED: centrosomal protein of 78 kDa-like [Acyrthosiphon pisum] |
| Unigene19346[a] | 8.00 | 6.29E-06 | - |

**(Continued)**

**Table E.2 (cont'd)** Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, log₂ Ratio, False discovery rate (FDR) and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.001 and the absolute value of the log₂ Ratio≥1 were thresholds for determining the significance of gene expression differences.

| GeneID | log₂ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Unigene22343[a] | 7.99 | 4.23E-05 | - |
| Unigene19540[a] | 7.98 | 0.000151 | - |
| Unigene21014[a] | 7.97 | 0.000151 | XP_003247256.1\|PREDICTED: hypothetical protein LOC100569128 [Acyrthosiphon pisum] |
| Unigene22887[a] | 7.78 | 2.24E-05 | XP_002161449.1\|PREDICTED: similar to SET domain and mariner transposase fusion [Hydra magnipapillata] |
| Unigene22994[a] | 7.77 | 7.98E-05 | XP_003247311.1\|PREDICTED: hypothetical protein LOC100574103 [Acyrthosiphon pisum] |
| CL1361.Contig3[a] | 7.65 | 4.27E-10 | XP_003242396.1\|PREDICTED: deoxynucleotidyltransferase terminal-interacting protein 1-like [Acyrthosiphon pisum] |
| Unigene19111[a] | 7.62 | 0.000284 | - |
| Unigene22875[a] | 7.57 | 0.000535 | - |
| Unigene20767[a] | 7.04 | 0.000535 | XP_003241394.1\|PREDICTED: hypothetical protein LOC100575926 [Acyrthosiphon pisum] |
| CL273.Contig1[a] | 7.00 | 3.32E-06 | NP_001155375.1\|glutaredoxin-like [Acyrthosiphon pisum] |

**(Continued)**

**Table E.2 (cont'd)** Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, log$_2$ Ratio, False discovery rate (FDR) and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.001 and the absolute value of the log$_2$ Ratio≥1 were thresholds for determining the significance of gene expression differences.

| GeneID | log$_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Unigene13938[b] | -11.58 | 1.36E-36 | - |
| Unigene13783[b] | -11.06 | 2.39E-52 | - |
| Unigene11861[b] | -10.95 | 4.36E-51 | - |
| Unigene15683[b] | -10.76 | 1.10E-40 | XP_003244579.1\|PREDICTED: hypothetical protein LOC100571774, partial [Acyrthosiphon pisum] |
| Unigene5239[b] | -10.71 | 1.30E-30 | XP_001946997.2\|PREDICTED: hypothetical protein LOC100162722 [Acyrthosiphon pisum] |
| Unigene16205[b] | -10.54 | 2.27E-40 | XP_003244577.1\|PREDICTED: nose resistant to fluoxetine protein 6-like [Acyrthosiphon pisum] |
| CL218.Contig3[b] | -10.39 | 4.48E-23 | CBY13234.1\|unnamed protein product [Oikopleura dioica] |
| Unigene19845[b] | -10.28 | 4.01E-17 | - |
| Unigene932[b] | -10.17 | 4.48E-23 | - |
| Unigene16990[b] | -10.11 | 1.95E-17 | - |
| Unigene16507[b] | -10.10 | 3.40E-21 | XP_003492800.1\|PREDICTED: bifunctional purine biosynthesis protein purH-like [Bombus impatiens] |

**(Continued)**

**Table E.2 (cont'd)** Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, log$_2$ Ratio, False discovery rate (FDR) and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate$\leq$0.001 and the absolute value of the log$_2$ Ratio$\geq$1 were thresholds for determining the significance of gene expression differences.

| GeneID | log$_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Unigene1872[b] | -10.07 | 1.69E-16 | - |
| Unigene18414[b] | -10.03 | 3.70E-27 | - |
| Unigene18254[b] | -9.99 | 3.95E-33 | XP_003243328.1\|PREDICTED: hypothetical protein LOC100574363 [Acyrthosiphon pisum] |
| Unigene18905[b] | -9.94 | 1.09E-13 | XP_003243746.1\|PREDICTED: x-ray radiation resistance-associated protein 1-like [Acyrthosiphon pisum] |
| Unigene18326[b] | -9.94 | 1.89E-22 | XP_001948736.2\|PREDICTED: probable multidrug resistance-associated protein lethal(2)03659-like [Acyrthosiphon pisum] |
| Unigene17020[b] | -9.83 | 5.32E-19 | - |
| Unigene18322[b] | -9.76 | 1.47E-15 | XP_001943231.1\|PREDICTED: phosphatidylinositol 3-kinase catalytic subunit type 3-like [Acyrthosiphon pisum] |
| Unigene19719[b] | -9.69 | 8.10E-12 | - |
| Unigene19097[b] | -9.67 | 4.62E-18 | XP_001950522.1\|PREDICTED: hypothetical protein LOC100163439 [Acyrthosiphon pisum] |
| Unigene17628[b] | -9.65 | 3.39E-11 | XP_001943487.1\|PREDICTED: hypothetical protein LOC100164810 [Acyrthosiphon pisum] |

**(Continued)**

**Table E.2 (cont'd)** Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, log$_2$ Ratio, False discovery rate (FDR) and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.001 and the absolute value of the log$_2$ Ratio≥1 were thresholds for determining the significance of gene expression differences.

| GeneID | log$_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Sus F 96 vs Carr | | | |
| Unigene13810[a] | 10.83 | 7.49E-174 | XP_003242198.1|PREDICTED: hypothetical protein LOC100573940 [Acyrthosiphon pisum] |
| Unigene5980[a] | 10.76 | 2.60E-21 | - |
| Unigene6303[a] | 10.67 | 2.73E-33 | - |
| Unigene14552[a] | 10.49 | 8.45E-101 | XP_002027279.1|GL24774 [Drosophila persimilis] |
| Unigene15492[a] | 10.41 | 4.98E-22 | XP_001948285.2|PREDICTED: maltase 2-like [Acyrthosiphon pisum] |
| Unigene1320[a] | 10.04 | 6.67E-25 | XP_003242199.1|PREDICTED: hypothetical protein LOC100574035 [Acyrthosiphon pisum] |
| Unigene1246[a] | 10.01 | 6.32E-19 | XP_001948285.2|PREDICTED: maltase 2-like [Acyrthosiphon pisum] |
| Unigene5979[a] | 9.86 | 1.46E-11 | - |
| Unigene16958[a] | 9.84 | 2.09E-14 | - |
| Unigene18503[a] | 9.82 | 5.65E-18 | XP_003242202.1|PREDICTED: hypothetical protein LOC100574264 isoform 1 [Acyrthosiphon pisum] |

**(Continued)**

**Table E.2 (cont'd)** Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, $\log_2$ Ratio, False discovery rate (FDR) and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate$\leq$0.001 and the absolute value of the $\log_2$ Ratio$\geq$1 were thresholds for determining the significance of gene expression differences.

| GeneID | $\log_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Unigene19840[a] | 9.62 | 1.31E-31 | XP_003242197.1\|PREDICTED: hypothetical protein LOC100573859 [Acyrthosiphon pisum] |
| Unigene20836[a] | 9.59 | 3.20E-13 | XP_003242199.1\|PREDICTED: hypothetical protein LOC100574035 [Acyrthosiphon pisum] |
| Unigene17389[a] | 9.58 | 4.53E-16 | - |
| Unigene4404[a] | 9.54 | 3.82E-10 | - |
| CL1560.Contig2[a] | 9.48 | 5.34E-60 | XP_003244804.1\|PREDICTED: hypothetical protein LOC100570532 [Acyrthosiphon pisum] |
| CL1027.Contig2[a] | 9.41 | 2.91E-08 | - |
| CL1708.Contig1[a] | 9.29 | 4.91E-12 | BAH70886.1\|ACYPI000014 [Acyrthosiphon pisum] |
| Unigene19111[a] | 9.28 | 1.66E-12 | - |
| Unigene22140[a] | 9.28 | 2.21E-10 | - |
| Unigene5141[a] | 9.26 | 1.66E-12 | - |
| Unigene13938[b] | -11.58 | 3.31E-42 | - |

**(Continued)**

**Table E.2 (cont'd)** Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, log$_2$ Ratio, False discovery rate (FDR) and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.001 and the absolute value of the log$_2$ Ratio≥1 were thresholds for determining the significance of gene expression differences.

| GeneID | log$_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Unigene16132[b] | -10.92 | 7.07E-50 | XP_003241320.1\|PREDICTED: hypothetical protein LOC100571804 [Acyrthosiphon pisum] |
| CL893.Contig3[b] | -10.87 | 2.34E-32 | - |
| Unigene4253[b] | -10.69 | 2.89E-31 | - |
| Unigene17599[b] | -10.66 | 1.02E-28 | - |
| Unigene16085[b] | -10.62 | 4.39E-25 | - |
| Unigene17405[b] | -10.44 | 2.89E-23 | - |
| Unigene185[b] | -10.42 | 1.25E-31 | - |
| Unigene17753[b] | -10.34 | 2.35E-24 | - |
| Unigene19845[b] | -10.28 | 1.23E-19 | - |
| Unigene18008[b] | -10.18 | 1.89E-21 | - |
| Unigene16661[b] | -10.18 | 6.51E-35 | AEO35729.1\|hypothetical protein [Amblyomma maculatum] |

**(Continued)**

**Table E.2 (cont'd)** Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, log$_2$ Ratio, False discovery rate (FDR) and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.001 and the absolute value of the log$_2$ Ratio≥1 were thresholds for determining the significance of gene expression differences.

| GeneID | log$_2$ Ratio[1] (Res/Sus) | FDR | Blast nr |
|---|---|---|---|
| Unigene17266[b] | -10.08 | 6.50E-19 | - |
| Unigene1872[b] | -10.07 | 6.50E-19 | - |
| Unigene18414[b] | -10.03 | 2.89E-31 | - |
| Unigene18907[b] | -9.97 | 4.17E-17 | - |
| Unigene951[b] | -9.92 | 6.09E-15 | - |
| Unigene19766[b] | -9.90 | 2.66E-15 | - |
| Unigene660[b] | -9.77 | 4.36E-21 | - |
| Unigene8883[b] | -9.71 | 2.43E-11 | - |
| Unigene19129[b] | -9.67 | 3.21E-14 | - |

[a]Up-regulated transcripts; [b]Down-regulated transcripts; [1]Ratio, RPKM of resistant/RPKM of susceptible samples. RPKM, reads per kilo bases per million reads.

**Table E.3** List of differentially expressed genes relating to the cytochrome P450 detoxification family among thiamethoxam resistant (F 101, Glen twn S and Carr) *Aphis gossypii* strains (compared to the reference susceptible Sus F 96). Transcript ID, orthologue gene name in the appropriate organism as retrieved from the GenBank (Blast nr) and $\log_2$ Ratio are indicated. False discovery rate$\leq$0.001 and the absolute value of the $\log_2$ Ratio$\geq$1 were thresholds for determining the significance of gene expression differences.

| GeneID | Blast nr | log2 Ratio[1] (Res/Sus) | | |
|---|---|---|---|---|
| | | F 101 | Glen twn S | Carr |
| Unigene12819 | XP_001948934.1|PREDICTED: probable cytochrome P450 6a13-like [Acyrthosiphon pisum] | - | 1.045520134 | 2.422280456 |
| Unigene12511 | XP_001948934.1|PREDICTED: probable cytochrome P450 6a13-like [Acyrthosiphon pisum] | - | 0.696480194[a] | 2.285020359 |
| CL1418.Contig1 | XP_001948421.1|PREDICTED: cytochrome P450 6k1-like [Acyrthosiphon pisum] | 0.044069122[a] | 1.217801839 | 2.404381888 |
| CL1190.Contig1 | XP_001948421.1|PREDICTED: cytochrome P450 6k1-like [Acyrthosiphon pisum] | 0.583157737[a] | 1.818017036 | 3.099165446 |
| CL627.Contig1 | XP_001952620.1|PREDICTED: probable cytochrome P450 305a1-like [Acyrthosiphon pisum] | - | 3.022800058 | 3.423523622 |
| Unigene15803 | XP_001945934.2|PREDICTED: probable cytochrome P450 305a1-like [Acyrthosiphon pisum] | - | 4.385370137 | 4.337108871 |
| Unigene4712 | XP_001952620.1|PREDICTED: probable cytochrome P450 305a1-like [Acyrthosiphon pisum] | - | 2.878410148 | 3.095290951 |

[a]Indicates genes which are not differentially expressed but were included for comparison; [1]Ratio, RPKM of resistant/RPKM of susceptible samples. RPKM, reads per kilo bases per million reads.

Table E.4 List of differentially expressed nicotinic acetylcholine receptor subunit genes among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, orthologue gene name in the appropriate organism as retrieved from the GenBank (Blast nr) and $log_2$ Ratio are indicated. False discovery rate≤0.001 and the absolute value of the $log_2$ Ratio≥1 were thresholds for determining the significance of gene expression differences.

| GeneID | Blast nr | $log_2$ Ratio[1] | | | |
|---|---|---|---|---|---|
| | | Sus F 96 | F 101 | Glen twn S | Carr |
| Unigene11848 | CAA57476.1\|/0/nicotinic acetylcholine receptor alpha subunit [Myzus persicae] | 23.72323 | 4.896363 | 6.761522 | 9.049615 |
| Unigene10018 | AFP55242.1\|/0/nicotinic acetylcholine receptor alpha 4 subunit [Aphis glycines] | 15.53073 | 3.691985 | 3.774305 | 6.830007 |
| Unigene12372 | AEV54111.1\|/0/nicotinic acetylcholine receptor alpha 2 subunit [Aphis gossypii] | 21.63213 | 2.951797 | 3.539873 | 7.879289 |
| CL1326.Contig2 | AFP55243.1\|/0/nicotinic acetylcholine receptor beta 1 subunit [Aphis glycines] | 3.810137 | 1.117733 | 1.642734 | 1.910338 |
| Unigene6313 | AEV54111.1\|/5.98093e-66/nicotinic acetylcholine receptor alpha 2 subunit [Aphis gossypii] | 16.03923 | 3.036168 | 2.950106 | 5.5476 |
| Unigene10203 | AFH00994.1\|/8.04063e-74/nicotinic acetylcholine receptor beta 1 subunit [Aphis gossypii] | 9.206973 | 2.114636 | 3.02445 | 3.922346 |
| Unigene12768 | ABR21379.1\|/1.31991e-12/nicotinic acetylcholine receptor alpha 3 subunit [Aphis gossypii] | 20.29044 | 5.893415 | 5.272609 | 8.498577 |
| CL834.Contig2 | AEO91541.1\|/0/nicotinic acetylcholine receptor alpha 2 subunit [Aphis glycines] | 1.891482 | 0.294408 | 0.752958 | 0.845874 |
| Unigene20148 | AEV54113.1\|/2.6702e-77/nicotinic acetylcholine receptor alpha 4-2 [Aphis gossypii] | 3.469474 | 1.103186 | 2.209797 | 2.448757 |
| CL1326.Contig1 | AFP55243.1\|/1.1358e-175/nicotinic acetylcholine receptor beta 1 subunit [Aphis glycines] | 0.362329 | 0.05965 | 0.194163 | 0.260799 |

**(Continued)**

**Table E.4 (cont'd)** List of differentially expressed nicotinic acetylcholine receptor subunit genes among thiamethoxam resistant (F 101, Glen twn S and Carr) and susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, orthologue gene name in the appropriate organism as retrieved from the GenBank (Blast nr) and log$_2$ Ratio are indicated. False discovery rate≤0.001 and the absolute value of the log$_2$ Ratio≥1 were thresholds for determining the significance of gene expression differences.

| GeneID | Blast nr | log$_2$ Ratio[1] | | | |
|---|---|---|---|---|---|
| | | Sus F 96 | F 101 | Glen twn S | Carr |
| Unigene1409 | AEV54113.1|/7.79165e-37/nicotinic acetylcholine receptor alpha 4-2 [Aphis gossypii] | 4.203021 | 2.126141 | 1.06472 | 2.002179 |
| Unigene20625 | AEV54113.1|/1.47694e-43/nicotinic acetylcholine receptor alpha 4-2 [Aphis gossypii] | 4.651564 | 1.422168 | 1.234458 | 1.616667 |
| CL834.Contig1 | CAA57477.1|/0/nicotinic acetyl choline receptor alpha-subunit [Myzus persicae] | - | - | - | 0.024128 |

[1] Ratio, RPKM of resistant/RPKM of susceptible samples. RPKM, reads per kilo bases per million reads.

174 | P a g e

**Table E.5** Four differentially expressed transcripts by RNA-Seq and qRT-PCR analysis among thiamethoxam resistant (F 101, Glen twn S and Carr) *Aphis gossypii* strains (compared to the reference susceptible Sus F 96).

| Gene ID | Description | $\log_2$ Ratio[a] | | | qRT-PCR fold[b] | | |
|---|---|---|---|---|---|---|---|
| | | F 101 | Glen twn S | Carr | F 101 | Glen twn S | Carr |
| CL1418 | cytochrome P450 6k1-like [Acyrthosiphon pisum] | 0.044069122* | 1.217801839 | 2.404381888 | 23.85518555 | 31.61857289 | 27.82101852 |
| CL1190 | cytochrome P450 6k1-like [Acyrthosiphon pisum] | 0.583157737* | 1.818017036 | 3.099165446 | 9.268142804 | 12.79010477 | 10.92957044 |
| Unigene10451 | Rhopalosiphum padi virus clone RhPV6 | 2.387079771 | 1.877274135 | 1.735050914 | - | 0.085260928 | 0.513416077 |
| Unigene10452 | Rhopalosiphum padi virus clone RhPV6 | 2.457089645 | 2.370491142 | 1.921851692 | 0.168513252 | 0.13612662 | 0.639917571 |

[a]Ratio, RPKM of resistant samples / RPKM of susceptible samples. RPKM: Reads per kilobases per million reads. FDR≤0.001 and the absolute value of log2 Ratio ≥ 1 were used as thresholds to determine the significance of gene expression differences; [b]Delta Delta C (T) method (2−ΔΔC T) was used for analysis of relative expression. *Denotes transcripts which were not significantly differentially expressed in RNA-Seq analysis.

# Bibliography

Abbott, W. S. (1925). "A method for computing the effectiveness of an insecticide." Journal of Economic Entomology **18**: 265-267.

Adams, M. D., S. E. Celniker, R. A. Holt, C. A. Evans, J. D. Gocayne, P. G. Amanatides, S. E. Scherer, P. W. Li, R. A. Hoskins, R. F. Galle, R. A. George, S. E. Lewis, S. Richards, M. Ashburner, S. N. Henderson, G. G. Sutton, J. R. Wortman, M. D. Yandell, Q. Zhang, L. X. Chen, R. C. Brandon, Y. H. Rogers, R. G. Blazej, M. Champe, B. D. Pfeiffer, K. H. Wan, C. Doyle, E. G. Baxter, G. Helt, C. R. Nelson, G. L. Gabor, J. F. Abril, A. Agbayani, H. J. An, C. Andrews-Pfannkoch, D. Baldwin, R. M. Ballew, A. Basu, J. Baxendale, L. Bayraktaroglu, E. M. Beasley, K. Y. Beeson, P. V. Benos, B. P. Berman, D. Bhandari, S. Bolshakov, D. Borkova, M. R. Botchan, J. Bouck, P. Brokstein, P. Brottier, K. C. Burtis, D. A. Busam, H. Butler, E. Cadieu, A. Center, I. Chandra, J. M. Cherry, S. Cawley, C. Dahlke, L. B. Davenport, P. Davies, B. de Pablos, A. Delcher, Z. Deng, A. D. Mays, I. Dew, S. M. Dietz, K. Dodson, L. E. Doup, M. Downes, S. Dugan-Rocha, B. C. Dunkov, P. Dunn, K. J. Durbin, C. C. Evangelista, C. Ferraz, S. Ferriera, W. Fleischmann, C. Fosler, A. E. Gabrielian, N. S. Garg, W. M. Gelbart, K. Glasser, A. Glodek, F. Gong, J. H. Gorrell, Z. Gu, P. Guan, M. Harris, N. L. Harris, D. Harvey, T. J. Heiman, J. R. Hernandez, J. Houck, D. Hostin, K. A. Houston, T. J. Howland, M. H. Wei, C. Ibegwam, M. Jalali, F. Kalush, G. H. Karpen, Z. Ke, J. A. Kennison, K. A. Ketchum, B. E. Kimmel, C. D. Kodira, C. Kraft, S. Kravitz, D. Kulp, Z. Lai, P. Lasko, Y. Lei, A. A. Levitsky, J. Li, Z. Li, Y. Liang, X. Lin, X. Liu, B. Mattei, T. C. McIntosh, M. P. McLeod, D. McPherson, G. Merkulov, N. V. Milshina, C. Mobarry, J. Morris, A. Moshrefi, S. M. Mount, M. Moy, B. Murphy, L. Murphy, D. M. Muzny, D. L. Nelson, D. R. Nelson, K. A. Nelson, K. Nixon, D. R. Nusskern, J. M. Pacleb, M. Palazzolo, G. S. Pittman, S. Pan, J. Pollard, V. Puri, M. G. Reese, K. Reinert, K. Remington, R. D. Saunders, F. Scheeler, H. Shen, B. C. Shue, I. Siden-

Kiamos, M. Simpson, M. P. Skupski, T. Smith, E. Spier, A. C. Spradling, M. Stapleton, R. Strong, E. Sun, R. Svirskas, C. Tector, R. Turner, E. Venter, A. H. Wang, X. Wang, Z. Y. Wang, D. A. Wassarman, G. M. Weinstock, J. Weissenbach, S. M. Williams, WoodageT, K. C. Worley, D. Wu, S. Yang, Q. A. Yao, J. Ye, R. F. Yeh, J. S. Zaveri, M. Zhan, G. Zhang, Q. Zhao, L. Zheng, X. H. Zheng, F. N. Zhong, W. Zhong, X. Zhou, S. Zhu, X. Zhu, H. O. Smith, R. A. Gibbs, E. W. Myers, G. M. Rubin and J. C. Venter (2000). "The genome sequence of *Drosophila melanogaster*." Science **287**(5461): 2185-2195.

Adessi, C., G. Matton, G. Ayala, G. Turcatti, J. J. Mermod, P. Mayer and E. Kawashima (2000). "Solid phase DNA amplification: characterisation of primer attachment and amplification mechanisms." Nucleic Acids Research **28**(20): 87.

Agarwala, B. K. and P. R. Choudhury (2013). "Host Races of the Cotton Aphid, *Aphis gossypii*, in Asexual Populations from Wild Plants of Taro and Brinjal." Journal of Insect Science **13**(34): 1-13.

Aggs, R. (2011). Near total resistance. Ag Today, New South Wales Department of Primary Industries: 3.

Ahmad, M., M. I. Arif and I. Denholm (2003). "High resistance of field populations of the cotton aphid *Aphis gossypii* Glover (Homoptera: Aphididae) to pyrethroid insecticides in Pakistan." Journal of Economic Entomology **96**(3): 875-878.

Ahmad, M. and A. R. McCaffery (1988). "Resistance to Insecticides in a Thailand Strain of *Heliothis armigera* (Hübner) (Lepidoptera: Noctuidae)." Journal of Economic Entomology **81**(1): 45-48.

Alyokhin, A., M. Baker, D. Mota-Sanchez, G. Dively and E. Grafius (2008). "Colorado Potato Beetle Resistance to Insecticides." American Journal of Potato Research **85**(6): 395-413.

Amichot, M., S. Tares, A. Brun-Barale, L. Arthaud, J. M. Bride and J. B. Berge (2004). "Point mutations associated with insecticide resistance in the Drosophila cytochrome P450 Cyp6a2 enable DDT metabolism." European Journal of Biochemistry **271**(7): 1250-1257.

Andrews, M. C., A. Callaghan, L. M. Field, M. S. Williamson and G. D. Moores (2004). "Identification of mutations conferring insecticide-insensitive AChE in the cotton-melon aphid, *Aphis gossypii* Glover." Insect Molecular Biology **13**(5): 555-561.

Anthony, N., T. Unruh, D. Ganser and R. ffrench-Constant (1998). "Duplication of the Rdl GABA receptor subunit gene in an insecticide-resistant aphid, *Myzus persicae*." Molecular Genetics and Genomics **260**: 165-175.

Apperson, C. S. and G. P. Georghiou (1975). "Mechanisms of resistance to organophosphorus insecticides in *Culex tarsalis*." Journal of Economic Entomology **68**(2): 153-157.

APVMA. (2013). "Overview Report: Neonicotinoids and the health of honey bees in Australia." February 2014. Retrieved March 9th, 2014, from https://archive.apvma.gov.au/news_media/chemicals/bee_and_neonicotinoids.php.

APVMA. (2014). "Agricultural and Veterinary Permits search." Retrieved 11th May, 2015, from https://portal.apvma.gov.au/permits.

Arakane, Y., S. Muthukrishnan, R. W. Beeman, M. R. Kanost and K. J. Kramer (2005). "Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning." Proceedings of the National Academy of Sciences of the United States of America **102**(32): 11337-11342.

Audic, S. and J. M. Claverie (1997). "The significance of digital gene expression profiles." Genome Research **7**(10): 986-995.

Barchia, I. (2001). Probit analysis and fiducial limits in Genstat. Genstat 2001 Program and Abstracts. V. Doogan, D. Mayer and T. Swain. Mercure Resort, Gold Coast, Australia. 31st January–2nd February: p. 3.

Bass, C. and L. M. Field (2011). "Gene amplification and insecticide resistance." Pest Management Science **67**(8): 886-890.

Bass, C., A. Puinean, M. Andrews, P. Cutler, M. Daniels, J. Elias, V. Paul, A. Crossthwaite, I. Denholm, L. Field, S. Foster, R. Lind, M. Williamson and R. Slater (2011). "Mutation of a nicotinic acetylcholine receptor beta subunit is associated with resistance to neonicotinoid insecticides in the aphid *Myzus persicae*." BMC Neuroscience **12**(51): 1-11.

Bass, C., C. T. Zimmer, J. M. Riveron, C. S. Wilding, C. S. Wondji, M. Kaussmann, L. M. Field, M. S. Williamson and R. Nauen (2013). "Gene amplification and microsatellite polymorphism underlie a recent insect host shift." Proceedings of the National Academy of Sciences **110**(48): 19460-19465.

Baxter, S. W., M. Chen, A. Dawson, J.-Z. Zhao, H. Vogel, A. M. Shelton, D. G. Heckel and C. D. Jiggins (2010). "Mis-Spliced Transcripts of Nicotinic Acetylcholine Receptor α6 Are Associated with Field Evolved Spinosad Resistance in *Plutella xylostella* (L.)." PLoS Genetics **6**(1): 1-10.

Benjamini, Y. and D. Yekutieli (2001). "The control of the false discovery rate in multiple testing under dependency." The Annals of Statistics **29**(4): 1165-1188.

Bennett, S. (2004). "Solexa Ltd." Pharmacogenomics **5**(4): 433-438.

Bennett, S. T., C. Barnes, A. Cox, L. Davies and C. Brown (2005). "Toward the 1,000 dollars human genome." Pharmacogenomics **6**(4): 373-382.

Benting, J. and R. Nauen (2004). "Biochemical evidence that an S431F mutation in acetylcholinesterase-1 of *Aphis gossypii* mediates resistance to pirimicarb and omethoate." Pest Management Science **60**(11): 1051-1055.

Bentley, D. R. (2006). "Whole-genome re-sequencing." Current Opinion in Genetics & Development **16**(6): 545-552.

Bentley, D. R., S. Balasubramanian, H. P. Swerdlow, G. P. Smith, J. Milton, C. G. Brown, K. P. Hall, D. J. Evers, C. L. Barnes, H. R. Bignell, J. M. Boutell, J. Bryant, R. J. Carter, R. Keira Cheetham, A. J. Cox, D. J. Ellis, M. R. Flatbush, N. A. Gormley, S. J. Humphray, L. J. Irving, M. S. Karbelashvili, S. M. Kirk, H. Li, X. Liu, K. S. Maisinger, L. J. Murray, B. Obradovic, T. Ost, M. L. Parkinson, M. R. Pratt, I. M. Rasolonjatovo, M. T. Reed, R. Rigatti, C. Rodighiero, M. T.

Ross, A. Sabot, S. V. Sankar, A. Scally, G. P. Schroth, M. E. Smith, V. P. Smith, A. Spiridou, P. E. Torrance, S. S. Tzonev, E. H. Vermaas, K. Walter, X. Wu, L. Zhang, M. D. Alam, C. Anastasi, I. C. Aniebo, D. M. Bailey, I. R. Bancarz, S. Banerjee, S. G. Barbour, P. A. Baybayan, V. A. Benoit, K. F. Benson, C. Bevis, P. J. Black, A. Boodhun, J. S. Brennan, J. A. Bridgham, R. C. Brown, A. A. Brown, D. H. Buermann, A. A. Bundu, J. C. Burrows, N. P. Carter, N. Castillo, E. C. M. Chiara, S. Chang, R. Neil Cooley, N. R. Crake, O. O. Dada, K. D. Diakoumakos, B. Dominguez-Fernandez, D. J. Earnshaw, U. C. Egbujor, D. W. Elmore, S. S. Etchin, M. R. Ewan, M. Fedurco, L. J. Fraser, K. V. Fuentes Fajardo, W. Scott Furey, D. George, K. J. Gietzen, C. P. Goddard, G. S. Golda, P. A. Granieri, D. E. Green, D. L. Gustafson, N. F. Hansen, K. Harnish, C. D. Haudenschild, N. I. Heyer, M. M. Hims, J. T. Ho, A. M. Horgan, K. Hoschler, S. Hurwitz, D. V. Ivanov, M. Q. Johnson, T. James, T. A. Huw Jones, G. D. Kang, T. H. Kerelska, A. D. Kersey, I. Khrebtukova, A. P. Kindwall, Z. Kingsbury, P. I. Kokko-Gonzales, A. Kumar, M. A. Laurent, C. T. Lawley, S. E. Lee, X. Lee, A. K. Liao, J. A. Loch, M. Lok, S. Luo, R. M. Mammen, J. W. Martin, P. G. McCauley, P. McNitt, P. Mehta, K. W. Moon, J. W. Mullens, T. Newington, Z. Ning, B. Ling Ng, S. M. Novo, M. J. O'Neill, M. A. Osborne, A. Osnowski, O. Ostadan, L. L. Paraschos, L. Pickering, A. C. Pike, A. C. Pike, D. Chris Pinkard, D. P. Pliskin, J. Podhasky, V. J. Quijano, C. Raczy, V. H. Rae, S. R. Rawlings, A. Chiva Rodriguez, P. M. Roe, J. Rogers, M. C. Rogert Bacigalupo, N. Romanov, A. Romieu, R. K. Roth, N. J. Rourke, S. T. Ruediger, E. Rusman, R. M. Sanches-Kuiper, M. R. Schenker, J. M. Seoane, R. J. Shaw, M. K. Shiver, S. W. Short, N. L. Sizto, J. P. Sluis, M. A. Smith, J. Ernest Sohna Sohna, E. J. Spence, K. Stevens, N. Sutton, L. Szajkowski, C. L. Tregidgo, G. Turcatti, S. Vandevondele, Y. Verhovsky, S. M. Virk, S. Wakelin, G. C. Walcott, J. Wang, G. J. Worsley, J. Yan, L. Yau, M. Zuerlein, J. Rogers, J. C. Mullikin, M. E. Hurles, N. J. McCooke, J. S. West, F. L. Oaks, P. L. Lundberg, D. Klenerman, R. Durbin and A. J. Smith (2008). "Accurate whole human genome sequencing using reversible terminator chemistry." Nature **456**(7218): 53-59.

Bingham, G., R. V. Gunning, G. Delogu, V. Borzatta, L. M. Field and G. D. Moores (2008). "Temporal synergism can enhance carbamate and neonicotinoid

insecticidal activity against resistant crop pests." Pest Management Science **64**(1): 81-85.

Bingham, G., C. Strode, L. Tran, P. T. Khoa and H. P. Jamet (2011). "Can piperonyl butoxide enhance the efficacy of pyrethroids against pyrethroid-resistant *Aedes aegypti*?" Tropical Medicine & International Health **16**(4): 492-500.

Blackman, R. L. (1987). Reproduction, Cytogenetics and Development. Aphids: Their Biology, Natural Enemies and Control. A. K. Minks and P. Harrewijn. Elsevier, Amsterdam.

Blackman, R. L. (2000). "The cloning experts." Antenna: Bulletin of the Royal Entomological Society **24**(4): 206-214.

Blackman, R. L. and V. F. Eastop (2000). Aphids on the World's Crops: An Identification and Information Guide (2nd Edition). Chichester, UK, John Wiley and Sons.

Breer, H. and D. B. Sattelle (1987). "Molecular properties and functions of insect acetylcholine receptors." Journal of Insect Physiology **33**(11): 771-790.

Brejc, K., W. J. van Dijk, R. V. Klaassen, M. Schuurmans, J. van der Oost, A. B. Smit and T. K. Sixma (2001). "Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors." Nature **411**(6835): 269-276.

Brown, J. (1992). Virus Diseases. Cotton diseases. R. Hillocks. Wallingford, UK., CAB International: 275-329.

Brown, T. M. and W. G. Brogdon (1987). "Improved detection of insecticide resistance through conventional and molecular techniques." Annual Review of Entomology **32**: 145-162.

Busvine, J. R. (1971). A critical review of the techniques for testing insecticides. Slough, England, Commonwealth Agricultural Bureaux.

CABI. (2005). "Invasive Species Compendium." Retrieved July 12th, 2014, from www.cabi.org.

Cacho, A., E. Smirnova, S. Huzurbazar and X. Cui (2015). "A Comparison of Base-calling Algorithms for Illumina Sequencing Technology." Briefings in Bioinformatics.

Campbell, P. M., R. D. Newcomb, R. J. Russell and J. G. Oakeshott (1998). "Two different amino acid substitutions in the ali-esterase, E3, confer alternative types of organophosphorus insecticide resistance in the sheep blowfly, *Lucilia cuprina*." Insect Biochemistry and Molecular Biology **28**(3): 139-150.

Campbell, P. M., J. F. Trott, C. Claudianos, K. A. Smyth, R. J. Russell and J. G. Oakeshott (1997). "Biochemistry of esterases associated with organophosphate resistance in *Lucilia cuprina* with comparisons to putative orthologues in other Diptera." Biochemical Genetics **35**(1): 17-40.

Carletto, J., E. Lombaert, P. Chavigny, T. Brevault, L. Lapchin and F. Vanlerberghe-Masutti (2009). "Ecological specialization of the aphid *Aphis gossypii* Glover on cultivated host plants." Molecular Ecology **18**(10): 2198-2212.

Carletto, J., T. Martin, F. Vanlerberghe-Masutti and T. Brevault (2010). "Insecticide resistance traits differ among and within host races in *Aphis gossypii*." Pest Management Science **66**(3): 301-307.

Celie, P. H. N., S. E. van Rossum-Fikkert, W. J. van Dijk, K. Brejc, A. B. Smit and T. K. Sixma (2004). "Nicotine and Carbamylcholine Binding to Nicotinic Acetylcholine Receptors as Studied in AChBP Crystal Structures." Neuron **41**(6): 907-914.

Chen, E. H., D. D. Wei, G. M. Shen, G. R. Yuan, P. P. Bai and J. J. Wang (2014). "De novo characterization of the *Dialeurodes citri* transcriptome: mining genes involved in stress resistance and simple sequence repeats (SSRs) discovery." Insect Molecular Biology **23**(1): 52-66.

Chen, H., X.-L. Xu, Y.-P. Li and J.-X. Wu (2014). "Characterization of heat shock protein 90, 70 and their transcriptional expression patterns on high temperature in adult of *Grapholita molesta* (Busck)." Insect Science **21**(4): 439-448.

Chen, Y. Z., F. Vanlerberghe-Masutti, L. J. Wilson, I. Barchia, M. O. McLoon, T. Smith and G. A. Herron (2013). "Evidence of superclones in Australian cotton aphid *Aphis gossypii* Glover (Aphididae: Hemiptera)." Pest Management Science **69**(8): 938-948.

Chimpanzee Sequencing and Analysis Consortium (2005). "Initial sequence of the chimpanzee genome and comparison with the human genome." Nature **437**(7055): 69-87.

Conesa, A., S. Gotz, J. M. Garcia-Gomez, J. Terol, M. Talon and M. Robles (2005). "Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research." Bioinformatics **21**(18): 3674-3676.

Corrêa, R. L., T. F. Silva, J. L. Simões-Araújo, P. A. V. Barroso, M. S. Vidal and M. F. S. Vaslin (2005). "Molecular characterization of a virus from the family Luteoviridae associated with cotton blue disease." Archives of Virology **150**(7): 1357-1367.

Corringer, P. J., N. Le Novere and J. Changeux (2000). "Nicotinic receptors at the amino acid level." Annual Review of Pharmacology and Toxicology **40**: 431-458.

CottASSIST. (2008). "Aphid Yield Loss Estimator." Retrieved November 17th, 2014, from https://www.cottassist.com.au.

Cotton Catchment Communities Cooperative Research Centre. (2008). "Cotton Insects: Aphid ecology in cotton." On Farm Series: How To Retrieved February 11th, 2015, from http://www.insidecotton.com/.

CottonInfo (2015). Cotton Pest Management Guide 2015-16. Toowoomba, QLD, Greenmount Press.

Crow, J. F. (1957). "Genetics of Insect Resistance to Chemicals." Annual Review of Entomology **2**(1): 227-246.

Davies, T. E., A. O. O'Reilly, L. M. Field, B. Wallace and M. S. Williamson (2008). "Knockdown resistance to DDT and pyrethroids: from target-site mutations to molecular modelling." Pest Management Science **64**(11): 1126-1130.

Dean, M., A. Rzhetsky and R. Allikmets (2001). "The human ATP-binding cassette (ABC) transporter superfamily." Genome Research **11**(7): 1156-1166.

Debnath, B., S. Gayen, S. K. Naskar, K. Roy and T. Jha (2003). "Quantitative structure-activity relationship study on some azidopyridinyl neonicotinoid insecticides for their selective affinity towards the drosophila nicotinic receptor over mammalian alpha4beta2 receptor using electrotopological state atom index." Drug Design Discovery **18**(2-3): 81-89.

Denholm, I., R. M. Sawicki and A. W. Farnham (1984). The relatioship between insecticide resistance and control failure. 1984 British Crop Protection Conference, Pests and Diseases, Brighton, British Crop Protection Council, Croydon, England.

Dermauw, W. and T. Van Leeuwen (2014). "The ABC gene family in arthropods: Comparative genomics and role in insecticide transport and resistance." Insect Biochemistry and Molecular Biology **45**: 89-110.

Devonshire, A. L. (1989). Resistance of Aphids to Insecticides. World Crop Pests: Aphids, Their Biology, Natural Enemies and Control. W. Helle. Amsterdam, The Netherlands, Elsevier. **Vol. C:** 123-139.

DeVries, D. H. and G. P. Georghiou (1981). "Decreased nerve sensitivity and decreased cuticular penetration as mechanisms of resistance to pyrethroids in a (1R)-trans-permethrin-selected strain of the house fly." Pesticide Biochemistry and Physiology **15**(3): 234-241.

Diguistini, S., N. Y. Liao, D. Platt, G. Robertson, M. Seidel, S. K. Chan, T. R. Docking, I. Birol, R. A. Holt, M. Hirst, E. Mardis, M. A. Marra, R. C. Hamelin, J. Bohlmann, C. Breuil and S. J. Jones (2009). "De novo genome sequence assembly of a filamentous fungus using Sanger, 454 and Illumina sequence data." Genome Biology **10**(9).

Ding, Z., Y. Wen, B. Yang, Y. Zhang, S. Liu, Z. Liu and Z. Han (2013). "Biochemical mechanisms of imidacloprid resistance in *Nilaparvata lugens*: over-expression of cytochrome P450 CYP6AY1." Insect Biochemistry and Molecular Biology **43**(11): 1021-1027.

Dixon, A. F. G. (1975). "Function of the siphunculi in aphids with particular reference to the sycamore aphid, *Drepanosiphum platanoides*." Journal of Zoology **175**(2): 279-289.

Dixon, A. F. G. (1988). Host specificity and speciation Aphid Ecology: An Optimization Approach Second Edition. London, United Kingdom, Chapman and Hall: 27-57.

Dixon, A. F. G. (1989). Parthenogenetic reproduction and the rate of increase in aphids. Aphids - their biology, natural enemies and control. A. K. Minks and P. Harrewijn. Amsterdam, Elsevier. **Volume A:** 269-287.

Dixon, A. F. G. (1992). "Constraints on the rate of parthenogenetic reproduction and pest status of aphids." Invertebrate Reproduction & Development **22**(1-3): 159-163.

Doyle, L. and D. D. Ross (2003). "Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2)." Oncogene **22**(47): 7340-7358.

Elbert, A., B. Becker, J. Hartwig and C. Erdelen (1991). "Imidacloprid - a new systemic insecticide." Pflanzenschutz-Nachrichten Bayer **44**(2): 113-136.

Elbert, A., M. Haas, B. Springer, W. Thielert and R. Nauen (2008). "Applied aspects of neonicotinoid uses in crop protection." Pest Management Science **64**(11): 1099-1105.

Elbert, T. A. and B. Cartwright (1997). "Biology and ecology of *Aphis gossypii* Glover (Homoptera: Aphididae)." Southwestern Entomologist **22**(1): 116-153.

Ellis, M. H., T. F. Silva, W. N. Stiller, L. J. Wilson, M. F. S. Vaslin, M. Sharman and D. J. Llewellyn (2013). "Identification of a new Polerovirus (family Luteoviridae)

274 of 308

associated with cotton bunchy top disease in Australia." Australasian Plant Pathology **42**(3): 261-269.

Ettre, L. S. and A. Guttman (1996). "The evolution of capillary gel electrophoresis: From proteins to DNA sequencing." LCGC North America **22**(9): 896-904.

Faucon, F., I. Dusfour, T. Gaude, V. Navratil, F. Boyer, F. Chandre, P. Sirisopa, K. Thanispong, W. Juntarajumnong, R. Poupardin, T. Chareonviriyaphap, R. Girod, V. Corbel, S. Reynaud and J. P. David (2015). "Identifying genomic changes associated with insecticide resistance in the dengue mosquito *Aedes aegypti* by deep targeted sequencing." Genome Research **25**(9): 1347-1359.

Feder, M. E. and G. E. Hofmann (1999). "Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology." Annual Review of Physiology **61**: 243-282.

Feng, H., L. Wang, Y. Liu, L. He, M. Li, W. Lu and C. Xue (2010). "Molecular characterization and expression of a heat shock protein gene (HSP90) from the carmine spider mite, *Tetranychus cinnabarinus* (Boisduval)." Journal of Insect Science **10**(112): 1-14.

Feng, Y. T., Q. J. Wu, B. Y. Xu, S. L. Wang, X. L. Chang, W. Xie and Y. J. Zhang (2009). "Fitness costs and morphological change of laboratory-selected thiamethoxam resistance in the B-type *Bemisia tabaci* (Hemiptera: Aleyrodidae)." Journal of Applied Entomology **133**(6): 466-472.

Ferre, J. and J. Van Rie (2002). "Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*." Annual Review of Entomology **47**: 501-533.

Feyereisen, R. (1995). "Molecular biology of insecticide resistance." Toxicology Letters **82–83**: 83-90.

Feyereisen, R. (1999). "INSECT P450 ENZYMES." Annual Review of Entomology **44**(1): 507-533.

Feyereisen, R. (2005). Insect Cytochrome P450. Comprehensive Molecular Insect Science: Biochemistry and Molecular Biology. Gilbert L.I., Iatrou K. and G. S.S. Amsterdam, Elsevier. **4:** 1-77.

ffrench-Constant, R., K. Aronstein, N. Anthony and C. Coustau (1995). "Polymerase chain reaction-based monitoring techniques for the detection of insecticide resistance-associated point mutations and their potential applications." Pesticide Science **43**(3): 195-200.

ffrench-Constant, R. H. and R. T. Roush (1990). Resistance Detection and Documentation: The Relative Roles of Pesticidal and Biochemical Assays. Pesticide Resistance in Arthropods. R. T. Roush and B. E. Tabashnik. Great Britain, Chapman and Hall: 4-38.

ffrench–Constant, R. H., B. Pittendrigh, A. Vaughan and N. Anthony (1998). "Why are there so few resistance–associated mutations in insecticide target genes?" Philosophical Transactions of the Royal Society of London B: Biological Sciences **353**(1376): 1685-1693.

Field, L. M., R. L. Blackman, C. Tyler-Smith and A. L. Devonshire (1999). "Relationship between amount of esterase and gene copy number in insecticide-resistant *Myzus persicae* (Sulzer)." Biochemical Journal **339** ( **Pt 3**): 737-742.

Finney, D. J. (1971). Probit Analysis. Cambridge, UK., Cambridge University Press.

Fitt, G., L. Wilson, D. Kelly and R. Mensah (2009). Advances with Integrated Pest Management as a Component of Sustainable Agriculture: The Case of the Australian Cotton Industry Integrated Pest Management: Dissemination and Impact. R. Peshin and A. K. Dhawan. Netherlands, Springer: 507-524.

Foster, S. P., G. Devine and A. L. Devonshire (2007). Insecticide Resistance. Aphids As Crop Pests. H. van Emden and R. Harrington. Wallingford, United Kingdom, CABI: 261-286.

Fournier, D. (2005). "Mutations of acetylcholinesterase which confer insecticide resistance in insect populations." Chemico-Biological Interactions **157–158**: 257-261.

Furk, C., D. F. Powell and S. Heyd (1980). "Pirimicarb resistance in the melon and cotton aphid, *Aphis gossypii* Glover." Plant Pathology **29**(4): 191-196.

Galzi, J. L., F. Revah, A. Bessis and J. P. Changeux (1991). "Functional Architecture of the Nicotinic Acetylcholine Receptor: From Electric Organ to Brain." Annual Review of Pharmacology and Toxicology **31**(1): 37-72.

Gao, C.-F., S.-Z. Ma, C.-H. Shan and S.-F. Wu (2014). "Thiamethoxam resistance selected in the western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae): Cross-resistance patterns, possible biochemical mechanisms and fitness costs analysis." Pesticide Biochemistry and Physiology **114**: 90-96.

Gao, C. F., S. Z. Ma, C. H. Shan and S. F. Wu (2014). "Thiamethoxam resistance selected in the western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae): cross-resistance patterns, possible biochemical mechanisms and fitness costs analysis." Pesticide Biochemistry and Physiology **114**: 90-96.

Gasteiger, E., A. Gattiker, C. Hoogland, I. Ivanyi, R. D. Appel and A. Bairoch (2003). "ExPASy: the proteomics server for in-depth protein knowledge and analysis." Nucleic Acids Research **31**(13): 3784-3788.

Georghiou, G. P. (1972). "The evolution of resistance to pesticides." Annual Review of Ecology and Systematics **3**: 133-168.

Georghiou, G. P. and R. B. Mellon (1983). Pesticide Resistance in Time and Space. Pest Resistance to Pesticides. G. P. Georghiou and T. Saito. New York Plenum Press: 1-22.

Gepner, J. I., L. M. Hall and D. B. Sattelle (1978). "Insect acetylcholine receptors as a site of insecticide action." Nature **276**(5684): 188-190.

Gerlach, J. H., N. Kartner, D. R. Bell and V. Ling (1986). "Multidrug resistance." Journal of Cancer Survivorship **5**(1): 25-46.

Gerwick, B. C. and T. C. Sparks (2014). "Natural products for pest control: an analysis of their role, value and future." Pest Management Science **70**(8): 1169-1185.

Ghong, K., G. Zhang and G. Zhai (1964). "Resistance of cotton aphid to demeton." Journal of Entomology **13**: 1-9.

Gilmour, A. R., B. J. Gogel, B. R. Cullis and R. Thompson (2009). ASReml User Guide Release 3.0. Hemel Hempstead, HP1 1ES, UK, VSN International Ltd.

Gore, J., D. Cook, A. Catchot, B. R. Leonard, S. D. Stewart, G. Lorenz and D. Kerns (2013). "Cotton aphid (Heteroptera: Aphididae) susceptibility to commercial and experimental insecticides in the southern United States." Journal of Economic Entomology **106**(3): 1430-1439.

Gorman, K., G. Devine, J. Bennison, P. Coussons, N. Punchard and I. Denholm (2007). "Report of resistance to the neonicotinoid insecticide imidacloprid in *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae)." Pest Management Science **63**(6): 555-558.

Gottesman, M. M., T. Fojo and S. E. Bates (2002). "Multidrug resistance in cancer: role of ATP-dependent transporters." Nature Reviews Cancer **2**(1): 48-58.

Grabherr, M. G., B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F. di Palma, B. W. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman and A. Regev (2011). "Full-length transcriptome assembly from RNA-Seq data without a reference genome." Nature Biotechnology **29**(7): 644-652.

Grada, A. and K. Weinbrecht (2013). "Next-Generation Sequencing: Methodology and Application." Journal of Investigative Dermatology **133**(8): 1-4.

Greenwood, R., D. W. Salt and M. G. Ford (2007). Pharmacokinetics: Computational Versus Experimental Approaches to Optimize Insecticidal Chemistry. Insecticides Design Using Advanced Technologies. Isaac Ishaaya, Ralf Nauen and A. R. Horowitz. Berlin Heidelberg, Springer-Verlag.

Guengerich, F. P. (1996). The chemistry of cytochrome P450 reactions. New York, CRC Press.

Guengerich, F. P. (2001). "Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity." Chemical Research in Toxicology **14**(6): 611-650.

Guillemaud, T., N. Makate, M. Raymond, B. Hirst and A. Callaghan (1997). "Esterase gene amplification in *Culex pipiens*." Insect Molecular Biology **6**(4): 319-327.

Guldemond, J. A., W. T. Tigges and P. W. F. De Vrijer (1994). "Host Races of *Aphis gossypii* (Homoptera: Aphididae) on Cucumber and Chrysanthemum." Environmental Entomology **23**(5): 1235-1240.

Gunning, R., S. Young, G. Bingham and G. Moores (2004). Synergists turn back the clock on insecticide resistance. The Australian Cottongrower. Toowoomba, Australia, Greenmount Press. **August-September:** 10-11.

Gunning, R. V. and G. D. Moores (2001). Insensitive Acetylcholinesterase as Sites for Resistance to Organophosphates and Carbamates in Insects: Insensitive Acetylcholinesterase Confers Resistance in Lepidoptera. Biochemical Sites of Insecticide Action and Resistance. I. Ishaaya. Berlin, Heidelberg, Springer-Verlag: 221-236.

Guo, Z., S. Kang, X. Zhu, J. Xia, Q. Wu, S. Wang, W. Xie and Y. Zhang (2015). "Down-regulation of a novel ABC transporter gene (Pxwhite) is associated with Cry1Ac resistance in the diamondback moth, *Plutella xylostella* (L.)." Insect Biochemistry and Molecular Biology **59**: 30-40.

Hake, S. J., K. D. Hake and T. A. Kerby (1996). Planting and Stand Establishment. Cotton Production Manual. S. J. Hake, T. A. Kerby and K. D. Hake. University of California, Oakland, CA, ANR Publications: 21-28.

Heckel, D. G., L. J. Gahan, S. W. Baxter, J.-Z. Zhao, A. M. Shelton, F. Gould and B. E. Tabashnik (2007). "The diversity of Bt resistance genes in species of Lepidoptera." Journal of Invertebrate Pathology **95**(3): 192-197.

Heimoana, S. C. (2012). The effects of aphids (*Aphis gossypii*) Glover on photosynthesis in cotton (*Gossypium hirsutum*). PhD Thesis, The University of Queensland.

Hemingway, J. (2000). "The molecular basis of two contrasting metabolic mechanisms of insecticide resistance." Insect Biochemistry and Molecular Biology **30**: 1009-1015.

Hemingway, J. and G. P. Georghiou (1984). "Differential suppression of organophosphorus resistance in Culex quinquefasciatus by the synergists IBP, DEF, and TPP." Pesticide Biochemistry and Physiology **21**(1): 1-9.

Hemingway, J., N. J. Hawkes, L. McCarroll and H. Ranson (2004). "The molecular basis of insecticide resistance in mosquitoes." Insect Biochemistry and Molecular Biology **34**(7): 653-665.

Henderson, C. F. and E. W. Tilton (1955). "Tests with acaricides against the brown wheat mite." Journal of Economic Entomology **48**: 157-161.

Henneberry, T. J., L. Forlow Jech, T. de la Torre and D. L. Hendrix (2000). "Cotton Aphid (Homoptera: Aphididae) Biology, Honeydew Production, Sugar Quality and Quantity and Relationships to Sticky Cotton." Southwestern Entomologist **25**(3).

Hequet, E., D. Ethridge, B. Cole and B. Wyatt (2000). How cotton stickiness measurements relate to spinning efficiency. Proceedings of the Engineered Fiber Selection System Conference (EFS).

Herron, G., K. Powis and J. Rophail (2000). "Baseline studies and preliminary resistance survey of Australian populations of cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae)." Australian Journal of Entomology **39**(1): 33-38.

Herron, G. A. (2012). Resistance in Aphids. 16th Australian Cotton Conference, Growing Better all the Time. Broadbeach, Queensland. 14-16th August, CRDC, Narrabri.

Herron, G. A., R. V. Gunning, E. L. Cottage, V. Borzatta and C. Gobbi (2014). "Spinosad resistance, esterase isoenzymes and temporal synergism in Frankliniella occidentalis (Pergande) in Australia." Pesticide Biochemistry and Physiology **114**: 32-37.

Herron, G. A., B. J. Langfield, D. R. Bogema and Y. Chen (2013). "Baseline susceptibility and cross-resistance in *Aphis gossypii* Glover (Aphididae: Hemiptera) to phorate and sulfoxaflor." Australian Journal of Entomology **53**(1): 32-35.

Herron, G. A., K. Powis and J. Rophail (2001). "Insecticide resistance in *Aphis gossypii* Glover (Hemiptera: Aphididae), a serious threat to Australian cotton." Australian Journal of Entomology **40**(1): 85–91.

Herron, G. A. and J. Rophail (2000). Insecticide resistance in cotton aphid linked to field-control failures in Australian cotton. The 10th Australian Cotton Conference, Gold Coast, N.S.W.

Herron, G. A. and L. J. Wilson (2011). "Neonicotinoid resistance in *Aphis gossypii* Glover (Aphididae: Hemiptera) from Australian cotton." Australian Journal of Entomology **50**(1): 93-98.

Higgins, C. F. (1992). "ABC transporters: from microorganisms to man." Annual Review of Cell Biology **8**: 67-113.

Hodgson, E. (1985). Microsomal mono-oxygenases. Oxford, Pergamon Press.

Hollingsworth, R. G., B. E. Tabashnik, D. E. Ullman, M. W. Johnson and R. Messing (1994). "Resistance of *Aphis gossypii* (Homoptera: Aphididae) to Insecticides in Hawaii: Spatial Patterns and Relation to Insecticide Use." Journal of Economic Entomology **87**(2): 293-300.

Hoskins, W. M. and R. Craig (1962). "Uses of Bioassay in Entomology." Annual Review of Entomology **7**(1): 437-464.

Hotelier, T., egrave, V. gre, P. Marchot and A. Chatonnet (2010). "Insecticide resistance through mutations in cholinesterases or carboxylesterases: data mining in the ESTHER database." Journal of Pesticide Science **35**(3): 315-320.

Hou, W., Q. Liu, L. Tian, Q. Wu, Y. Zhang, W. Xie, S. Wang, K. S. Miguel, J. Funderburk and J. G. Scott (2014). "The alpha6 nicotinic acetylcholine receptor

subunit of *Frankliniella occidentalis* is not involved in resistance to spinosad." Pesticide Biochemistry and Physiology **111**: 60-67.

Hoyer, R. F. and F. W. Plapp, Jr. (1968). "Insecticide resistance in the house fly: identification of a gene that confers resistance to organotin insecticides and acts as an intensifier of parathion resistance." Journal of Economic Entomology **61**(5): 1269-1276.

Ihara, M., K. Matsuda, M. Otake, M. Kuwamura, M. Shimomura, K. Komai, M. Akamatsu, V. Raymond and D. B. Sattelle (2003). "Diverse actions of neonicotinoids on chicken α7, α4β2 and Drosophila–chicken SADβ2 and ALSβ2 hybrid nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes." Neuropharmacology **45**(1): 133-144.

Illumina, I. (2010). "Technology Spotlight: Illumina Sequencing Technology." Retrieved January 3rd, 2015, from https://www.illumina.com/documents/products/techspotlights/techspotlight_sequencing.pdf.

IRAC (1990). "Proposed Insecticide/Acaricide Susceptibility Tests " EPPO Bulletin **20**(2): 389-404.

IRAC. (2009). "IRAC Susceptibility Test Methods Series Version 3." Retrieved November 12th, 2014, from http://www.irac-online.org/content/uploads/Intro_v3_june09.pdf.

IRAC. (2015). "Cotton Aphid (*Aphis gossypii*)." Retrieved May 12th, 2015, from http://www.irac-online.org/documents/aphis-gossypii-irm-poster/?ext=pdf.

Iseli, C., C. V. Jongeneel and P. Bucher (1999). ESTScan: a program for detecting, evaluating, and reconstructing potential coding regions in EST sequences. Proceedings of the Seventh International Conference on Intelligent Systems for Molecular Biology, Heidelberg, Germany, AAAI Press,.

Isley, D. (1946). "The cotton aphid." Arkansas Agricultural Experimentation Station Bulletin **462**.

Isman, M. B. (2006). "Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world." Annual Review of Entomology **51**: 45-66.

Jeschke, P., K. Moriya, R. Lantzsch, H. Seifert, W. Linder, A. Gohrt, M. E. Beck and W. Etzel (2001). "Thiacloprid (BAY YRC2894) - a new member of the chloronicotinyl insecticide (CNI) family." Pflanzenschutz-Nachrichten Bayer **54**: 147-160.

Jeschke, P. and R. Nauen (2008). "Neonicotinoids—from zero to hero in insecticide chemistry." Pest Management Science **64**(11): 1084-1098.

Jeschke, P., R. Nauen, M. Schindler and A. Elbert (2011). "Overview of the Status and Global Strategy for Neonicotinoids." Journal of Agricultural and Food Chemistry **59**(7): 2897-2908.

Jones, A. K., P. Davis, J. Hodgkin and D. B. Sattelle (2007). "The nicotinic acetylcholine receptor gene family of the nematode *Caenorhabditis elegans*: an update on nomenclature." Invertebrate Neuroscience **7**(2): 129-131.

Jones, A. K., M. Grauso and D. B. Sattelle (2005). "The nicotinic acetylcholine receptor gene family of the malaria mosquito, *Anopheles gambiae*." Genomics **85**(2): 176-187.

Jones, A. K., V. Raymond-Delpech, S. H. Thany, M. Gauthier and D. B. Sattelle (2006). "The nicotinic acetylcholine receptor gene family of the honey bee, *Apis mellifera*." Genome Research **16**(11): 1422-1430.

Jones, A. K. and D. B. Sattelle (2007). "The cys-loop ligand-gated ion channel gene superfamily of the red flour beetle, *Tribolium castaneum*." BMC Genomics **8**(327): 1-16.

Jones, A. K. and D. B. Sattelle (2010). Diversity of Insect Nicotinic Acetylcholine Receptor Subunits. Insect Nicotinic Acetylcholine Receptors. S. H. Thany, Springer New York. **683**: 25-43.

Jones, W. A. and L. Zhao (2012). "Expression of heat shock protein genes in insect stress responses." Invertebrate Survival Journal **9**(1): 93-101.

Kagabu, S. (2011). "Discovery of imidacloprid and further developments from strategic molecular designs." Journal of Agricultural and Food Chemistry **59**(7): 2887-2896.

Kagabu, S. and S. Medej (1995). "Stability Comparison of Imidacloprid and Related Compounds under Simulated Sunlight, Hydrolysis Conditions, and to Oxygen." Bioscience, Biotechnology, and Biochemistry **59**(6): 980-985.

Karlin, A. (2002). "Emerging structure of the Nicotinic Acetylcholine receptors." Nature Reviews Neuroscience **3**(2): 102-114.

Karunker, I., J. Benting, B. Lueke, T. Ponge, R. Nauen, E. Roditakis, J. Vontas, K. Gorman, I. Denholm and S. Morin (2008). "Over-expression of cytochrome P450 CYP6CM1 is associated with high resistance to imidacloprid in the B and Q biotypes of *Bemisia tabaci* (Hemiptera: Aleyrodidae)." Insect Biochemistry and Molecular Biology **38**(6): 634-644.

Karunker, I., E. Morou, D. Nikou, R. Nauen, R. Sertchook, B. J. Stevenson, M. J. Paine, S. Morin and J. Vontas (2009). "Structural model and functional characterization of the *Bemisia tabaci* CYP6CM1vQ, a cytochrome P450 associated with high levels of imidacloprid resistance." Insect Biochemistry and Molecular Biology **39**(10): 697-706.

Kennedy, G. G., D. L. McLean and M. G. Kinsey (1978). "Probing Behavior of *Aphis gossypii* on Resistant and Susceptible Muskmelon." Journal of Economic Entomology **71**(1): 13-16.

Kerns, D. L. and M. J. Gaylor (1992). "Insecticide Resistance in Field Populations of the Cotton Aphid (Homoptera: Aphididae)." Journal of Economic Entomology **85**(1): 1-8.

Kersting, U., S. Satar and N. Uygun (1999). "Effect of temperature on development rate and fecundity of apterous *Aphis gossypii* Glover (Hom., Aphididae) reared on *Gossypium hirsutum* L." Journal of Applied Entomology **123**(1): 23-27.

Khan, H. A. A., W. Akram, J. Iqbal and U. Naeem-Ullah (2015). "Thiamethoxam Resistance in the House Fly, *Musca domestica* L.: Current Status, Resistance Selection, Cross-Resistance Potential and Possible Biochemical Mechanisms." PLoS One **10**(5).

Kim, D. H., S.-C. Lee, D.-Y. Kwak and K.-Y. Lee (2008). "Cloning of heat shock protein genes from the brown planthopper, *Nilaparvata lugens*, and the small brown planthopper, *Laodelphax striatellus*, and their expression in relation to thermal stress." Insect Science **15**(5): 415-422.

Kim, J. I., M. Kwon, G.-H. Kim, S. Y. Kim and S. H. Lee (2015). "Two mutations in nAChR beta subunit is associated with imidacloprid resistance in the *Aphis gossypii*." Journal of Asia-Pacific Entomology **18**(2): 291-296.

Kleier, D., I. Holden, J. E. Casida and L. O. Ruzo (1985). "Novel photoreactions of an insecticidal nitromethylene heterocycle." Journal of Agricultural and Food Chemistry **33**(5): 998-1000.

Koganemaru, R., D. M. Miller and Z. N. Adelman (2013). "Robust cuticular penetration resistance in the common bed bug (*Cimex lectularius* L.) correlates with increased steady-state transcript levels of CPR-type cuticle protein genes." Pesticide Biochemistry and Physiology **106**(3): 190-197.

Kollmeyer, W. D., R. F. Flattum, J. P. Foster, J. E. Powell, M. E. Schroeder and S. B. Soloway (1999). Discovery of the Nitromethylene Heterocycle Insecticides. Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor. I. Yamamoto and J. E. Casida. Toyko, Springer-Verlag: 71-89.

Koo, H.-N., J.-J. An, S.-E. Park, J.-I. Kim and G.-H. Kim (2014). "Regional susceptibilities to 12 insecticides of melon and cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae) and a point mutation associated with imidacloprid resistance." Crop Protection **55**: 91-97.

Koonin, E. V. and M. Y. Galperin (2003). Principles and Methods of Sequence Analysis. Sequence - Evolution - Function: Computational Approaches in Comparative Genomics. Boston, Kluwer Academic.

Kramer, K. J., M. R. Kanost, T. L. Hopkins, H. Jiang, Y. C. Zhu, R. Xu, J. L. Kerwin and F. Turecek (2001). "Oxidative conjugation of catechols with proteins in insect skeletal systems." Tetrahedron **57**(2): 385-392.

Kranthi, K. (2005). Insecticide Resistance - Monitoring, Mechanisms and Management Manual. Nagpur, India and Washington DC, CICR & ICAC.

Kshirsagar, S. D., N. S. Satpute and M. P. Moharil (2012). "Monitoring of insecticide resistance in Cotton leafhoppers, *Amrasca bigutulla bigutulla* (Ishida)." Annals of Plant Protection Sciences **20**(2): 283-286.

Kumar, S., G. K. Christophides, R. Cantera, B. Charles, Y. S. Han, S. Meister, G. Dimopoulos, F. C. Kafatos and C. Barillas-Mury (2003). "The role of reactive oxygen species on Plasmodium melanotic encapsulation in Anopheles gambiae." Proceedings of the National Academy of Sciences of the United States of America **100**(24): 14139-14144.

Kundu, D. (1994). "Ecology of host alternation in aphids." European Journal of Entomology **91**(1): 63-70.

Kundu, R. and A. F. G. Dixon (1995). "Evolution of Complex Life Cycles in Aphids." Journal of Animal Ecology **64**(2): 245-255.

Le Goff, G., S. Boundy, P. J. Daborn, J. L. Yen, L. Sofer, R. Lind, C. Sabourault, L. Madi-Ravazzi and R. H. ffrench-Constant (2003). "Microarray analysis of cytochrome P450 mediated insecticide resistance in Drosophila." Insect Biochemistry and Molecular Biology **33**(7): 701-708.

Le Goff, G., A. Hamon, J. B. Berge and M. Amichot (2005). "Resistance to fipronil in *Drosophila simulans*: influence of two point mutations in the RDL GABA receptor subunit." Journal of Neurochemistry **92**(6): 1295-1305.

Leather, S. R. and A. F. G. Dixon (1984). "Aphid growth and reproductive rates." Entomologia Experimentalis et Applicata **35**(2): 137-140.

Leclant, F. and J.-P. Deguine (1994). Cotton aphids. Insect Pests of Cotton. G. A. Matthews and J. P. Tunstall. Wallingford, CAB International: 285-323.

Lester, H. A., M. I. Dibas, D. S. Dahan, J. F. Leite and D. A. Dougherty (2004). "Cys-loop receptors: new twists and turns." Trends in Neurosciences **27**(6): 329-336.

Li, F. and Z.-J. Han (2002). "Two different genes encoding acetylcholinesterase existing in cotton aphid (*Aphis gossypii*)." Genome **45**(6): 1134-1141.

Li, F. and Z. Han (2004). "Mutations in acetylcholinesterase associated with insecticide resistance in the cotton aphid, *Aphis gossypii* Glover." Insect Biochemistry and Molecular Biology **34**(4): 397-405.

Li, R., C. Yu, Y. Li, T. W. Lam, S. M. Yiu, K. Kristiansen and J. Wang (2009). "SOAP2: an improved ultrafast tool for short read alignment." Bioinformatics **25**(15): 1966-1967.

Li, X., M. A. Schuler and M. R. Berenbaum (2006). "Molecular Mechanisms of Metabolic Resistance to Synthetic and Natural Xenobiotics." Annual Review of Entomology **52**(1): 231-253

Li, X., M. A. Schuler and M. R. Berenbaum (2007). "Molecular Mechanisms of Metabolic Resistance to Synthetic and Natural Xenobiotics." Annual Review of Entomology **52**(1): 231-253.

Liu, B., G. Jiang, Y. Zhang, J. Li, X. Li, J. Yue, F. Chen, H. Liu, H. Li, S. Zhu, J. Wang and C. Ran (2011). "Analysis of Transcriptome Differences between Resistant and Susceptible Strains of the Citrus Red Mite *Panonychus citri* (Acari: Tetranychidae)." PLoS One **6**(12).

Liu, Y.-p., K.-j. Lin, Y. Liu, F.-r. Gui and G.-r. Wang (2013). "Nicotinic Acetylcholine Receptor Gene Family of the Pea Aphid, *Acyrthosiphon pisum*." Journal of Integrative Agriculture **12**(11): 2083-2091.

Liu, Z. and Z. Han (2006). "Fitness costs of laboratory-selected imidacloprid resistance in the brown planthopper, *Nilaparvata lugens* Stal." Pest Management Science **62**(3): 279-282.

Liu, Z., M. S. Williamson, S. J. Lansdell, I. Denholm, Z. Han and N. S. Millar (2005). "A nicotinic acetylcholine receptor mutation conferring target-site resistance to

imidacloprid in *Nilaparvata lugens* (brown planthopper)." Proceedings of the National Academy of Sciences of the United States of America **102**(24): 8420-8425.

Liu, Z., M. S. Williamson, S. J. Lansdell, Z. Han, I. Denholm and N. S. Millar (2006). "A nicotinic acetylcholine receptor mutation (Y151S) causes reduced agonist potency to a range of neonicotinoid insecticides." Journal of Neurochemistry **99**(4): 1273-1281.

Liu, Z., X. Yao and Y. Zhang (2008). "Insect nicotinic acetylcholine receptors (nAChRs): Important amino acid residues contributing to neonicotinoid insecticides selectivity and resistance." African Journal of Biotechnology **7**(25): 4935-4939.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Llaca, V. (2012). Sequencing Technologies and Their Use in Plant Biotechnology and Breeding. DNA Sequencing - Methods and Applications. A. Munshi. Croatia, InTech.

Lumjuan, N., L. McCarroll, L. A. Prapanthadara, J. Hemingway and H. Ranson (2005). "Elevated activity of an Epsilon class glutathione transferase confers DDT resistance in the dengue vector, *Aedes aegypti*." Insect Biochemistry and Molecular Biology **35**(8): 861-871.

Lumjuan, N., S. Rajatileka, D. Changsom, J. Wicheer, P. Leelapat, L. A. Prapanthadara, P. Somboon, G. Lycett and H. Ranson (2011). "The role of the *Aedes aegypti* Epsilon glutathione transferases in conferring resistance to DDT and pyrethroid insecticides." Insect Biochemistry and Molecular Biology **41**(3): 203-209.

Maienfisch, P., M. Angst, F. Brandl, W. Fischer, D. Hofer, H. Kayser, W. Kobel, A. Rindlisbacher, R. Senn, A. Steinemann and H. Widmer (2001). "Chemistry and biology of thiamethoxam: a second generation neonicotinoid." Pest Management Science **57**(10): 906-913.

Maienfisch, P., F. Brandl, W. Kobel, A. Rindlisbacher and R. Senn (1999). CGA 293'343: A Novel, Broad-Spectrum Neonicotinoid Insecticide. Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor. I. Yamamoto and J. E. Casida. Tokyo, Springer-Verlag: 177-182.

Mallet, J. (1989). "The evolution of insecticide resistance: Have the insects won?" Trends in Ecology & Evolution **4**(11): 336-340.

Mannervik, B. and U. H. Danielson (1988). "Glutathione transferases--structure and catalytic activity." Critical Reviews in Biochemistry and Molecular Biology **23**(3): 283-337.

Mansfield, S., M. L. Dillon and M. E. A. Whitehouse (2006). "Are arthropod communities in cotton really disrupted? An assessment of insecticide regimes and evaluation of the beneficial disruption index." Agriculture, Ecosystems & Environment **113**(1–4): 326-335.

Mardis, E. R. (2008). "The impact of next-generation sequencing technology on genetics." Trends in Genetics **24**(3): 133-141.

Margaritopoulos, J. T., M. Tzortzi, K. D. Zarpas, J. A. Tsitsipis and R. L. Blackman (2006). "Morphological discrimination of *Aphis gossypii* (Hemiptera: Aphididae) populations feeding on Compositae." Bulletin of Entomological Research **96**(2): 153-165.

Markussen, M. D. K. and M. Kristensen (2010). "Cytochrome P450 monooxygenase-mediated neonicotinoid resistance in the house fly *Musca domestica* L." Pesticide Biochemistry and Physiology **98**(1): 50-58.

Markussen, M. D. K. and M. Kristensen (2010). "Low expression of nicotinic acetylcholine receptor subunit Mdα2 in neonicotinoid-resistant strains of *Musca domestica* L." Pest Management Science **66**(11): 1257-1262.

Marshall, K. L., C. Moran, Y. Chen and G. A. Herron (2012). "Detection of kdr pyrethroid resistance in the cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae), using a PCR-RFLP assay." Journal of Pesticide Science **37**(2): 169-172.

Martinez-Carrillo, J. L. and H. T. Reynolds (1983). Dosage-Mortality Studies with Pyrethroids and Other Insecticides on the Tobacco Budworm (Lepidoptera: Noctuidae) from the Imperial Valley, California.

Martinez-Torres, D., S. P. Foster, L. M. Field, A. L. Devonshire and M. S. Williamson (1999). "A sodium channel point mutation is associated with resistance to DDT and pyrethroid insecticides in the peach-potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae)." Insect Molecular Biology **8**(3): 339-346.

Mass, S. (2012). Cotton Pest Management Guide 2011-12. Toowoomba, Australia, Greenmount Press.

Mass, S. (2014). Cotton Pest Management Guide 2014-15. Toowoomba, Australia, Greenmount Press.

Matsuda, K., S. D. Buckingham, J. C. Freeman, M. D. Squire, H. A. Baylis and D. B. Sattelle (1998). "Effects of the alpha subunit on imidacloprid sensitivity of recombinant nicotinic acetylcholine receptors." British Journal of Pharmacology **123**(3): 518-524.

Matsuda, K., S. D. Buckingham, D. Kleier, J. J. Rauh, M. Grauso and D. B. Sattelle (2001). "Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors." Trends in Pharmacological Sciences **22**(11): 573-580.

Matsuda, K., S. Kanaoka, M. Akamatsu and D. B. Sattelle (2009). "Diverse actions and target-site selectivity of neonicotinoids: structural insights." Molecular Pharmacology **76**(1): 1-10.

Matsuura, A. and M. Nakamura (2014). "Development of neonicotinoid resistance in the cotton aphid *Aphis gossypii* (Hemiptera: Aphididae) in Japan." Applied Entomology and Zoology **49**(4): 535-540.

Maxam, A. M. and W. Gilbert (1977). "A new method for sequencing DNA." Proceedings of the National Academy of Sciences of the United States of America **74**(2): 560-564.

McKenzie, J. A. (1985). Genetics of resistance to chemotherapeutic agents. <u>Resistance in Nematodes to Anthelmintic Drugs</u>. N. Anderson and P. J. Waller. Sydney, Australia, CSIRO & Australian Wool Corporation: 89-95.

McLoon, M. O. and G. A. Herron (2009). "PCR detection of pirimicarb resistance in Australian field isolates of *Aphis gossypii* Glover (Aphididae: Hemiptera)." <u>Australian Journal of Entomology</u> **48**(1): 65-72.

Metzker, M. L. (2010). "Sequencing technologies - the next generation." <u>Nature Reviews Genetics</u> **11**(1): 31-46.

Millar, N. S. (1999). "Heterologous expression of mammalian and insect neuronal nicotinic acetylcholine receptors in cultured cell lines." <u>Biochemical Society Transactions</u> **27**(6): 944-950.

Millar, N. S. (2003). "Assembly and subunit diversity of nicotinic acetylcholine receptors." <u>Biochemical Society Transactions</u> **31**(Pt 4): 869-874.

Miller, W. B., E. Peralta, D. R. Ellis and H. H. Perkins (1994). "Stickiness Potential of Individual Insect Honeydew Carbohydrates on Cotton Lint." <u>Textile Research Journal</u> **64**(6): 344-350.

Mitani, Y., K. Suzuki, K. Kondo, K. Okumura and T. Tamura (2006). "Gene expression analysis using a modified HiCEP method applicable to prokaryotes: a study of the response of Rhodococcus to isoniazid and ethambutol." <u>Journal of Biotechnology</u> **123**(3): 259-272.

Montella, I. R., R. Schama and D. Valle (2012). "The classification of esterases: an important gene family involved in insecticide resistance--a review." <u>Memórias do Instituto Oswaldo Cruz</u> **107**(4): 437-449.

Moores, G. (2015). "Insecticide boost." <u>Chemistry & Industry</u> **79**(9): 26-29.

Moores, G. D., X. W. Gao, I. Denholm and A. L. Devonshire (1996). "Characterisation of insensitive acetylcholinesterase in insecticide-resistant cotton aphids, *Aphis gossypii* glover (Homoptera: Aphididae)." <u>Pesticide Biochemistry and Physiology</u> **56**(2): 102-110.

Moran, N. A. (1992). "The Evolution of Aphid Life Cycles." Annual Review of Entomology **37**(1): 321-348.

Moriya, K., K. Shibuya, Y. Hattori, S.-i. Tsuboi, K. Shiokawa and S. Kagabu (1993). "Structural Modification of the 6-Chloropyridyl Moiety in the Imidacloprid Skeleton: Introduction of a Five-membered Heteroaromatic Ring and the Resulting Insecticidal Activity." Bioscience, Biotechnology, and Biochemistry **57**(1): 127-128.

Mortazavi, A., B. A. Williams, K. McCue, L. Schaeffer and B. Wold (2008). "Mapping and quantifying mammalian transcriptomes by RNA-Seq." Nature Methods **5**(7): 621-628.

Mota-Sanchez, D., R. M. Hollingworth, E. J. Grafius and D. D. Moyer (2006). "Resistance and cross-resistance to neonicotinoid insecticides and spinosad in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)." Pest Management Science **62**(1): 30-37.

Murray, C. L., M. Quaglia, J. T. Arnason and C. E. Morris (1994). "A putative nicotine pump at the metabolic blood-brain barrier of the tobacco hornworm." Journal of Neurobiology **25**(1): 23-34.

Mutero, A., J. M. Bride, M. Pralavorio and D. Fournier (1994). "*Drosophila melanogaster* acetylcholinesterase: identification and expression of two mutations responsible for cold- and heat-sensitive phenotypes." Molecular Genetics and Genomics **243**(6): 699-705.

Nabeshima, T., T. Kozaki, T. Tomita and Y. Kono (2003). "An amino acid substitution on the second acetylcholinesterase in the pirimicarb-resistant strains of the peach potato aphid, *Myzus persicae*." Biochemical and Biophysical Research Communications **307**(1): 15-22.

Nagalakshmi, U., K. Waern and M. Snyder (2010). "RNA-Seq: a method for comprehensive transcriptome analysis." Current Protocols in Molecular Biology **11**(Unit 4): 1-13.

Narahashi, T. (1996). "Neuronal ion channels as the target sites of insecticides." Pharmacology and Toxicology **79**(1): 1-14.

Nauen, R. and I. Denholm (2005). "Resistance of insect pests to neonicotinoid insecticides: Current status and future prospects." Archives of Insect Biochemistry and Physiology **58**(4): 200-215.

Nauen, R., A. Elbert, A. McCaffery, R. Slater and T. C. Sparks (2012). IRAC: Insecticide Resistance, and Mode of Action Classification of Insecticides. Modern Crop Protection Compounds, Wiley-VCH Verlag GmbH & Co. KGaA: 935-955.

Nauen, R., P. Jeschke and L. Copping (2008). "In Focus: Neonicotinoid insecticides Editorial." Pest Management Science **64**(11): 1081-1081.

Nauen, R., N. Stumpf and A. Elbert (2002). "Toxicological and mechanistic studies on neonicotinoid cross resistance in Q-type *Bemisia tabaci* (Hemiptera: Aleyrodidae)." Pest Management Science **58**(9): 868-875.

Nazir, A., I. Mukhopadhyay, D. K. Saxena and D. Kar Chowdhuri (2001). "Chlorpyrifos-induced hsp70 expression and effect on reproductive performance in transgenic Drosophila melanogaster (hsp70-lacZ) Bg9." Archives of Environmental Contamination and Toxicology **41**(4): 443-449.

Newcomb, R. D., P. M. Campbell, D. L. Ollis, E. Cheah, R. J. Russell and J. G. Oakeshott (1997). "A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly." Proceedings of the National Academy of Sciences of the United States of America **94**(14): 7464-7468.

Ng, P. C. and E. F. Kirkness (2010). Whole Genome Sequencing. Genetic Variation: Methods and Protocols. M. Barnes, R., and G. Breen. **628**: 215-226.

Niu, J. Z., W. Dou, T. B. Ding, G. M. Shen, K. Zhang, G. Smagghe and J. J. Wang (2012). "Transcriptome analysis of the citrus red mite, *Panonychus citri*, and its gene expression by exposure to insecticide/acaricide." Insect Molecular Biology **21**(4): 422-436.

Noda, M., H. Takahashi, T. Tanabe, M. Toyosato, Y. Furutani, T. Hirose, M. Asai, S. Inayama, T. Miyata and S. Numa (1982). "Primary structure of alpha-subunit precursor of *Torpedo californica* acetylcholine receptor deduced from cDNA sequence." Nature **299**(5886): 793-797.

Noda, M., H. Takahashi, T. Tanabe, M. Toyosato, S. Kikyotani, Y. Furutani, T. Hirose, H. Takashima, S. Inayama, T. Miyata and S. Numa (1983). "Structural homology of *Torpedo californica* acetylcholine receptor subunits." Nature **302**(5908): 528-532.

Noda, M., H. Takahashi, T. Tanabe, M. Toyosato, S. Kikyotani, T. Hirose, M. Asai, H. Takashima, S. Inayama, T. Miyata and S. Numa (1983). "Primary structures of beta- and delta-subunit precursors of *Torpedo californica* acetylcholine receptor deduced from cDNA sequences." Nature **301**(5897): 251-255.

Noppun, V., T. Saito and T. Miyata (1989). "Cuticular penetration of S-fenvalerate in fenvalerate-resistant and susceptible strains of the diamondback moth, *Plutella xylostella* (L.)." Pesticide Biochemistry and Physiology **33**(1): 83-87.

O'Brien, P. J., Y. A. Abdel-Aal, J. A. Ottea and J. B. Graves (1992). "Relationship of Insecticide Resistance to Carboxylesterases In *Aphis gossypii* (Homoptera: Aphididae) from Midsouth Cotton." Journal of Economic Entomology **85**(3): 651-657.

Oakeshott, J. G., C. Claudianos, P. M. Campbell, R. D. Newcomb and R. J. Russell (2010). Biochemical genetics and genomics of insect esterases. Insect pharmacology: Channels, receptors, toxins and enzymes. L. I. Gilbert and S. S. Gill. London, U.K, Elsevier: 229-301.

Ohkawara, Y., A. Akayama, K. Matsuda and W. Andersch (2002). Clothianidin: a novel broad spectrum neonicotinoid insecticide. Brighton Crop Protection Conference-Pests and Diseases, Farnham, Surrey, UK.

Oppenoorth, F. J. (1971). "Resistance in insects: the role of metabolism and the possible use of synergists." Bulletin of the World Health Organization **44**(1-3): 195-202.

Ortelli, F., L. C. Rossiter, J. Vontas, H. Ranson and J. Hemingway (2003). "Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide-resistance locus from the malaria vector *Anopheles gambiae*." The Biochemical Journal **373**(Pt 3): 957-963.

Paddock, F. B. (1919). The cotton or melon louse: life history studies College Station, Texas., Texas Agricultural Experiment Station.

Pan, C., Y. Zhou and J. Mo (2009). "The clone of laccase gene and its potential function in cuticular penetration resistance of *Culex pipiens pallens* to fenvalerate." Pesticide Biochemistry and Physiology **93**(3): 105-111.

Pan, Y., T. Peng, X. Gao, L. Zhang, C. Yang, J. Xi, X. Xin, R. Bi and Q. Shang (2015). "Transcriptomic comparison of thiamethoxam-resistance adaptation in resistant and susceptible strains of *Aphis gossypii* Glover." Comparative Biochemistry and Physiology Part D Genomics and Proteomics **13**: 10-15.

Pareek, C. S., R. Smoczynski and A. Tretyn (2011). "Sequencing technologies and genome sequencing." Journal of Applied Genetics **52**(4): 413-435.

Parsell, D. A. and S. Lindquist (1993). "The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins." Annual Review of Genetics **27**: 437-496.

Perry, T., J. Q. Chan, P. Batterham, G. B. Watson, C. Geng and T. C. Sparks (2012). "Effects of mutations in Drosophila nicotinic acetylcholine receptor subunits on sensitivity to insecticides targeting nicotinic acetylcholine receptors." Pesticide Biochemistry and Physiology **102**(1): 56-60.

Perry, T., D. G. Heckel, J. A. McKenzie and P. Batterham (2008). "Mutations in Dα1 or Dβ2 nicotinic acetylcholine receptor subunits can confer resistance to neonicotinoids in *Drosophila melanogaster*." Insect Biochemistry and Molecular Biology **38**(5): 520-528.

Pittendrigh, B. R., V. M. Margam, L. Sun and J. E. Huesing (2008). Resistance in the Post-Genomics Age. Insect Resistance Management: Biology Economics and Prediction. David W. Onstad. Oxford, UK, Elsevier.

Plapp, F. W., Jr. and R. F. Hoyer (1968). "Insecticide resistance in the house fly: decreased rate of absorption as the mechanism of action of a gene that acts as an intensifier of resistance." Journal of Economic Entomology **61**(5): 1298-1303.

Plapp, F. W., Jr. and T. C. Wang (1983). Genetic Origins of Insecticide Resistance. Pest Resistance to Insecticides. P. Georghiou and T. Saito. New York, Plenum Press: 47-70.

Porter, T. D. (2002). "The roles of cytochrome b5 in cytochrome P450 reactions." Journal of Biochemical and Molecular Toxicology **16**(6): 311-316.

Pournourmahammadi, S. and M. Abdollahi (2011). Gene Expression. Anticholinesterase Pesticides: Metabolism, Neurotoxicity, and Epidemiology. T. Satoh and R. C. Gupta. Hoboken, New Jersey, John Wiley & Sons: 175-188.

Powell, G. F., D. A. Ward, M. C. Prescott, D. G. Spiller, M. R. H. White, P. C. Turner, F. G. P. Earley, J. Phillips and H. H. Rees (2011). "The molecular action of the novel insecticide, Pyridalyl." Insect Biochemistry and Molecular Biology **41**(7): 459-469.

Prasanna, A. R., M. Bheemanna and B. V. Patil (2004). "Evaluation of Thiamethoxam 70WS as Seed Treatment Against Leaf Miner and Early Sucking Pests on Hybrid Cotton." Karnataka Journal of Agricultural Science **17**(2): 238-241.

Puinean, A. M., J. Elias, R. Slater, A. Warren, L. M. Field, M. S. Williamson and C. Bass (2013). "Development of a high-throughput real-time PCR assay for the detection of the R81T mutation in the nicotinic acetylcholine receptor of neonicotinoid-resistant Myzus persicae." Pest Management Science **69**(2): 195-199.

Puinean, A. M., S. P. Foster, L. Oliphant, I. Denholm, L. M. Field, N. S. Millar, M. S. Williamson and C. Bass (2010). "Amplification of a cytochrome P450 gene is associated with resistance to neonicotinoid insecticides in the aphid *Myzus persicae*." PLoS Genetics **6**(6).

Puinean, A. M., S. J. Lansdell, T. Collins, P. Bielza and N. S. Millar (2013). "A nicotinic acetylcholine receptor transmembrane point mutation (G275E)

associated with resistance to spinosad in *Frankliniella occidentalis*." Journal of Neurochemistry **124**(5): 590-601.

Raffa, K. F. and T. M. Priester (1985). "Synergist as research tools and control agents in agriculture." Journal of Agricultural Entomology **2**(1): 27-45.

Ranson, H. and J. Hemingway (2005). "Mosquito glutathione transferases." Methods in Enzymology **401**: 226-241.

Rauch, N. and R. Nauen (2003). "Identification of biochemical markers linked to neonicotinoid cross resistance in *Bemisia tabaci* (Hemiptera: Aleyrodidae)." Archives of Insect Biochemistry and Physiology **54**(4): 165-176.

Raymond, M., D. G. Heckel and J. G. Scott (1989). "Interactions between pesticide genes: model and experiment." Genetics **123**(3): 543-551.

Reddall, A., A. Ali, J. A. Able, J. Stonor, L. Tesoriero, P. R. Wright, M. A. Rezaian and L. J. Wilson (2004). "Cotton bunchy top: an aphid and graft transmitted cotton disease." Australasian Plant Pathology **33**(2): 197-202.

Reinhardt, J. A., D. A. Baltrus, M. T. Nishimura, W. R. Jeck, C. D. Jones and J. L. Dangl (2009). "De novo assembly using low-coverage short read sequence data from the rice pathogen *Pseudomonas syringae* pv. oryzae." Genome Research **19**(2): 294-305.

Rinkevich, F. D., M. Chen, A. M. Shelton and J. G. Scott (2010). "Transcripts of the nicotinic acetylcholine receptor subunit gene Pxylalpha6 with premature stop codons are associated with spinosad resistance in diamondback moth, *Plutella xylostella*." Invertebrate Neuroscience **10**(1): 25-33.

Roberts, A., C. Trapnell, J. Donaghey, J. L. Rinn and L. Pachter (2011). "Improving RNA-Seq expression estimates by correcting for fragment bias." Genome Biology **12**(3).

Robertson, J. L. and H. K. Preisler (1992). Pesticide bioassays with arthropods. Boca Raton, CRC Press.

Robertson, J. L., R. M. Russell, H. K. Preisler and N. E. Savin (2007). Bioassays with Arthropods 2nd ed. Boca Raton, CRC Press.

Roush, R. T. and J. A. McKenzie (1987). "Ecological genetics of insecticide and acaricide resistance." Annual Review of Entomology **32**: 361-380.

Roush, R. T. and G. L. Miller (1986). "Considerations for Design of Insecticide Resistance Monitoring Programs." Journal of Economic Entomology **79**(2): 293-298.

Russell, R. J., C. Claudianos, P. M. Campbell, I. Horne, T. D. Sutherland and J. G. Oakeshott (2004). "Two major classes of target site insensitivity mutations confer resistance to organophosphate and carbamate insecticides." Pesticide Biochemistry and Physiology **79**(3): 84-93.

Russell, R. J., C. Scott, C. J. Jackson, R. Pandey, G. Pandey, M. C. Taylor, C. W. Coppin, J.-W. Liu and J. G. Oakeshott (2011). "The evolution of new enzyme function: lessons from xenobiotic metabolizing bacteria versus insecticide-resistant insects." Evolutionary Applications **4**(2): 225-248.

Saito, T. (1989). "Insecticide Resistance of the Cotton Aphid, *Aphis gossypii* Glover (Homoptera: Aphididae) I. Susceptibility to Several Insecticides and Esterase Activity of Field Populations Collected in Shizuoka Prefecture." Japanese Journal of Applied Entomology and Zoology **33**(4): 204-210.

Sanger, F. and A. R. Coulson (1975). "A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase." Journal of Molecular Biology **94**(3): 441-448.

Sanger, F., S. Nicklen and A. R. Coulson (1977). "DNA sequencing with chain-terminating inhibitors." Proceedings of the National Academy of Sciences **74**(12): 5463-5467.

Sawicki, R. M. (1987). Definition, detection and documentation of insecticide resistance Combating resistance to xenobiotics: biological and chemical approaches. M. G. Ford, D. W. Holloman, B. P. S. Khambay and R. M. Sawicki. Chichester, England, Ellis Horwood.

Schepers, A. (1989). Chemical conrol. <u>World Crop Pests: Aphids, Their Biology, Natural Enemies and Control</u>. A. K. Minks and P. Harrewijn. Amsterdam, Elsevier. **C:** 89-122.

Schuler, M. A. (2011). "P450s in plant-insect interactions." <u>Biochimica et Biophysica Acta</u> **1814**(1): 36-45.

Schulze, K. J. and A. R. Tomkins (2002). <u>Cotton Pest Management Guide 2002-2003</u>. Orange, NSW Agriculture.

Scott, J. A. (1995). "The molecular genetics of resistance: resistance as a response to stress." <u>Florida Entomologist</u> **78**(3): 399-413.

Scott, J. G. (1990). Investigating Mechanisms of Insecticide Resistance: Methods, Strategies and Pitfalls. <u>Pesticide Resistance in Arthropods</u>. R. T. Roush and B. E. Tabashnik. Great Britain, Chapman and Hall: 39-57.

Scott, J. G. and Z. Wen (2001). "Cytochromes P450 of insects: the tip of the iceberg." <u>Pest Management Science</u> **57**(10): 958-967.

Shao, Y.-M., K. Dong and C.-X. Zhang (2007). "The nicotinic acetylcholine receptor gene family of the silkworm, *Bombyx mori*." <u>BMC Genomics</u> **8**(324).

Shi, X.-B., X.-G. Shi, H.-Y. Wang, X.-M. Xia and K.-Y. Wang (2011). "Cross-resistance of the imidacloprid-resistant population of *Aphis gossypii* Glover (Homoptera: Aphididae) to other neonicotinoid insecticides and changes in activities of related enzymes." <u>Acta Entomologica Sinica</u> **54**(9): 1027-1033.

Shi, X., L. Jiang, H. Wang, K. Qiao, D. Wang and K. Wang (2011). "Toxicities and sublethal effects of seven neonicotinoid insecticides on survival, growth and reproduction of imidacloprid-resistant cotton aphid, *Aphis gossypii*." <u>Pest Management Science</u> **67**(12): 1528-1533.

Shi, X. G., Y. K. Zhu, X. M. Xia, K. Qiao, H. Y. Wang and K. Y. Wang (2012). "The mutation in nicotinic acetylcholine receptor beta 1 subunit may confer resistance to imidacloprid in *Aphis gossypii* (Glover)." <u>Journal of Food Agriculture & Environment</u> **10**(2): 1227-1230.

299 of 308

Shimomura, M., M. Yokota, M. Ihara, M. Akamatsu, D. B. Sattelle and K. Matsuda (2006). "Role in the selectivity of neonicotinoids of insect-specific basic residues in loop D of the nicotinic acetylcholine receptor agonist binding site." Molecular Pharmacology **70**(4): 1255-1263.

Shingleton, A. W., G. C. Sisk and D. L. Stern (2003). "Diapause in the pea aphid (*Acyrthosiphon pisum*) is a slowing but not a cessation of development." BMC Developmental Biology **3**(1): 1-12.

Silva, A. X., G. Jander, H. Samaniego, J. S. Ramsey and C. C. Figueroa (2012). "Insecticide resistance mechanisms in the green peach aphid *Myzus persicae* (Hemiptera: Aphididae) I: A transcriptomic survey." PLoS One **7**(6).

Simon-Delso, N., V. Amaral-Rogers, L. P. Belzunces, J. M. Bonmatin, M. Chagnon, C. Downs, L. Furlan, D. W. Gibbons, C. Giorio, V. Girolami, D. Goulson, D. P. Kreutzweiser, C. H. Krupke, M. Liess, E. Long, M. McField, P. Mineau, E. A. D. Mitchell, C. A. Morrissey, D. A. Noome, L. Pisa, J. Settele, J. D. Stark, A. Tapparo, H. Van Dyck, J. Van Praagh, J. P. Van der Sluijs, P. R. Whitehorn and M. Wiemers (2015). "Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites." Environmental Science and Pollution Research International **22**: 5-34.

Sivilotti, L. G., D. Colquhoun and N. S. Millar (2000). Comparison of native and recombinant neuronal nicotinic receptors: problems of measurement and expression. Handbook of Experimental Pharmacology. F. Clementi, D. Fornasari and C. Gotti. Berlin, Springer-Verlag. **144:** 379-416.

Škerl, M. and A. Gregorc (2010). "Heat shock proteins and cell death in situ localisation in hypopharyngeal glands of honeybee (*Apis mellifera carnica*) workers after imidacloprid or coumaphos treatment." Apidologie **41**(1): 73-86.

Slosser, J. E., M. N. Parajulee, D. L. Hendrix, T. J. Henneberry and D. R. Rummel (2002). "Relationship between *Aphis gossypii* (Homoptera: Aphididae) and sticky lint in cotton." Journal of Economic Entomology **95**(2): 299-306.

Slosser, J. E., W. E. Pinchak and D. R. Rummel (1989). "A review of known and potential factors affecting the population dynamics of the cotton aphid." Southwestern Entomologist **14**(3): 302-313.

Smith, L. M., J. Z. Sanders, R. J. Kaiser, P. Hughes, C. Dodd, C. R. Connell, C. Heiner, S. B. Kent and L. E. Hood (1986). "Fluorescence detection in automated DNA sequence analysis." Nature **321**(6071): 674-679.

Smith, T., L. Wilson, S. Heimoana, G. Herron and B. Franzmann (2006). Overwinter host plants of cotton aphid (*Aphis gossypii*) and implications for managing abundance and resistance. Proceedings 13th Australian Cotton Growers Conference. Gold Coast. 8th-10th August

Soderlund, D. M. and J. R. Bloomquist (1990). Molecular Mechanisms of Insecticide Resistance. Pesticide Resistance in Arthropods. R. T. Roush and B. E. Tabashnik. New York, Chapman and Hall: 58-96.

Sogorb, M. A. and E. Vilanova (2002). "Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis." Toxicology Letters **128**(1-3): 215-228.

Soloway, S. B., A. C. Henry, W. D. Kollmeyer, W. M. Padgett, J. E. Powell, S. A. Roman, C. H. Tieman, R. A. Corey and C. A. Horne (1978). Nitromethylene heterocycles as insecticides. Pesticide and Venom Neurotoxicity. D. L. Shankland, R. M. Hollingworth and T. Smith. New York, Plenum: 153-158.

Soloway, S. B., A. C. Henry, W. D. Kollmeyer, W. M. Padgett, J. E. Powell, S. A. Roman, C. H. Tieman, R. A. Corey and C. A. Horne (1979). Nitromethylene Insecticides. Advances in Pesticide Science. H. Geissbüehler. Oxford, Pergamon: 206-217.

Sørensen, J. G., T. N. Kristensen and V. Loeschcke (2003). "The evolutionary and ecological role of heat shock proteins." Ecology Letters **6**(11): 1025-1037.

Sorensen, J. G., P. Michalak, J. Justesen and V. Loeschcke (1999). "Expression of the heat-shock protein HSP70 in *Drosophila buzzatii* lines selected for thermal resistance." Hereditas **131**(2): 155-164.

Sparks, T. C., G. J. DeBoer, N. X. Wang, J. M. Hasler, M. R. Loso and G. B. Watson (2012). "Differential metabolism of sulfoximine and neonicotinoid insecticides by *Drosophila melanogaster* monooxygenase CYP6G1." Pesticide Biochemistry and Physiology **103**(3): 159-165.

Sparks, T. C. and R. Nauen (2015). "IRAC: Mode of action classification and insecticide resistance management." Pesticide Biochemistry and Physiology **121**: 122-128.

Stern, D. L., A. M. Puinean, S. P. Foster, L. Oliphant, I. Denholm, L. M. Field, N. S. Millar, M. S. Williamson and C. Bass (2010). "Amplification of a Cytochrome P450 Gene Is Associated with Resistance to Neonicotinoid Insecticides in the Aphid *Myzus persicae*." PLoS Genetics **6**(6).

Sumitomo Chemical Australia Pty Ltd. (2010). "Shield Cotton Brochure." Retrieved March 12th, 2014, from http://www.sumitomo-chem.com.au/sites/default/files/literature/shield_cotton_brochure.pdf.

Sun, Y. P. and E. R. Johnson (1960). "Synergistic and Antagonistic Actions of Insecticide-Synergist Combinations and Their Mode of Action." Journal of Agricultural and Food Chemistry **8**(4): 261-266.

Syngenta. (2012). "2011 Full Year Results." Retrieved 12th November 2014, from http://www.syngenta.com/global/corporate/en/news-center/news-releases/pages/120208.aspx.

Syngenta. (2013). "2012 Full Year Results." Retrieved 12th November 2014, from http://www.syngenta.com/global/corporate/SiteCollectionDocuments/pdf/media-releases/en/20130206-analyst-presentation-fyr2012.pdf.

Syvanen, M., Z. Zhou, J. Wharton, C. Goldsbury and A. Clark (1996). "Heterogeneity of the glutathione transferase genes encoding enzymes responsible for insecticide degradation in the housefly." Journal of Molecular Evolution **43**(3): 236-240.

302 of 308

Tabashnik, B. E. (1989). "Managing Resistance with Multiple Pesticide Tactics: Theory, Evidence, and Recommendations." Journal of Economic Entomology **82**(5): 1263-1269.

Tabashnik, B. E. (1990). Modeling and Evaluation of Resistance Management Tactics. Pesticide Resistance in Arthropods. R. T. Roush and B. E. Tabashnik. New York, Chapman and Hall: 152-182.

Testa, B. and S. D. Kramer (2007). "The biochemistry of drug metabolism-an introduction: part 3. Reactions of hydrolysis and their enzymes." Chemistry & Biodiversity **4**(9): 2031-2122.

Thany, S. H. (2010). Neonicotinoid Insecticides Historical Evolution and Resistance Mechanisms. Insect Nicotinic Acetylcholine Receptors. Berlin, Springer-Verlag **683**: 75-83.

Tiwari, S., R. S. Mann, M. E. Rogers and L. L. Stelinski (2011). "Insecticide resistance in field populations of Asian citrus psyllid in Florida." Pest Management Science **67**(10): 1258-1268.

Tiwari, S., L. L. Stelinski and M. E. Rogers (2012). "Biochemical basis of organophosphate and carbamate resistance in Asian citrus psyllid." Journal of Economic Entomology **105**(2): 540-548.

Toda, S., S. Komazaki, T. Tomita and Y. Kono (2004). "Two amino acid substitutions in acetylcholinesterase associated with pirimicarb and organophosphorous insecticide resistance in the cotton aphid, *Aphis gossypii* Glover (Homoptera : Aphididae)." Insect Molecular Biology **13**(5): 549-553.

Tomizawa, M. and J. E. Casida (2003). "Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors." Annual Review of Entomology **48**: 339-364.

Tomizawa, M. and J. E. Casida (2005). "Neonicotinoid insecticide toxicology: mechanisms of selective action." Annual Review of Pharmacology and Toxicology **45**: 247-268.

Tomizawa, M. and J. E. Casida (2009). "Molecular recognition of neonicotinoid insecticides: the determinants of life or death." Accounts of Chemical Research **42**(2): 260-269.

Toshima, K., S. Kanaoka, A. Yamada, K. Tarumoto, M. Akamatsu, D. B. Sattelle and K. Matsuda (2009). "Combined roles of loops C and D in the interactions of a neonicotinoid insecticide imidacloprid with the α4β2 nicotinic acetylcholine receptor." Neuropharmacology **56**(1): 264-272.

Toutant, J.-P. (1989). "Insect acetylcholinesterase: Catalytic properties, tissue distribution and molecular forms." Progress in Neurobiology **32**(5): 423-446.

Untergasser, A., H. Nijveen, X. Rao, T. Bisseling, R. Geurts and J. A. M. Leunissen (2007). "Primer3Plus, an enhanced web interface to Primer3." Nucleic Acids Research **35**.

Unwin, N. (2005). "Refined structure of the nicotinic acetylcholine receptor at 4A resolution." Journal of Molecular Biology **346**(4): 967-989.

Velculescu, V. E., L. Zhang, B. Vogelstein and K. W. Kinzler (1995). "Serial analysis of gene expression." Science **270**(5235): 484-487.

Vontas, J. G., G. J. Small and J. Hemingway (2001). "Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*." The Biochemical Journal **357**(Pt 1): 65-72.

Vontas, J. G., G. J. Small, D. C. Nikou, H. Ranson and J. Hemingway (2002). "Purification, molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the rice brown planthopper, *Nilaparvata lugens*." The Biochemical Journal **362**(Pt 2): 329-337.

Wakita, T., K. Kinoshita, E. Yamada, N. Yasui, N. Kawahara, A. Naoi, M. Nakaya, K. Ebihara, H. Matsuno and K. Kodaka (2003). "The discovery of dinotefuran: a novel neonicotinoid." Pest Management Science **59**(9): 1016-1022.

Wall, R. E. (1933). "A Study of Color and Color-Variation in *Aphis Gossypii* Glover." Annals of the Entomological Society of America **26**(3): 425-463.

304 of 308

Wang, K. I., Q. L. Guo, X. M. Xia, H. Y. Wang and T. X. Liu (2007). "Resistance of *Aphis gossypii* (Homoptera: Aphididae) to selected insecticides on cotton from five cotton production regions in Shandong, China." Journal of Pesticide Science **32**(4): 372-378.

Wang, K. Y., T. X. Liu, C. H. Yu, X. Y. Jiang and M. Q. Yi (2002). "Resistance of *Aphis Gossypii* (Homoptera: Aphididae) to fenvalerate and imidacloprid and activities of detoxification enzymes on cotton and cucumber." Journal of Economic Entomology **95**(2): 407-413.

Wang, M., D. L. Roberts, R. Paschke, T. M. Shea, B. S. S. Masters and J.-J. P. Kim (1997). "Three-dimensional structure of NADPH–cytochrome P450 reductase: Prototype for FMN- and FAD-containing enzymes." Proceedings of the National Academy of Sciences of the United States of America **94**(16): 8411-8416.

Wang, Z., M. Gerstein and M. Snyder (2009). "RNA-Seq: a revolutionary tool for transcriptomics." Nature Reviews Genetics **10**(1): 57-63.

Watt, M. and D. F. Hales (1996). "Dwarf Phenotype of the Cotton Aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae)." Australian Journal of Entomology **35**(2): 153-159.

Wellings, P. W., S. R. Leather and A. F. G. Dixon (1980). "Seasonal Variation in Reproductive Potential: A Programmed Feature of Aphid Life Cycles." Journal of Animal Ecology **49**(3): 975-985.

Whalon, M. E., D. Mota-Sanchez and R. M. Hollingworth. (2008). "The MSU Arthropod Pesticide Resistance Database." Retrieved March 12th 2015, from http://www.pesticideresistance.org.

WHO (1957). "Seventh report of the WHO expert committee on insecticides." World Health Organ Techical Report Series **125**: 1-32.

Wilhoit, L. R. and J. A. Rosenheim (1993). The yellow dwarf form of the cotton aphid, *Aphis gossypii*. Proceedings of Beltwide Cotton Conference.

305 of 308

Williams, I. S. and A. F. G. Dixon (2007). Life Cycles and Polymorphism. Aphids as Crop Pests. H. Van Emden and R. Harrington. Wallingford, United Kingdom, CABI.

Williamson, M. S., D. Martinez-Torres, C. A. Hick and A. L. Devonshire (1996). "Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides." Molecular Genetics and Genomics 252(1-2): 51-60.

Wilson, L., S. Downes, M. Khan, M. Whitehouse, G. Baker, P. Grundy and S. Maas (2013). "IPM in the transgenic era: a review of the challenges from emerging pests in Australian cotton systems." Crop and Pasture Science 64(8): 737-749.

Wilson, L. J., L. R. Bauer and D. A. Lally (1999). "Insecticide-induced increases in aphid abundance in cotton." Australian Journal of Entomology 38(3): 242-243.

Wilson, L. J., R. K. Mensah and G. P. Fitt (2004). Implementing Integrated Pest Management in Australian Cotton. Insect Pest Management: Field and Protected Crops. A. R. Horowitz and I. Ishaaya. Berlin, Heidelberg, Spinger-Verlag: 97-118.

Wool, D. and D. F. Hales (1997). "Phenotypic Plasticity in Australian Cotton Aphid (Homoptera: Aphididae): Host Plant Effects on Morphological Variation." Annals of the Entomological Society of America 90(3): 316-328.

Yamada, T., H. Takahashi and R. Hatano (1999). A Novel Insecticide, Acetamiprid. Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor. I. Yamamoto and J. Casida, Springer Japan: 149-176.

Yamamoto, I. (1999). Nicotine to Nicotinoids: 1962 to 1997. Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor. I. Yamamoto and J. E. Casida, Springer Japan: 3-27.

Yang, X., W. Xie, S. L. Wang, Q. J. Wu, H. P. Pan, R. M. Li, N. N. Yang, B. M. Liu, B. Y. Xu, X. Zhou and Y. J. Zhang (2013). "Two cytochrome P450 genes are involved in imidacloprid resistance in field populations of the whitefly, *Bemisia tabaci,* in China." Pesticide Biochemistry and Physiology 107(3): 343-350.

Yao, X., F. Song, F. Chen, Y. Zhang, J. Gu, S. Liu and Z. Liu (2008). "Amino acids within loops D, E and F of insect nicotinic acetylcholine receptor beta subunits influence neonicotinoid selectivity." Insect Biochemistry and Molecular Biology **38**(9): 834-840.

Ye, J., L. Fang, H. Zheng, Y. Zhang, J. Chen, Z. Zhang, J. Wang, S. Li, R. Li, L. Bolund and J. Wang (2006). "WEGO: a web tool for plotting GO annotations." Nucleic Acids Research **34**.

Yilmaz, M., O. Ozic and I. Gok (2012). Principles of Nucleic Acid Separation by Agarose Gel Electrophoresis Gel Electrophoresis - Principles and Basics. S. Magdeldin, InTech.

Yu, S., J. (2008). Insecticide Resistance. The Toxicology and Biochemistry of Insecticides. Boca Raton, CRC Press: 201-230.

Yu, S. J. (2014). Insecticide Resistance. The Toxicology and Biochemistry of Insecticides. Boca Raton, CRC Press.

Zewen, L., H. Zhaojun, W. Yinchang, Z. Lingchun, Z. Hongwei and L. Chengjun (2003). "Selection for imidacloprid resistance in *Nilaparvata lugens*: cross-resistance patterns and possible mechanisms." Pest Management Science **59**(12): 1355-1359.

Zhang, Q. R. and D. L. Denlinger (2010). "Molecular characterization of heat shock protein 90, 70 and 70 cognate cDNAs and their expression patterns during thermal stress and pupal diapause in the corn earworm." Journal of Insect Physiology **56**(2): 138-150.

Zhang, Y., R. Jiang, H. Wu, P. Liu, J. Xie, Y. He and H. Pang (2012). "Next-generation sequencing-based transcriptome analysis of *Cryptolaemus montrouzieri* under insecticide stress reveals resistance-relevant genes in ladybirds." Genomics **100**(1): 35-41.

Zhang, Y., X. Wang, B. Yang, Y. Hu, L. Huang, C. Bass and Z. Liu (2015). "Reduction in mRNA and protein expression of a nicotinic acetylcholine receptor alpha8

subunit is associated with resistance to imidacloprid in the brown planthopper, *Nilaparvata lugens*." Journal of Neurochemistry **135**(4): 686-694.

Zhu, K. Y. (2008). Synergism. Encyclopedia of Entomology. J. L. Capinera. Berlin, Heidelberg, Springer-Verlag.

Zidan, L. T. M. (2012). "Bio-Efficacy of Three New Neonicotinoid Insecticides as Seed Treatment Against Four Early Sucking Pests of Cotton." American-Eurasian Journal Agricutural and Environmental Science **12**(4): 535-540.

Zil'bermints, I. V. and L. M. Zhuravela (1984). "Response of melon and greenhouse aphids to Ambush and Actellic." Khimiya v Sel'skom Khozyaistva (in Russian) **3**: 37-40.