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 Sydney

By

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Bachelor of Animal and Veterinary Bioscience (Hons)

School of Life Sciences

APRIL 2017

Certificate of Original Authorship

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Refereed Journal Publications

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

Chapter 3:

Marshall, **K.L.,** Collins, D., Wilson, L.J. & Herron, G.A. 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, G.A. & 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

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 husband 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.

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List of Abbreviations and Acronyms

ABC: Adenosine triphosphate-binding cassette

Ace: Acetylcholinesterase gene

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

ACh: Acetylcholine

ATP: Adenosine triphosphate

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

FPKM: Fragments aligned per thousand bases per million reads

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

RR: Resistance ratio

RFLP: Restriction fragment length polymorphism

RNA-Seq: RNA-Sequencing

SNP: Single nucleotide polymorphism

UGT: UDP-glucuronosyltransferase

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 ratios 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 cytochrome P450 gene *6K1*-like) 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 characterised 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).

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.

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).

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 (Figure 1.2).

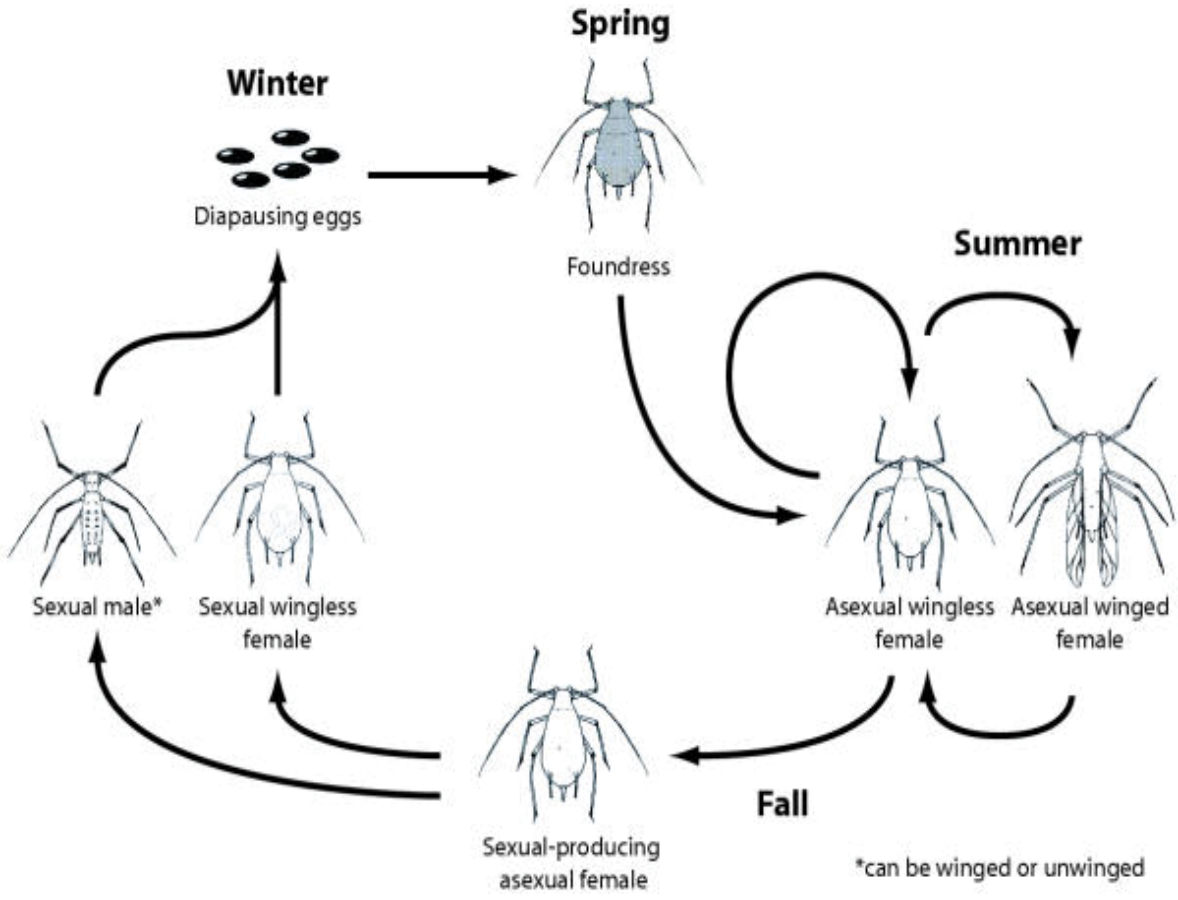

Figure 1.2 Life cycle of aphids (Shingleton et al. 2003).

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).

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 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 (Maas 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 (Maas 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%) (Maas 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 (CottonInfo 2015, IRAC 2016).

Group	Mode of Action	Subgroup	Chemical group	Trade names
1	Acetylcholine esterase inhibitors	A	Carbamates	Pirimicarb
		B	Organophosphates	Dimethoate, omethoate, phorate, chlorpyrifos
2	GABA-gated chloride channel antagonists	A	Cyclodiene organochlorines	Endosulfan
		B	Phenylpyrazoles	Fipronil
3	Sodium channel modulators	A	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	Chordotonal organ TRPV channel modulators	B	Pyridine azomethine derivatives	Pymetrozine
		C		Flonicamid
12	Inhibitors of mitochondrial ATP synthase	A		Diafenthiuron
22	Inhibitors of lipid synthesis			Spirotetramat
28	Ryanodine receptor modulators		Diamides	Cyantraniliprole
29	Chordotonal organ Modulators		Flonicamid	Flonicamid

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 (RRs) 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 RRs 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
29	Flonicamid	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 nicotinic acetylcholine receptors (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 commercialised for use (Kleier et al. 1985). In the 1980's, continued research on the chemical structure of nithiazine led to the discovery of l-(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 l-(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 (Nippon Soda) (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).

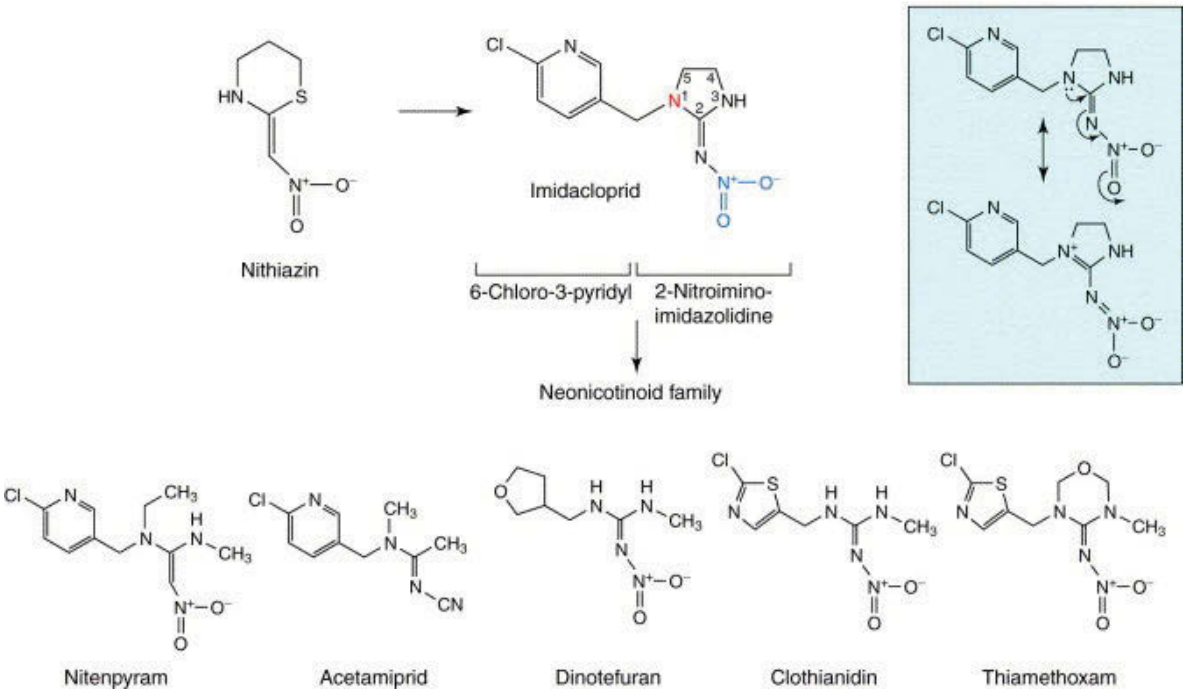

Figure 1.3 The structure of the synthetic insecticide imidacloprid. Also shown are other synthetic insecticides that are related to imidacloprid: nithiazin, nitenpyram, acetamiprid, dinotefuran, clothianidin and thiamethoxam. The two main moieties of the imidacloprid molecule are shown; the tertiary 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 synthesised (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 (nithiazine and nitenpyram); and cyanoimines (acetamiprid and thiacloprid). The newly developed insecticide sulfoxaflor also acts as a nAChR agonist but because of the novel way it interacts with the nAChR (due to structural differences), 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 et al. 2013).

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® (clothianidin), Mospilan® (acetamiprid), Actara® (thiamethoxam) and Confidor® (imidacloprid) (Maas 2014) (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 (Maas 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®	Gauncho®
			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. 1983a, Noda et al. 1983b, 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).

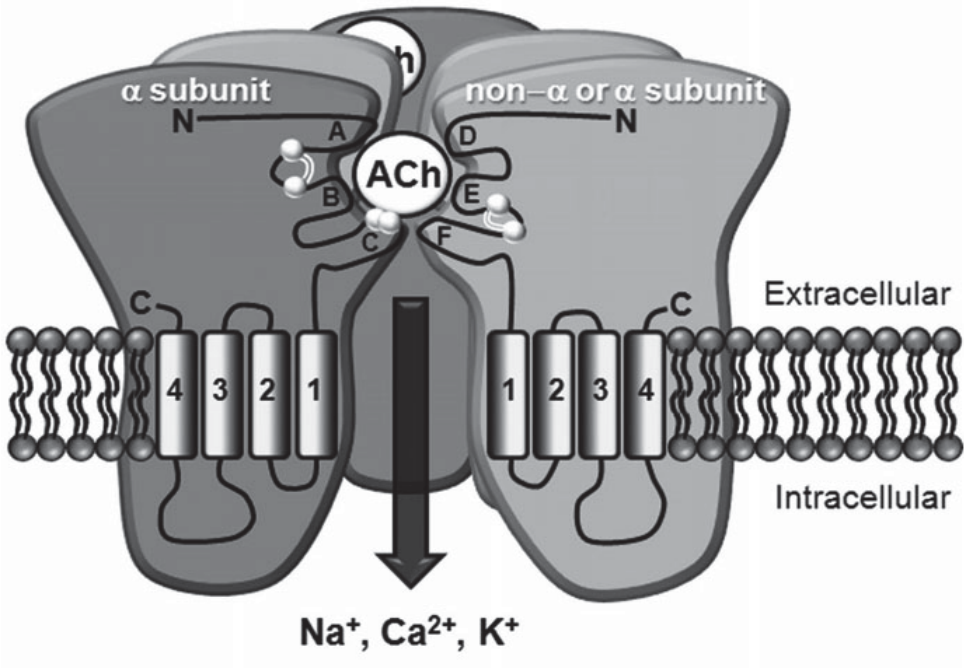

Figure 1.4 A schematic representation of the arrangement of a heteromeric acetylcholine receptor consisting of two α (dark grey) and three non-α (light grey) subunits arranged around a central cation-permeable channel. Acetylcholine binding sites: ACh; Four transmembrane domains: 1-4; Six binding loops: A-F; Cys-loop: two white circles connected by a white double line (Jones and Sattelle 2010).

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 specialised enzyme, acetylcholinesterase (AChE) (Toutant 1989). Neonicotinoid insecticides mimic the action of ACh but are unable to be broken down by AChE, the result of which is prolonged binding to and overstimulation of the receptor (Tomizawa and Casida 2005, Tomizawa and Casida 2009).

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, at least in part, their selectivity for insect nAChRs over vertebrate 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 ionised and interact with a subsite consisting of cationic amino acid residue(s) in the insect nAChR, while ionised 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.

Selectivity of neonicotinoid insecticides towards insects versus some spider and mite species, including the pond wolf spider *Pardosa pseudoannulata* (Boesenberg & Strand) (Song et al. 2009) and two spotted spider mite *Tetranychus urticae* Koch (Dermauw et al. 2012) has also centred on the role of β-nAChR subunits. In *P. pseudoannulata* a β1-nAChR subunit was cloned (Ppβ1) with high identity (74-78%) to insect β1 subunits (Song et al.

2009), although several amino acid differences between Ppβ1 and β1 subunits were found within loops D-F. The effects of these amino acid differences were evaluated by introducing loop D-F subunit chimeras with the green peach aphid *Myzus persicae* Sulzer and rat β2 nAChRs. In particular, replacement of the positively charged arginine residue in loop D with an uncharged glutamine (Q81) caused a right-ward shift to the imidacloprid dose-response curve (Song et al. 2009). This glutamine residue has also been found in several tick species, and thus highlights the role this amino acid might play in conferring insensitivity of arachnids to neonicotinoid insecticides (Erdmanis et al. 2012). In *T. urticae*, polymorphism of the same arginine residue (81E) is likely contributing to insensitivity to neonicotinoid insecticides (Dermauw et al. 2012).

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).

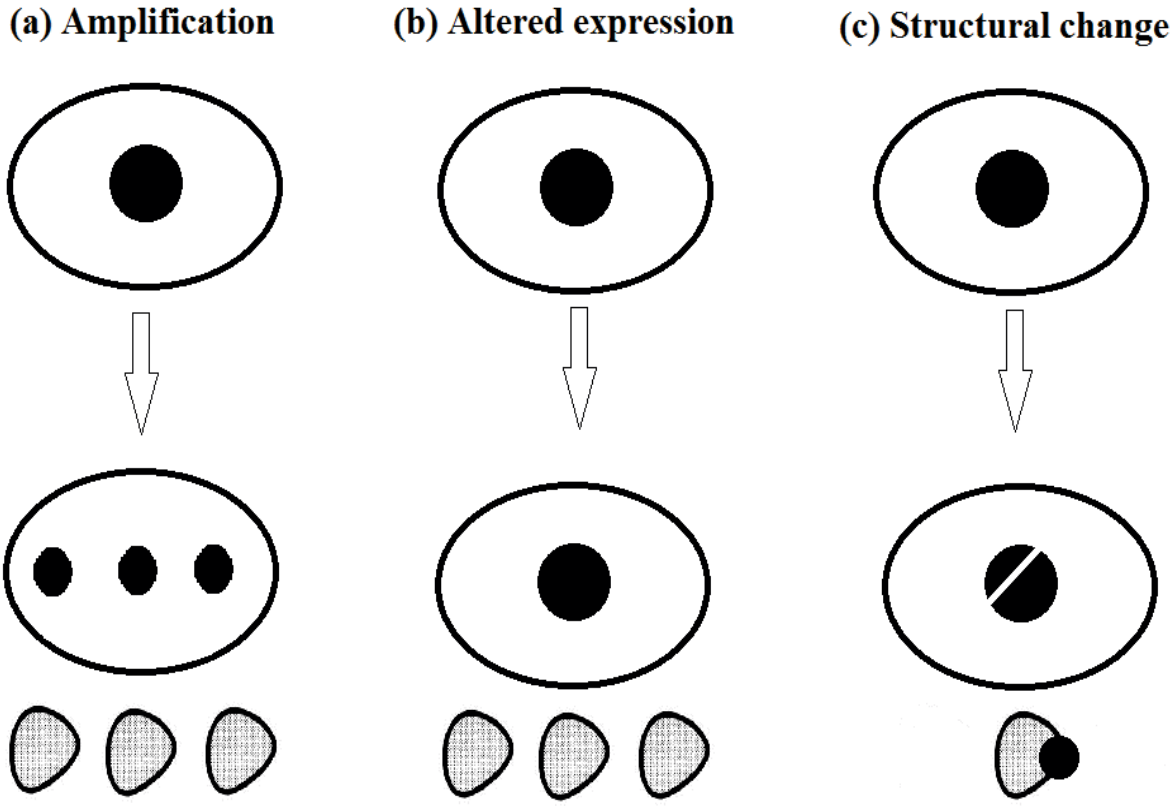

Figure 1.5 Graphic representation of the types of genetic mutations which occur and cause resistance. (a) the gene is amplified to produce more copies of itself and thus increase the amount of gene product made (b) the regulatory expression of a gene is modified so that the amount of gene product made 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 receptor subunit genes 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 (Davies et al. 2008, Dong et al. 2014). This mutation was first documented in *M. domestica* (Williamson et al. 1996) but has since been identified in almost all agriculturally important arthropod pests and diseases vectors, worldwide (Soderlund 2012, Rinkevich et al. 2013, Dong et al. 2014). To date, more than 50 sodium channel mutations or combinations of mutations associated with pyrethroid resistance in arthropod species have been elucidated (see Dong et al. (2014) for review). 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).

Co-occurrence of more than one *kdr* mutation often leads to increased sensitivity of the sodium channel to pyrethroid insecticides. The most prominent secondary mutation, termed super-*kdr* is located within the intracellular domain II S4-S5 loop and results in a methionine to threonine replacement (M918T) (Rinkevich et al. 2013, Dong et al. 2014). 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). Similarly, in *M. persicae*, co-occurrence of the L1014F and M918T mutations confers very high level resistance to a range of Type I and Type II pyrethroid insecticides (Eleftherianos et al. 2008). 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 Nicotinic acetylcholine receptor

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 *M. 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 resistance factor 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 *Nicotinic acetylcholine receptor channel blockers*

Also acting on nAChRs, are nereistoxin analogues including the insecticides cartap hydrochloride, thiocyclam, bensultap and thiosultap-sodium. Unlike group 4 and 5 MoA insecticides, nereistoxin analogues (group 14 MoA) act as antagonists of nAChRs and cause paralysis via a ganglionic blocking action on the central nervous system.

1.3.1.1.4 Gamma aminobutyric-gated chloride channels

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 chloride channel subunit (Thompson et al. 1993). In *D. melanogaster*, besides directly affecting the binding site, replacement of alanine 302 also destabilises the preferred confirmation of the receptor (ffrench-Constant et al. 1993). 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).

1.3.1.2 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).

1.3.1.3 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

Metabolic resistance is based on enzymatic detoxification systems that enable insects to metabolise, and thereby degrade toxins, into a form more suitable for elimination from the body. In insects, resistance is conferred by mutations affecting catalytic activities of detoxifying enzymes or leading to higher quantities of the enzymes as a consequence of increased transcription or gene amplification. Enzymes catalysing phase I elimination reactions, consisting of hydrolysis and oxidation, include P450s and esterases (ESTs), and phase II reactions, consisting of conjugation of phase I products with endogenous compounds and their subsequent excretion from the body, include glutathione-S-transferases (GSTs) (Li et al. 2007, Hollingworth and Dong 2008). Lastly, ABC (ATP-binding cassette) transporters are essential components of phase III elimination reactions, that is, the transfer of xenobiotic compounds or their modified forms from the cell to counter accumulation (Glavinas et al. 2004, Sarkadi et al. 2006).

1.3.2.1 Esterases

The carboxylesterase (CE) family from which ESTs belong to is an extremely versatile enzyme group characterised by an α / β 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). A vast number of conventional insecticides still 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 catalyse 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, dichlorodiphenyltrichloroethane (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 monoxygenase

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 synthesised 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 oxidised 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. 2007). 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 piperonyl butoxide (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 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 RR 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 RR 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 ABC Transporters

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). 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, G and H 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). Furthermore, in *M. persicae*, genes encoding an ABCG and ABCH transporter were found to be upregulated (although not validated by qRT-PCR) in adults exposed to pirimicarb (Silva et al. 2012). Finally in a thiamethoxam resistant strain of *B. tabaci* several ATP-binding cassette transporters of the ABCG subfamily were highly over-expressed in the adult stage and may play a role in resistance (Yang et al. 2013, Yang et al. 2013).

1.3.2.5 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 metabolise 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 sclerotisation by oxidizing catechols in the cuticle to their corresponding quinines, which then catalyse 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 characterise 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 characterise the causal mechanism.

1.4.1 Bioassay

Laboratory bioassay is utilised 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 realise 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 RR (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 (α, i = 1, 2) and the slopes (ε, 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 P450s (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). Elsewhere, DEM (diethyl maleate) has been commonly used as a synergist to suppress GST activity (Ahmad and Hollingworth 2004). 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 synthesise DNA in this manner, a single stranded DNA template, a DNA primer, a DNA polymerase, deoxynucleotidetriphosphates (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 visualised 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 utilises a unique solid phase 'bridged' amplification reaction that allows hybridised 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). Specialised adapters are ligated onto the ends of DNA fragments and one of these adapters is hybridised 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-Sequencing (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 characterised in Chapter 2. This was initiated to identify and characterise genes encoding detoxification enzymes and insecticide target proteins. Those genes found differentially expressed between susceptible and resistant strains were 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 RRs 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, RRs 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 characterise 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, Queensland (QLD) and Toobeah (QLD), respectively (Figure 2.1). In 2012, a third field strain termed Carr was

collected off cotton in Moree, New South Wales (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 ± 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®).

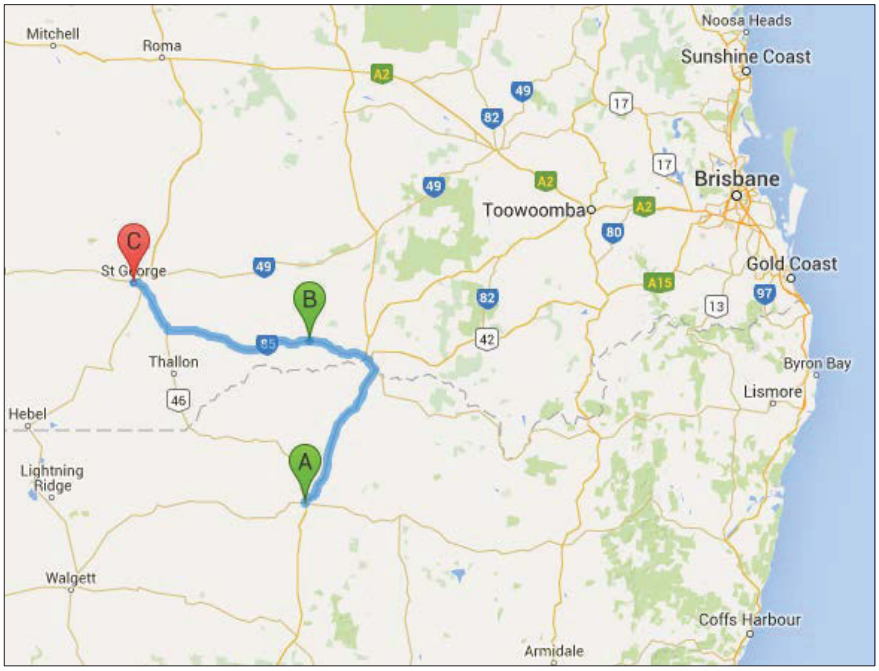

Figure 2.1 Location of aphid collections: A, Moree; B, Toobeah; C, St George.

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 ± 2ºC. 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 ± 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 ± 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 RRs 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 χ^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. RRs 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

PCR amplification of the L1014F mutation site was carried out 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. Positive and negative controls were also included. 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

PCR amplification of the S431F site was successfully carried out by employing the 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. Positive and negative controls were also included. 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 S431	Pyr L1014	Thia 0.02%*	Clo 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 RRs 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 χ^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 χ^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

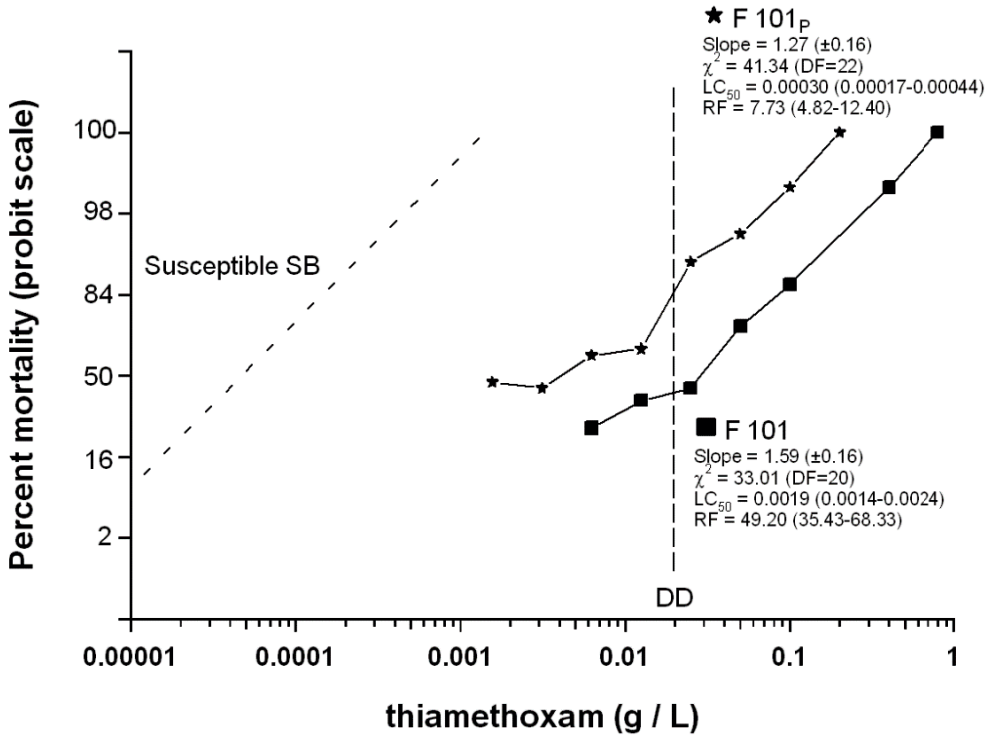

Figure 2.2 Dose–response for *Aphis gossypii* against thiamethoxam (F 101) and following three years of continual laboratory selection and maintenance (F 101_P) (Susceptible SB has been redrawn from Table 2.2 to add clarity).

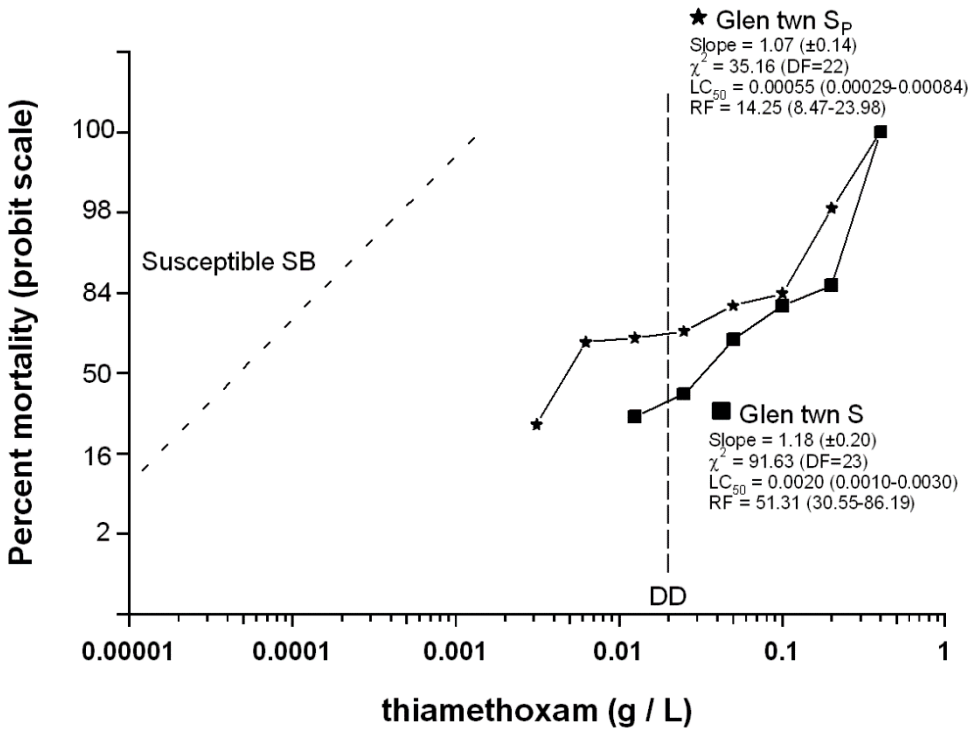

Figure 2.3 Dose–response for *Aphis gossypii* against thiamethoxam (Glen twn S) and following three years of continual laboratory selection and maintenance (Glen twn S_P) (Susceptible SB has been redrawn from Table 2.2 to add clarity).

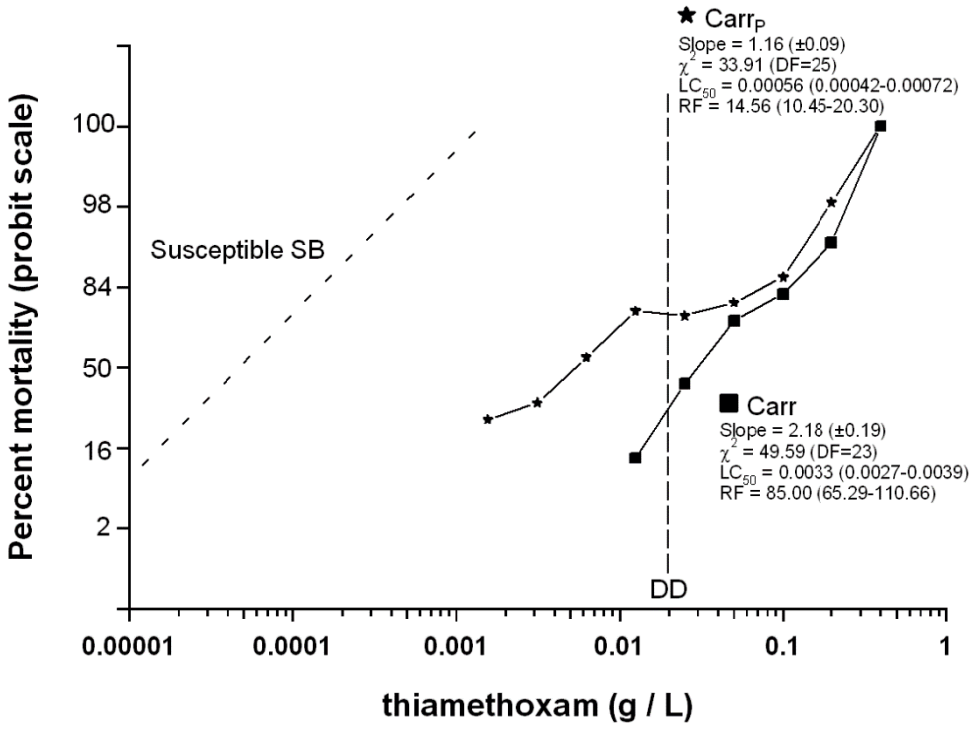

Figure 2.4 Dose–response for *Aphis gossypii* against thiamethoxam (Carr) and following three years of continual laboratory selection and maintenance ($Carr_P$) (Susceptible SB has been redrawn from Table 2.2 to add clarity).

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 RR 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 RR 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 RR 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 RR 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 RRs observed in this or other studies will indeed result in field control failures as hypothesised.

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 "K.L., Marshall, Collins, D., Wilson, L.J. & Herron, G.A. (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, RRs 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 (Maas 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, 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 ± 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 ± 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 ± 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"). 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)

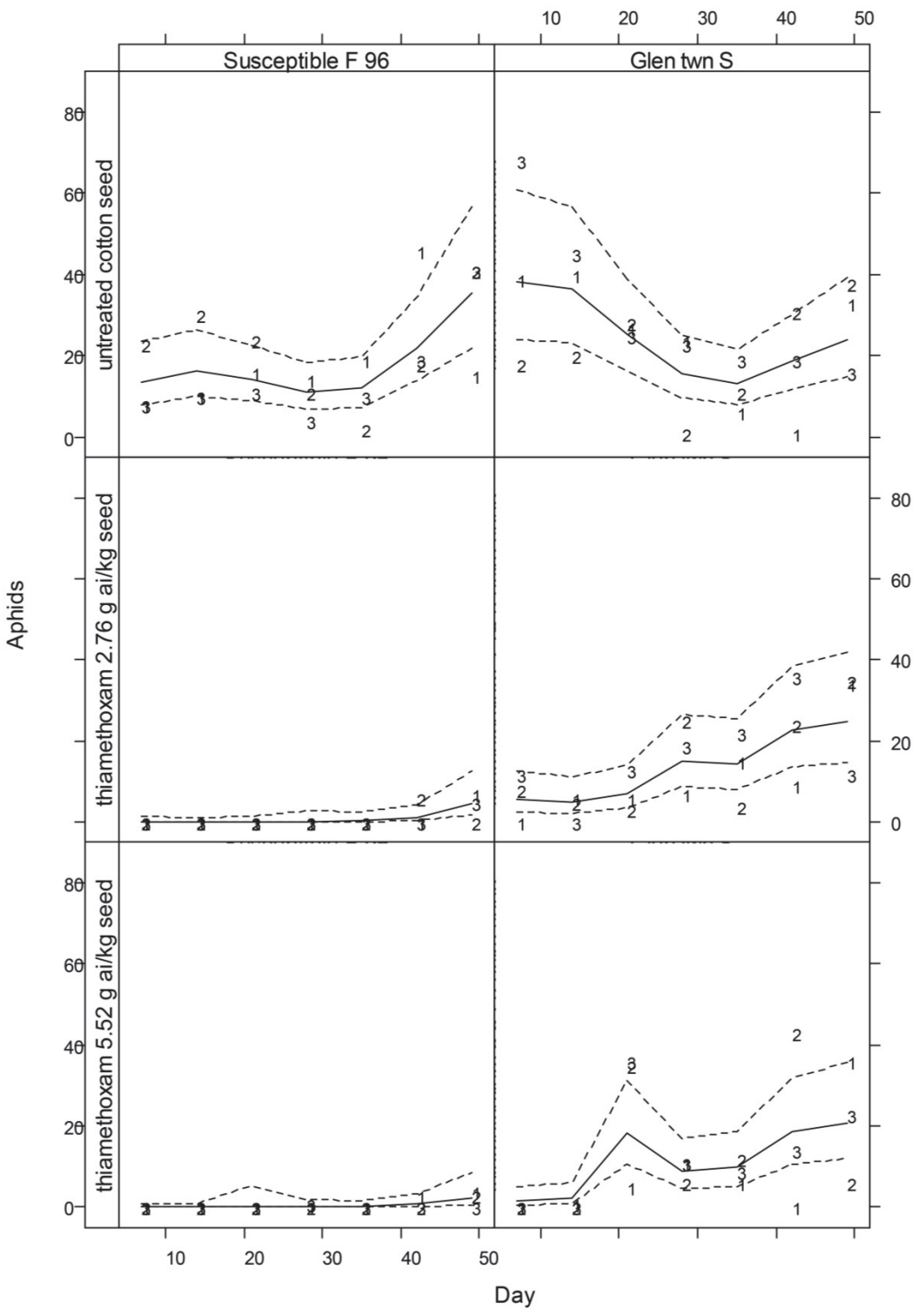
Susceptible F 96
Glen twn S
untreated cotton seed
thiamethoxam 2.76 g ai/kg seed
thiamethoxam 5.52 g ai/kg seed
Aphids
Day
0
20
40
60
80
10
20
30
40
50

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).

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)

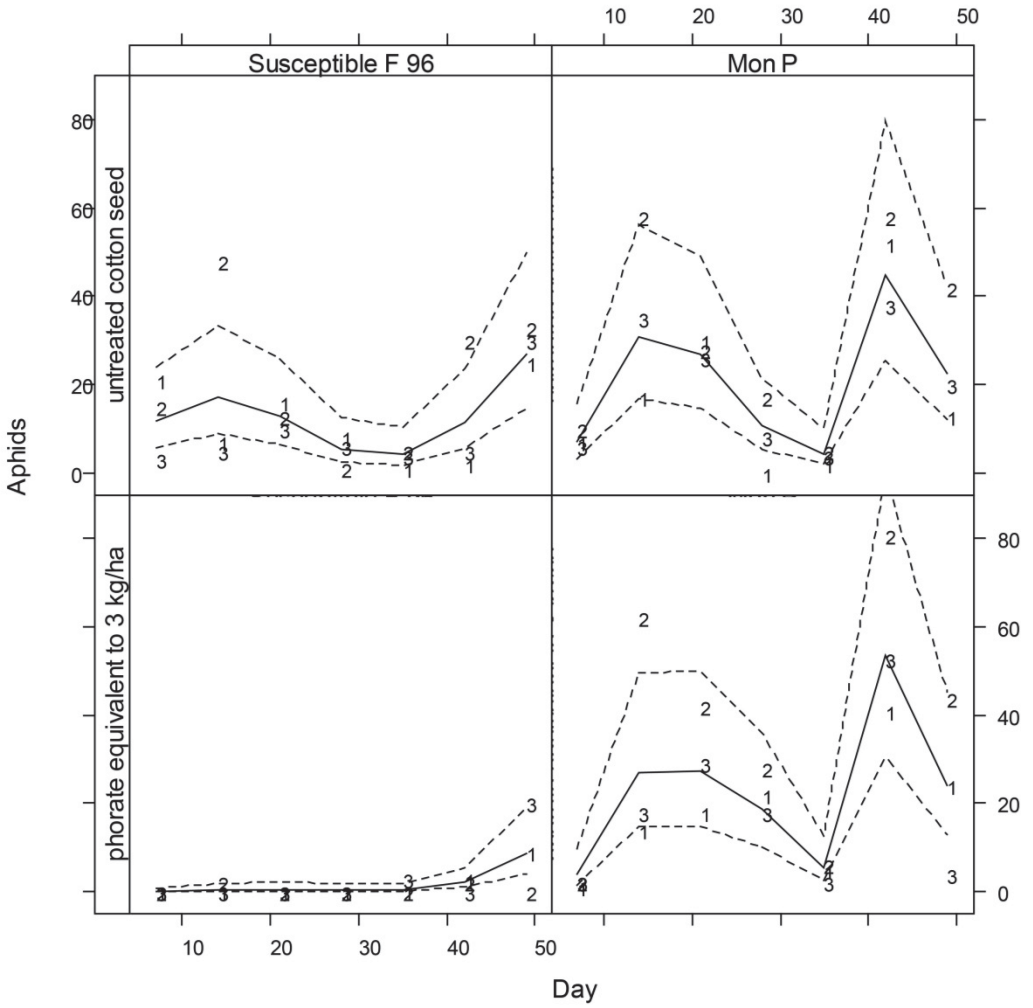

Figure 3.2 Fitted trends for the phorate analysis, for each strain by treatment combination (phorate equivalent to 3 kg/ha, Thimet® 200 G Systemic Granular Insecticide; untreated cotton seed, variety Sicot 71). The solid line represents the fitted trend, with dotted lines representing the 95% confidence 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* (Maas 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 (Maas 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 (Maas 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 RRs 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 ± 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 χ^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). RRs 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 RR 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 (prior to routine pressuring, i.e. not "p" variants) 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 98ºC, followed by 35 cycles at 98ºC for 30 s, 51ºC for 30 s and 72ºC 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 RRs 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 β1 subunit gene produced 350 bp of quality cDNA sequence in strains Sus F 96, F 101, Glen twn S and Carr (Figure 4.1).

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.

Amplification of the loop D region of the nAChR β1 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).

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 field populations of *A. gossypii* from Korea (Koo et al. 2014), and *M. persicae* originating from Southern France (Bass et al. 2011, Puinean et al. 2013), Italy (Panini et al. 2014) and Spain (Slater et al. 2012). 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.

Possibly, the varying resistance spectrum seen in my strains between thiamethoxam and imidacloprid may relate to each insecticides use pattern in Australian cotton. Although both are available as pre germination seed treatments, 80% of cotton seed planted is coated with thiamethoxam (Herron and Wilson 2011). For that reason, I consider the

higher usage of thiamethoxam in Australian cotton may be responsible for the metabolic resistance detected. (Herron and Wilson 2011). For that reason, I consider the higher usage of thiamethoxam in Australian cotton may be responsible for the metabolic resistance detected. Although further validation is recommended, in my study there is evidence to suggest that thiamethoxam resistance is likely conferred, at least in part, 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 ($P \leq 0.001$). Expression levels of P450s, UDP-glucuronosyltransferase (UGT) enzymes and proteins with choline or glucose dehydrogenase activity were significantly up-regulated in the resistant strains compared to the susceptible. Significant DEGs were subsequently assigned to known Gene Ontology (GO) categories to predict their functional roles and associated biological processes. Transcripts (CL1190 and CL1418), matched to cytochrome P450 gene *6K1*-like from *A. pisum* were up-regulated in all three resistant strains compared to the susceptible. 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 gene *6K1*-like 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). RRs 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 β1-subunit of the nAChR 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 (*CYP6CY3*) 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 strain (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 ± 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 RRs 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 (prior to routine pressuring, i.e. not "p" variants) 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 5 min 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 (25ºC), 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 synthesise 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), Clusters of Orthologous Groups (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

FPKM (fragments aligned per thousand bases per million reads) values for each contig were calculated, and differential expression statistical analysis was conducted using the DESeq package in the statistical software 'R' (Anders & Huber, 2010). The raw counts of each gene were normalised to adjust for different sequencing depths across samples using DESeq. After estimating the dispersion of each gene, DESeq analysis identified DEGs between thiamethoxam resistant and susceptible *A. gossypii* using adjusted p-value threshold p<0.001.

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 ("p" variants used) 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).

All qRT-PCR analyses were performed in 96-well plates on a 7500 Real-Time PCR Detection System (Applied Biosystems, Australia) using the primers listed in Table B.3. Gene specific primers were designed using Primer3Plus (Untergasser et al. 2007) and synthesised by Sigma Aldrich®, Australia. qRT-PCR reaction mixtures (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). qRT-PCR reactions were performed in triplicate with different RNA preparations for each strain. 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. Fold changes in gene expression between resistant and susceptible strains were derived by the comparative C_T method (Livak and Schmittgen 2001) using the endogenous control β-actin to standardise expression.

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).

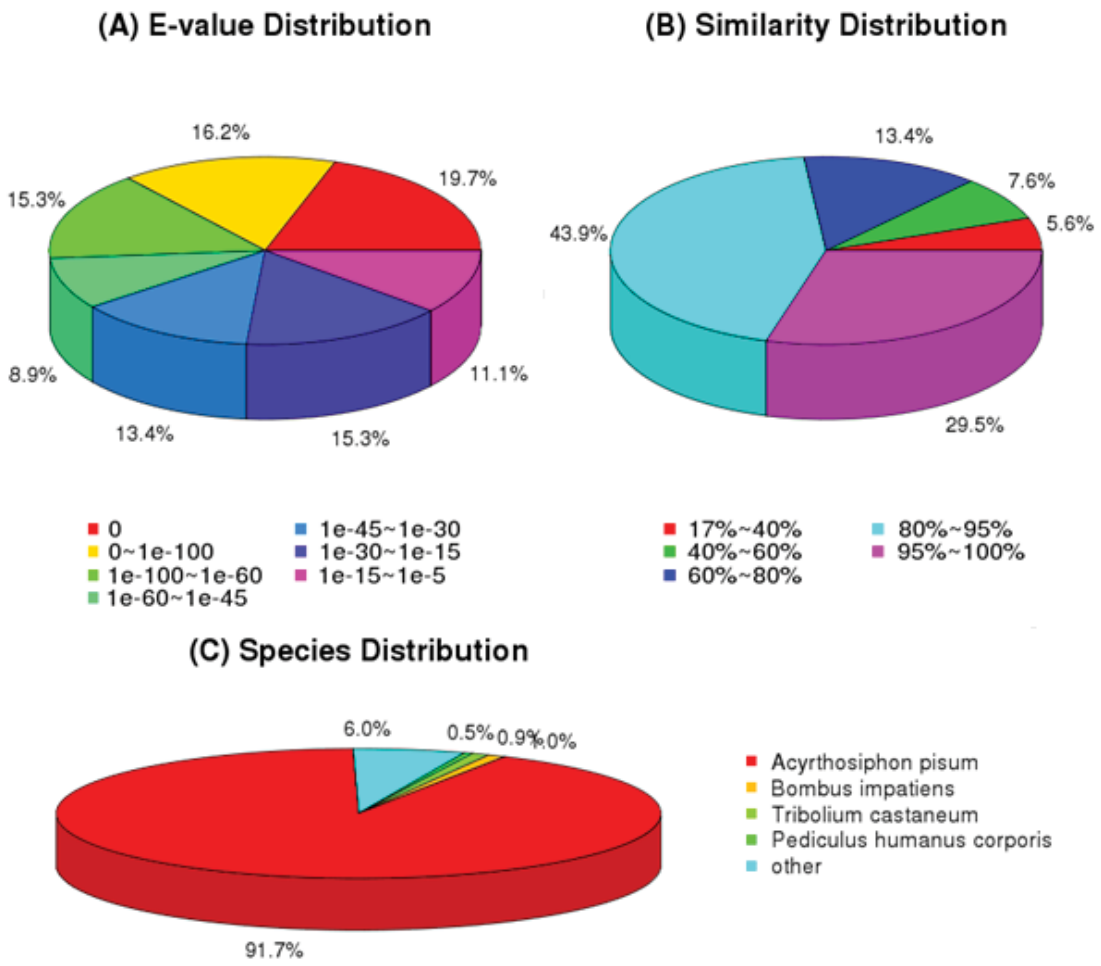

Figure 5.1 Pie-charts showing distributions from BLASTx matches of pooled *Aphis gossypii* transcriptome unigenes with respect to (A) E-values (B) gene identity and (C) insect species from which the homologous genes were matched to.

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.

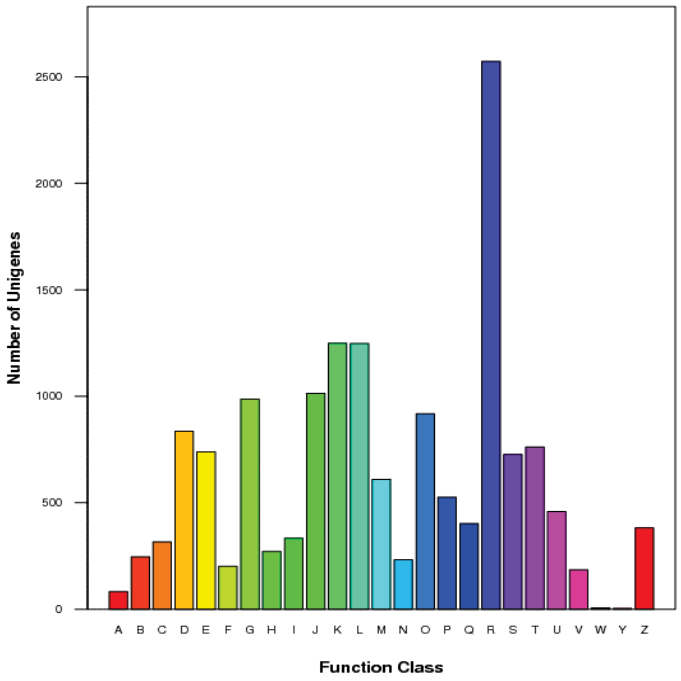

Figure 5.2 Clusters of Orthologous Groups (COG) of protein function classification of *Aphis gossypii* unigene sequences (a total of 7633 unigenes were grouped into COG function classifications). A: RNA processing and modification, B: Chromatin structure and dynamics, C: Energy production and conversion, 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 categorised 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.

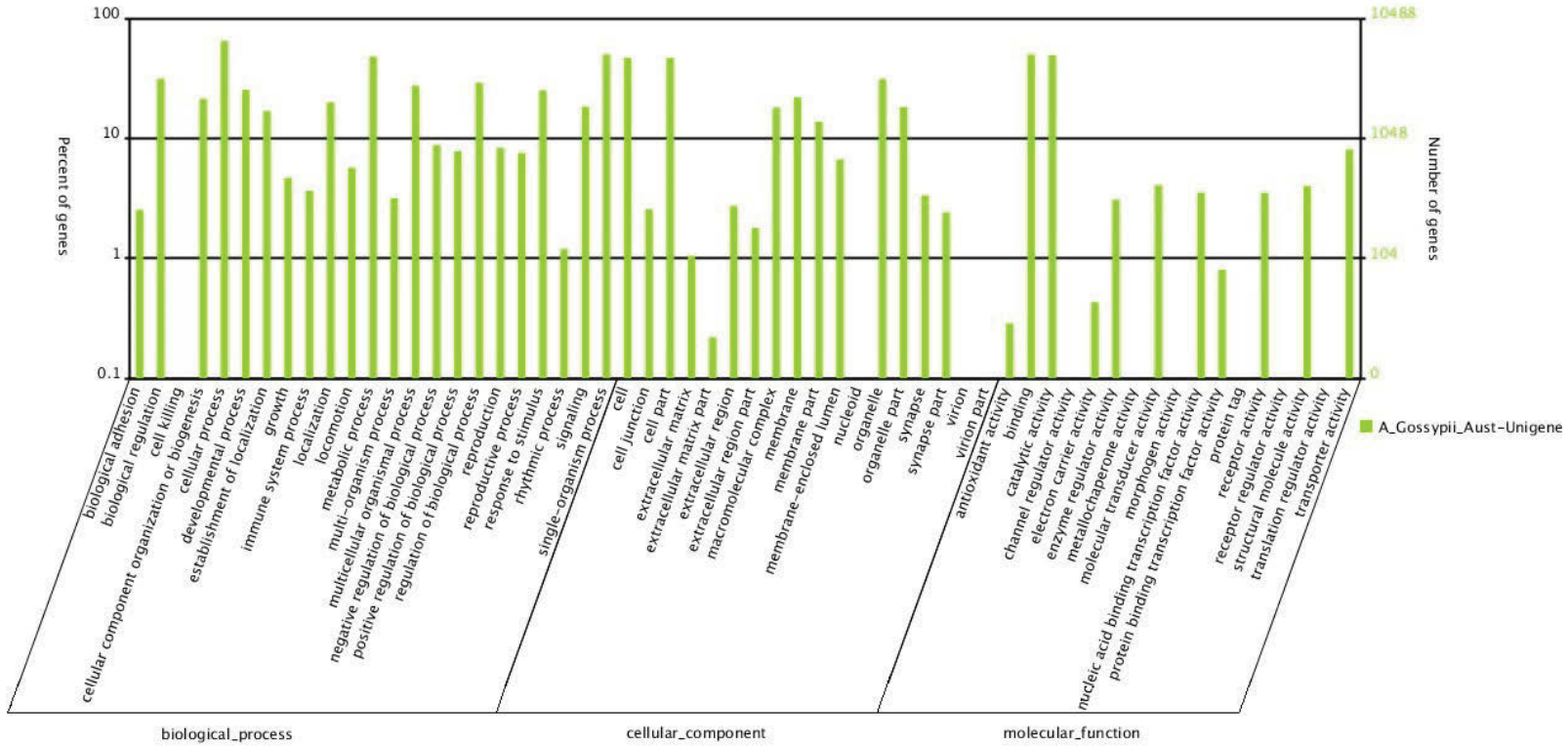

Figure 5.3 GO annotations of all combined unigenes and DEG sequences. GO categories shown in the x axis are grouped into three main ontologies: biological process, cellular component and molecular function. The right y-axis indicates the number of genes in each category, while the left y-axis indicates 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

To determine the changes in gene expression at the transcriptional level between thiamethoxam susceptible and resistant *A. gossypii*, we employed DESeq (Anders and Huber 2010) analysis. We were able to identify 81 DEGs with adjusted *p*-values <0.001 that were differentially expressed in all three resistant strains (F 101, Glen twn S and Carr) compared to the reference susceptible strain (Sus F 96). Of the 81 DEGs, 60 were up-regulated in resistant strains compared to Sus F 96, and 21 down-regulated (Table E.2).

5.4.5 Candidate resistance (detoxification) genes

The expression of transcripts encoding potential resistance genes is shown in Table E.2. All resistant strains contained DEGs relating to known insecticide detoxification mechanisms, when compared to Sus F 96. Of these, four belonged to the P450 family; two had predicted similarity to P450 gene *CYP6K1*-like of *A. pisum* (CL1190 and CL1418) and two to P450 gene *CYP6A13*-like of *A. pisum* (Unigene12511 and Unigene12819). One transcript belonging to the GST family, identified as GST sigma 1 (CL1795) was found significantly differentially expressed in resistant strains compared

to Sus F 96 (Table E.2). Finally, no CEs were significantly differentially expressed in resistant strains compared to Sus F 96.

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 5.4 and Table E.3).

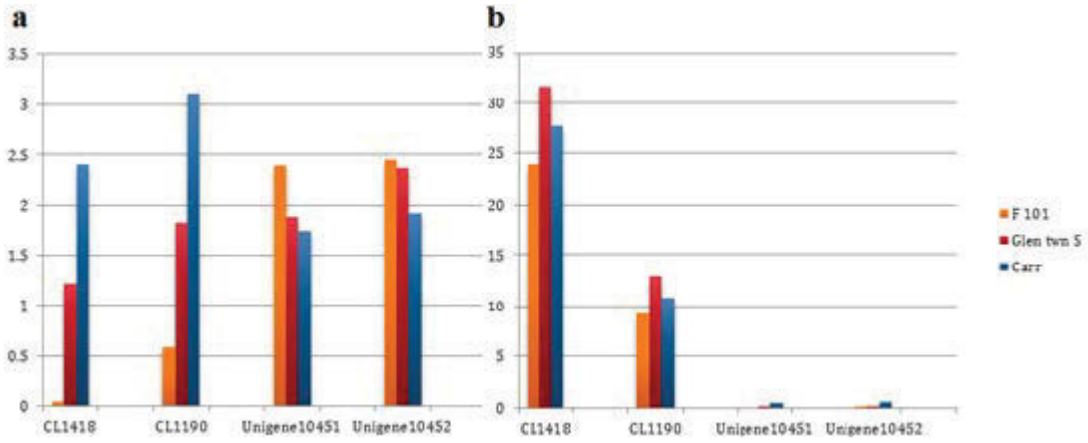

Figure 5.4 Validation of gene expression of four transcripts selected from RNA-Seq analysis. **(a)** The fold change (log_2Ratio) for genes from RNA-Seq analysis between strain comparisons: F 101/Sus F 96; Glen twn S/Sus F 96; and Carr/Sus F 96 **(b)** The relative expression of four transcripts between strain comparisons: F 101/Sus F 96; Glen twn S/Sus F 96; and Carr/Sus F 96, 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 characterised 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. 2007, 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 four differentially expressed transcripts (Contig ID 1190, 1418, 12511 and 12819) belonging to the CYP2 and CYP3 clans that were up-regulated in strains Carr, Glen twn S and F 101 when compared to Sus F 96. In this study, the role of contigs 1190 and 1418 were further evaluated. These transcripts were predicted as the P450 gene *CYP6K1*-like, and when blast searched against the NCBI database showed 68% and 82% similarity in amino acid sequence to *CYP6K1*-like 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. 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.

Several other genes with notable links to detoxification were also found up-regulated in resistant strains. In particular, a single gene encoding a GST (Contig ID 1795) belonging to the sigma class was found up-regulated in all resistant strains compared to the susceptible strain. GSTs are one of the major families of detoxifying enzymes that have frequently been associated with resistance to insecticides in a range of different arthropod species (Li et al. 2007). Elevated GST activity is well documented as a mechanism for resistance to DDT and organophosphate insecticides but to date has not yet been associated with resistance to neonicotinoid compounds (Enayati et al. 2005). Despite this, previous research has found up-regulation of a sigma-type GST may contribute to resistance to thiamethoxam. In *B. tabaci,* a single sigma-type GST gene was found over-expressed in all three life-stages of a thiamethoxam-selected strain (Yang et al. 2013). UDP-glucuronosyltransferases (UGTs) are another class of metabolizing enzymes that play an important role in phase II detoxification (Tephly and Burchell 1990). UGTs are responsible for the biological process of glucuronidation, whereby a glucuronic acid moiety is added to a xenobiotic substance to increase its water solubility, thus making it easier to excrete. In this study, three UGT transcripts (Contig ID 24, 1197 and 5652) were identified as up-regulated in resistant strains. Similarly, Yang et al. (2013) also found UGT transcripts were increased in response to thiamethoxam exposure in a strain of *B. tabaci*. Elsewhere, Højland and Kristensen (2017) found several UGTs which were up-regulated in a neonicotinoid resistant strain of *M. domestica*. Taken together, this evidence suggests that up-regulation of UGT transcripts may be involved in thiamethoxam resistance.

Among the 61 up-regulated genes found in the resistant strains, several corresponded to catalytic/oxidoreductase activity (such as proteins with choline or glucose dehydrogenase activity), suggesting a possible relationship between the insecticide resistance phenotype and these physiological processes (Contig ID 324, 934 and 13767). 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).

Another important group of DEGs included those genes coding for peptidases. Most notably, the aminopeptidase (Contig ID 5190) and cathepsin B (Contig ID 81) showed up-regulation in resistant strains when under thiamethoxam stress. This was consistent with the elevated proteolytic activities noted in insecticide resistant strains of *M. domestica* (Ahmed et al. 1998). Additionally, in insecticide resistant *Drosophila* (Pedra et al. 2004) and *A. gambiae* (Vontas et al. 2005) strains several genes belonging to the peptidase family were found over-expressed. It is uncertain what direct role peptidase activity may have in insecticide resistant strains, however, peptidases may be involved in protein degradation as a means to meet energy demands during stress (Pedra et al. 2004).

In insects, the majority of ABC genes encode ABC transporters which enable the transport of substrates across biological membranes by binding to and hydrolysing ATP (Dermauw and Van Leeuwen 2014). In particular, multidrug resistance-associated proteins (MRPs) are members of the MRP/ABCC subfamily of ABC transporters and have repeatedly been shown to confer resistance to xenobiotics, including insecticides (Labbe et al. 2011, Dermauw and Van Leeuwen 2014). Kang et al. (2016) found that exposure of *R. padi* to imidacloprid and chlorpyrifos increased the expression of MRP1 (encoded by the ABCC1 gene), indicating a role for ABCC1 in the efflux of insecticides in *R. padi*. In the present study, up-regulation of a single transcript (Contig ID 5321) that codes for a MRP and includes the ABC transporter cassette motif in its structures, is likely to contribute to thiamethoxam resistance in *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 UGTs, catalytic/oxidoreductase activity (such as proteins with choline or glucose dehydrogenase activity), ABC transporters and P450s might play crucial roles in conferring resistance to neonicotinoid compounds. To further strengthen the association of these genes with the resistant phenotypes observed, future work should seek to validate those DEGs using qRT-PCR. Among the DEGs, up-regulation of cytochrome P450 gene *6K1*-like and the role it plays in detoxifying thiamethoxam should be further investigated.

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. RRs 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 (Maas 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 (Maas 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 (Maas 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 β1 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, RRs 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 RRs 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*-like 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 hypothesised 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*-like, 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	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

Austral Entomology

Austral Entomology (2015) **54**, 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)

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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®.

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doi:10.1111/aen.12136

Appendix D. Supplementary material from Chapter 4

NEONICOTINOID RESISTANCE IN COTTON APHID FROM AUSTRALIA

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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 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

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.

Acknowledgements

Funding for this study is provided by Cotton Research and Development Corporation project DAN1201.

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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.

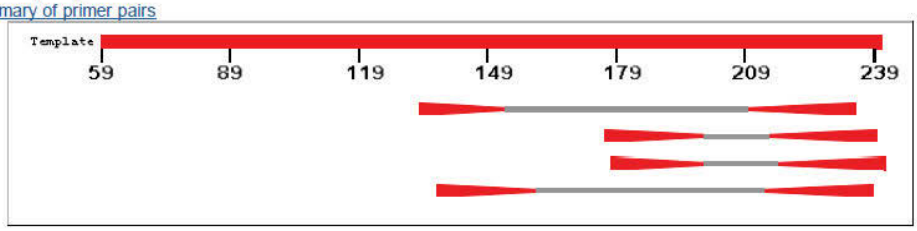
Other reports

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									

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

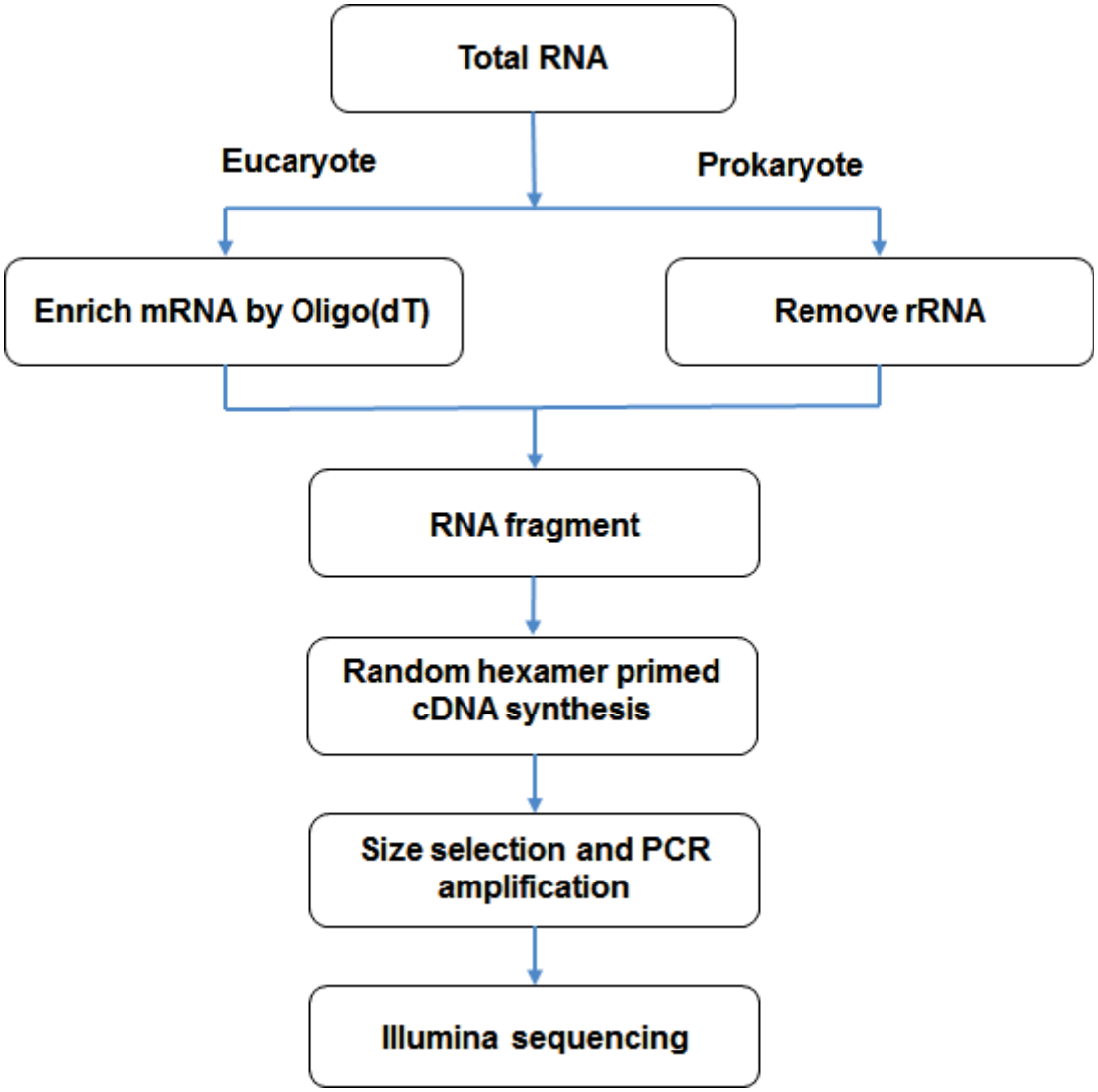


Figure E.1 Schematic diagram illustrating the experimental pipeline of transcriptome assembly used in this study.

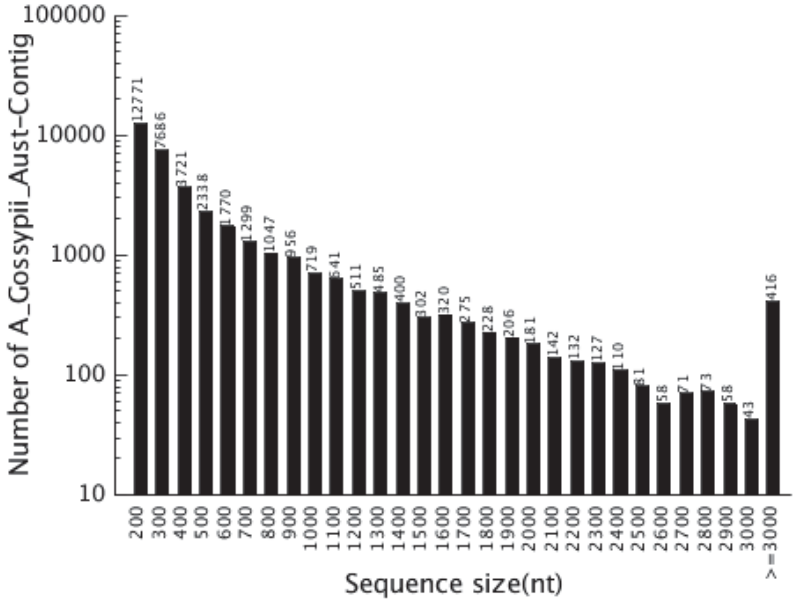


Figure E.2 Length distribution of contigs. 'A_Gossypii_Aust-Contig' indicates that the contigs were those assembled from reads from the pooled transcriptome of four *Aphis gossypii* strains [including the reference susceptible (Sus F 96) and thiamethoxam resistant (F 101, Glen twn S and Carr)].

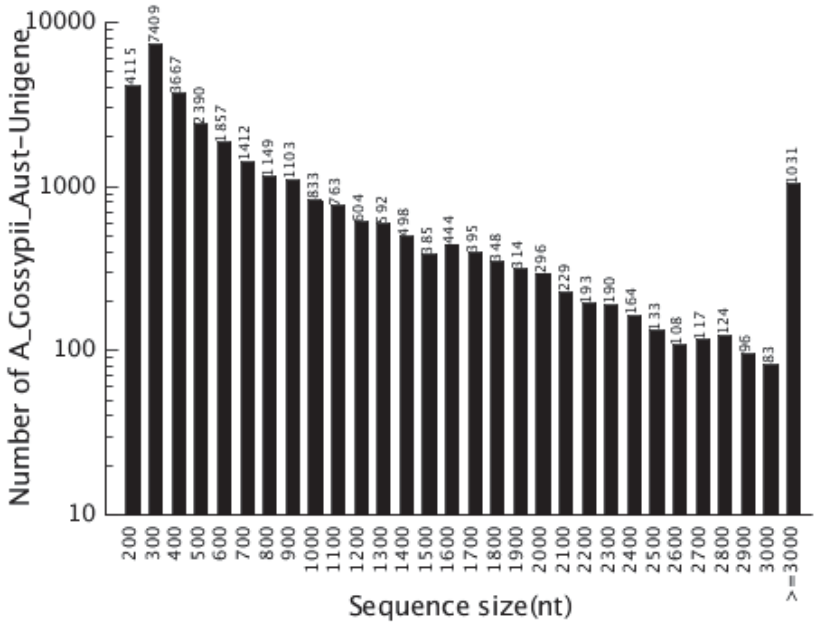


Figure E.3 Length distribution of unigenes. 'A_Gossypii_Aust_Unigene' indicates that the unigenes were those assembled from reads from the pooled transcriptomes of four *Aphis gossypii* strains [including the reference susceptible (Sus F 96) and thiamethoxam resistant (F 101, Glen twn S and Carr)].

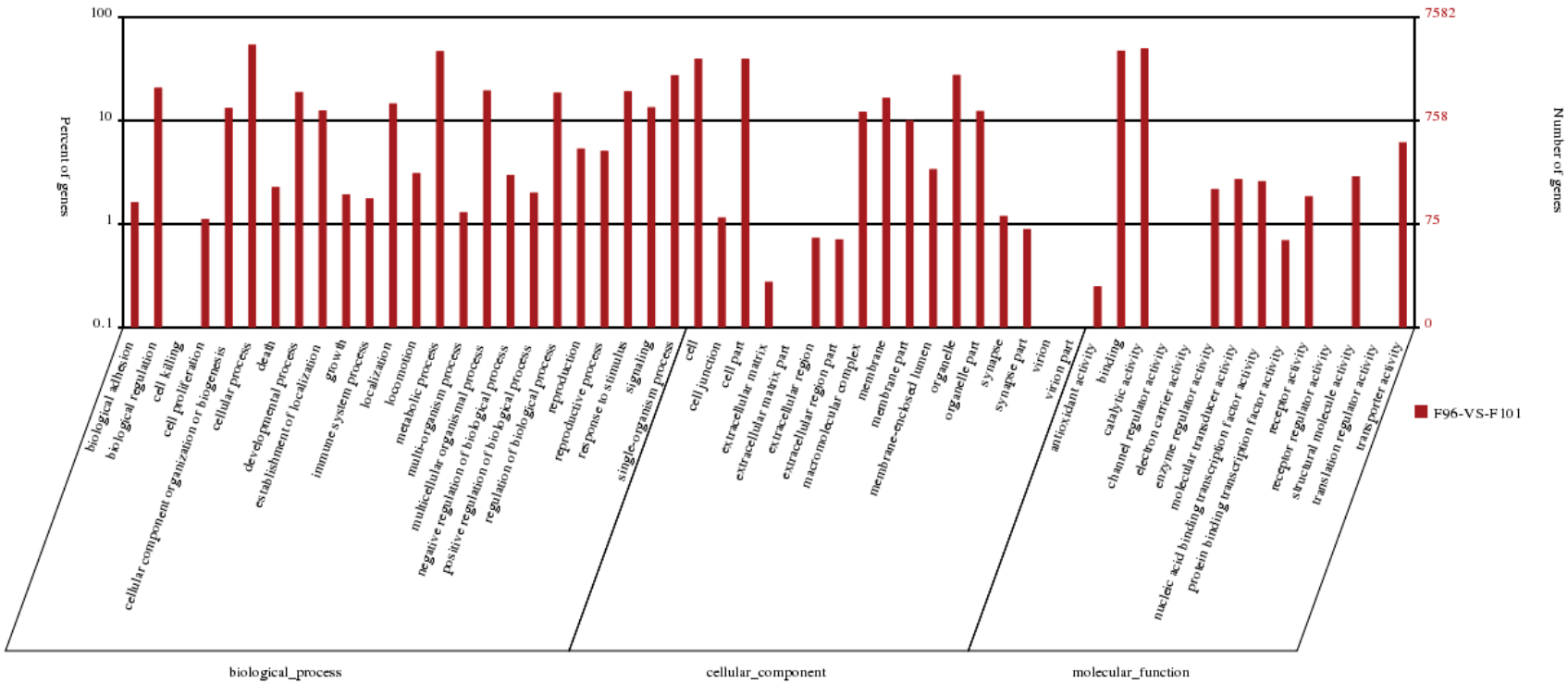


Figure E.4 Histogram presentation of the gene ontology classification**.** GO categories, shown in the x-axis, are grouped into three main ontologies: biological process, cellular component and molecular function. The right y-axis indicates the number of genes in each category, while the left y-axis 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.

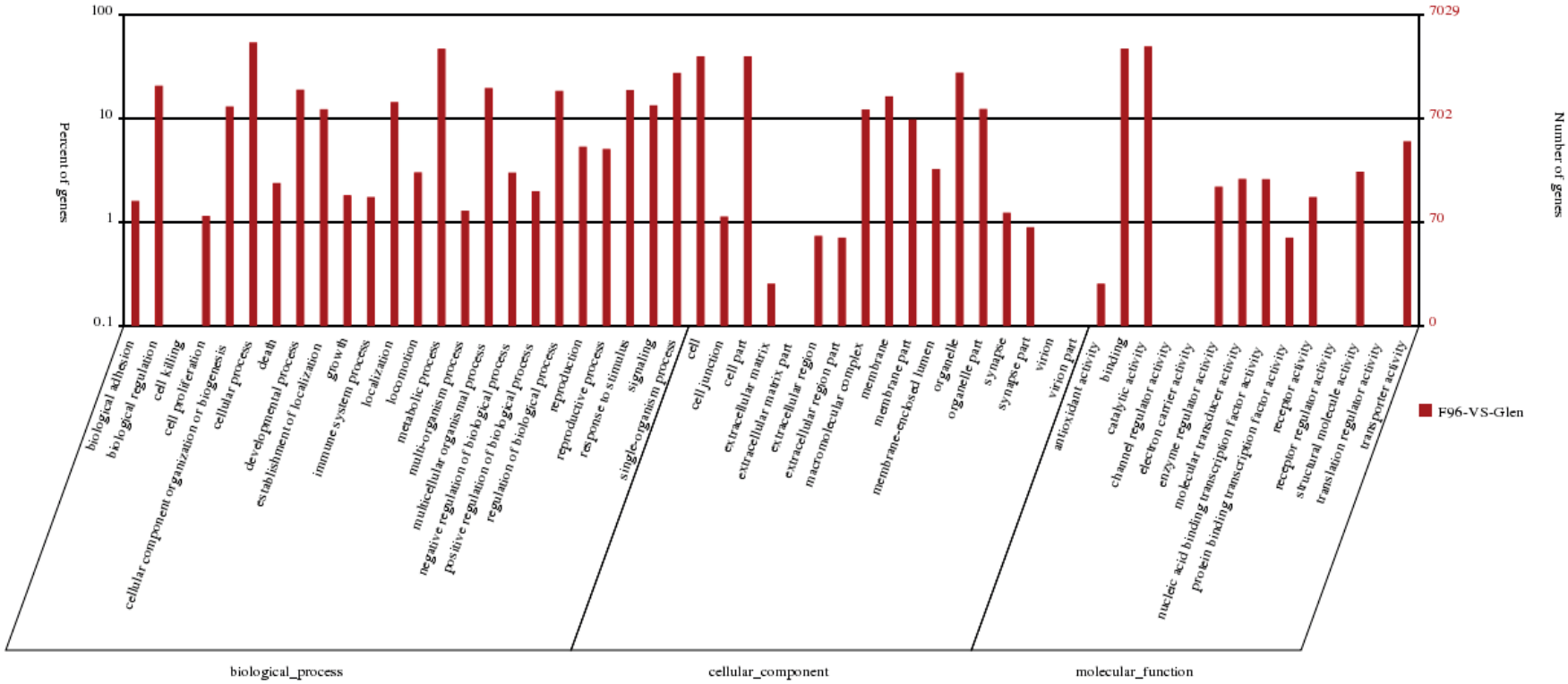


Figure E.5 Histogram presentation of the gene ontology classification. GO categories, shown in the x-axis, are grouped into three main ontologies: biological process, cellular component and molecular function. The right y-axis indicates the number of genes in each category, while the left y-axis indicates 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.

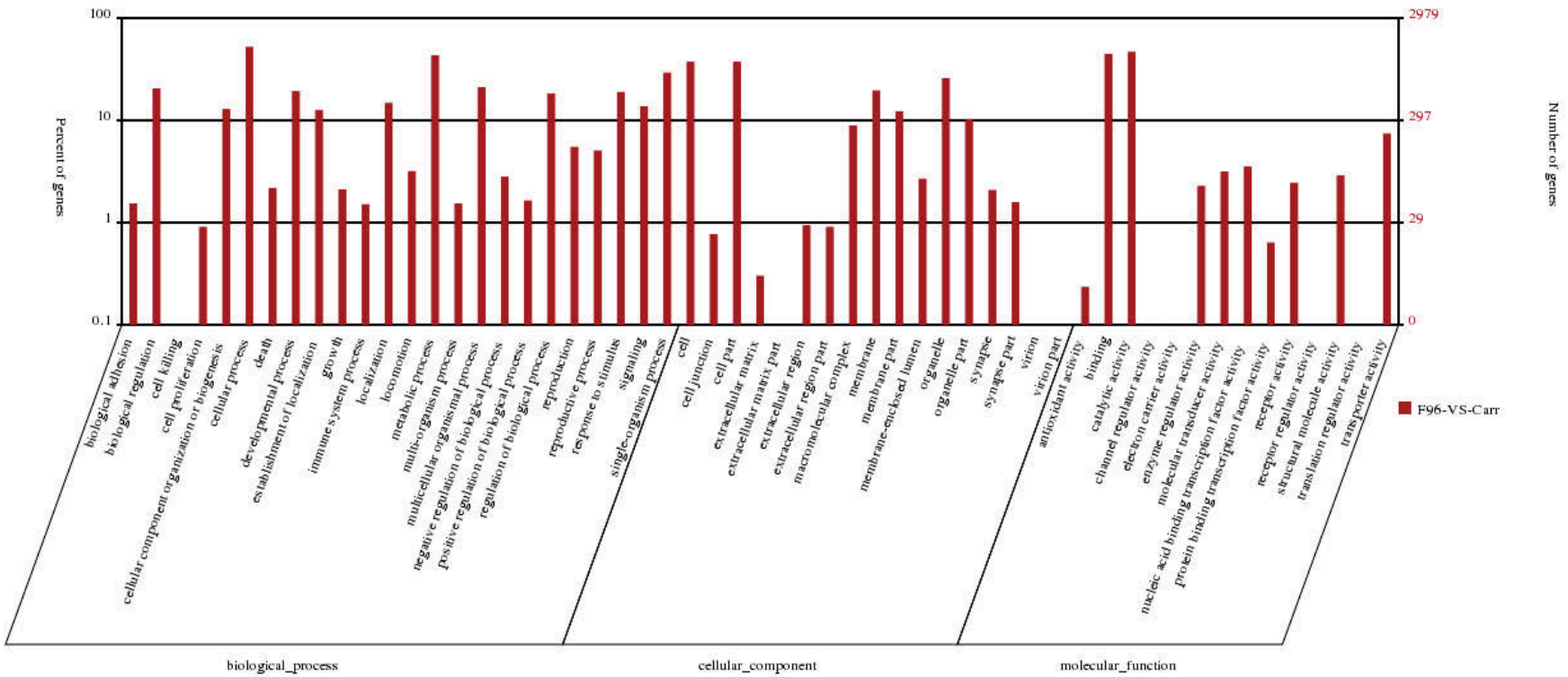


Figure E.6 Histogram presentation of the gene ontology classification. GO categories, shown in the x-axis, are grouped into three main ontologies: biological process, cellular component and molecular function. The right y-axis indicates the number of genes in each category, while the left y-axis indicates 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.

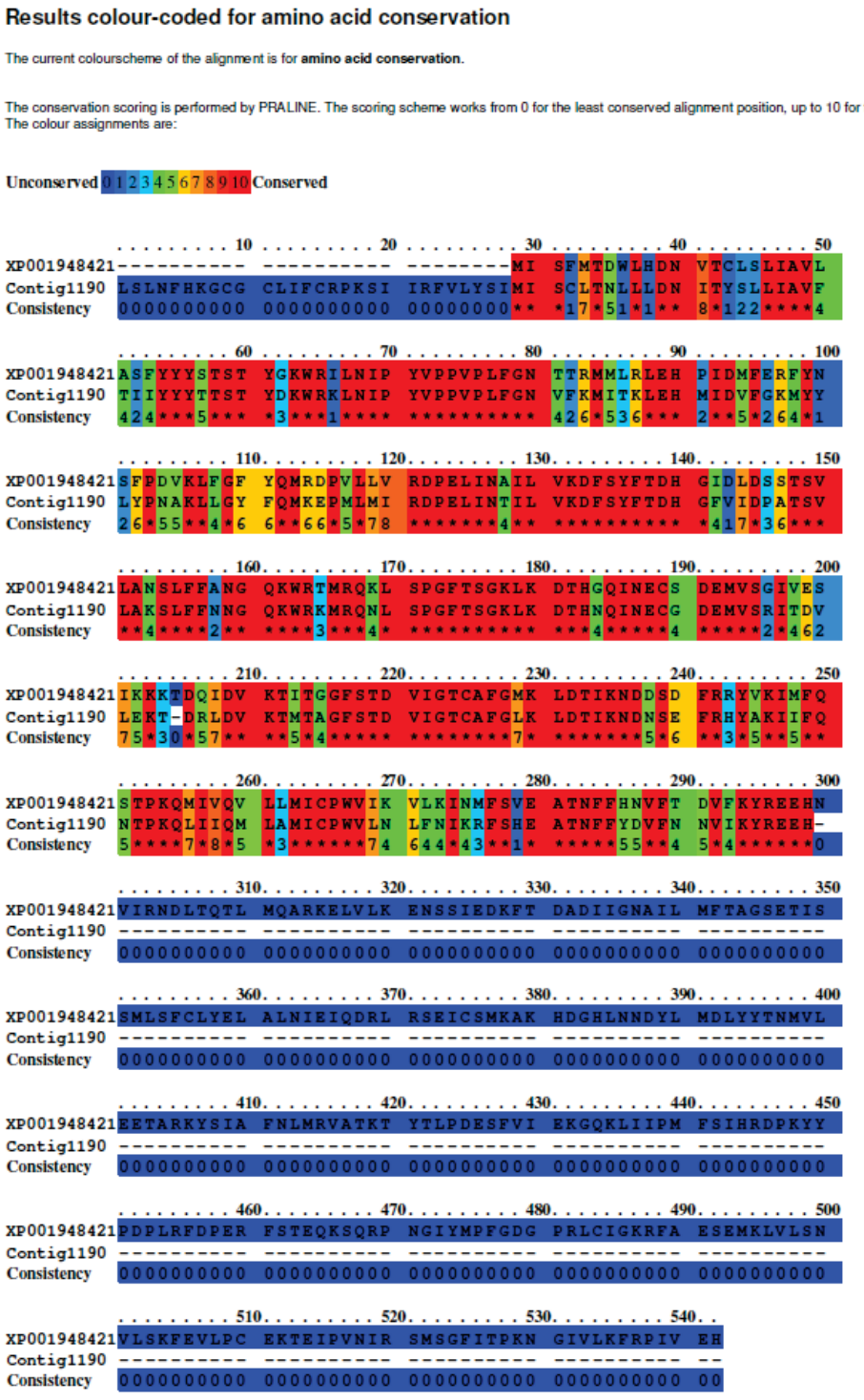


Figure E.7 PRALINE alignment of the predicted cytochrome P450 gene 6k1-like of the pea aphid *Acyrthosiphon pisum* (Accession number: XP001948421.1) and *Aphis gossypii* sequence Contig 1190 (firstly translated using ExPASy (Gasteiger et al. 2003)).

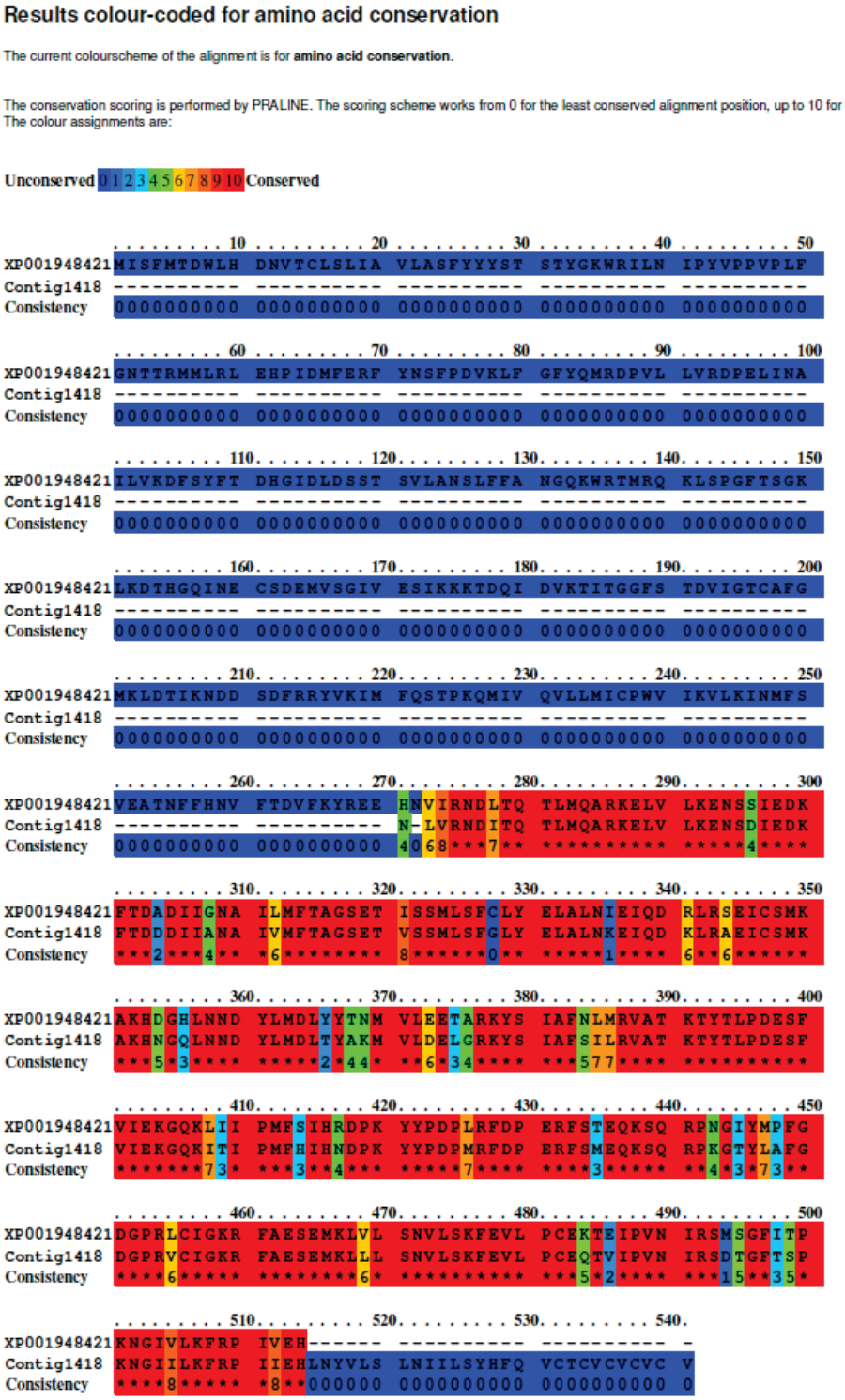


Figure E.8 PRALINE alignment of the predicted cytochrome P450 gene 6k1-like of the pea aphid *Acyrthosiphon pisum* (Accession number: XP001948421.1) and *Aphis gossypii* sequence Contig 1418 (firstly translated using ExPASy (Gasteiger et al. 2003)).

Table E.1 Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'.

#	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
24	Influenza A	283	ko05164
25	Dilated cardiomyopathy	278	ko05414

(Continued)

Table E.1 (cont'd) Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'.

#	Pathway	Count (15460)	Pathway ID
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
46	Tuberculosis	196	ko05152
47	ABC transporters	196	ko02010
48	Lysine degradation	195	ko00310
49	Alcoholism	193	ko05034

(Continued)

Table E.1 (cont'd) Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'.

#	Pathway	Count (15460)	Pathway ID
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
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

(Continued)

Table E.1 (cont'd) Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'.

#	Pathway	Count (15460)	Pathway ID
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
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

(Continued)

Table E.1 (cont'd) Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'.

#	Pathway	Count (15460)	Pathway ID
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
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

(Continued)

Table E.1 (cont'd) Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'.

#	Pathway	Count (15460)	Pathway ID
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
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

(Continued)

Table E.1 (cont'd) Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'.

#	Pathway	Count (15460)	Pathway ID
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
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

(Continued)

Table E.1 (cont'd) Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'.

#	Pathway	Count (15460)	Pathway ID
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
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

(Continued)

Table E.1 (cont'd) Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'.

#	Pathway	Count (15460)	Pathway ID
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
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

(Continued)

Table E.1 (cont'd) Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'.

#	Pathway	Count (15460)	Pathway ID
218	Taste transduction	31	ko04742
219	Histidine metabolism	30	ko00340
220	RIG-I-like receptor signaling pathway	30	ko04622
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

(Continued)

Table E.1 (cont'd) Summary of KEGG pathway mapping of 'A_Gossypii_Aust-Unigene'.

#	Pathway	Count (15460)	Pathway ID
242	Glycosphingolipid biosynthesis - globo series	11	ko00603
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 reference susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, $\log_2$ FC, p-value and orthologue gene name in the appropriate organism as retrieved from GenBank (Blast nr) are indicated. False discovery rate≤0.05 and p-value≤0.001 were thresholds for determining the significance of gene expression differences.

GeneID	$\log_2$ FC (Res/Sus)	p-value	Blast nr
CL324.Contig1	3.0919	< 1e-4	XP_001949949.2|PREDICTED: glucose dehydrogenase [acceptor]-like [Acyrthosiphon pisum]
CL1926.Contig2	2.6201	< 1e-4	XP_001951035.1|PREDICTED: hypothetical protein LOC100168536 [Acyrthosiphon pisum]
CL1190.Contig1	2.6744	< 1e-4	XP_001948421.1|PREDICTED: cytochrome P450 6k1-like [Acyrthosiphon pisum]
Unigene3424	2.1305	< 1e-4	XP_001947588.2|PREDICTED: protein SGT1 homolog ecdysoneless-like [Acyrthosiphon pisum]
CL81.Contig1	2.5187	< 1e-4	cathepsin B [EC:3.4.22.1]
CL1418.Contig1	2.3481	< 1e-4	XP_001948421.1|PREDICTED: cytochrome P450 6k1-like [Acyrthosiphon pisum]
Unigene6303	2.8181	< 1e-4	-
CL24.Contig4	1.8995	< 1e-4	glucuronosyltransferase [EC:2.4.1.17]
Unigene11676	1.8118	< 1e-4	XP_003243439.1|PREDICTED: RNA-directed DNA polymerase mobile element jockey-like [Acyrthosiphon pisum]
Unigene10452	2.2981	< 1e-4	ACY69873.1|/5.38887e-37/polyprotein-like protein [Glossina morsitans morsitans]

(Continued)

Table E.2 (cont'd) Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and reference susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, $\log_2$ FC, p-value and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.05 and p-value≤0.001 were thresholds for determining the significance of gene expression differences.

GeneID	$\log_2$ FC (Res/Sus)	p-value	Blast nr
Unigene3425	1.7741	< 1e-4	GO:0048598//embryonic morphogenesis;GO:0048513//organ development;GO:0009791//post-embryonic
Unigene163	1.2921	< 1e-4	XP_003246800.1|PREDICTED: hypothetical protein LOC100575145 [Acyrthosiphon pisum]
CL934.Contig1	1.5169	< 1e-4	aldehyde dehydrogenase (NAD+) [EC:1.2.1.3]
CL1025.Contig2	1.6128	< 1e-4	alkaline phosphatase [EC:3.1.3.1]
Unigene7812	-1.32	< 1e-4	cytochrome c oxidase subunit 2
Unigene13767	1.9111	< 1e-4	dehydrogenase/reductase SDR family member 4 [EC:1.1.-.-]
CL1435.Contig1	1.2254	< 1e-4	snRNA-activating protein complex subunit 3
Unigene6512	1.7579	< 1e-4	-
Unigene19346	1.7952	< 1e-4	-
Unigene8199	-1.6515	< 1e-4	-
Unigene12225	1.5772	< 1e-4	-

(Continued)

Table E.2 (cont'd) Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and reference susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, $\log_2$ FC, p-value and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.05 and p-value≤0.001 were thresholds for determining the significance of gene expression differences.

GeneID	$\log_2$ FC (Res/Sus)	p-value	Blast nr
CL1523.Contig1	1.2539	< 1e-4	elongator complex protein 3 [EC:2.3.1.48]
CL22.Contig1	1.5718	< 1e-4	XP_001949714.2|PREDICTED: major facilitator domain-containing protein 6-like isoform 1 [Acyrthosiphon pisum]
CL1660.Contig2	1.4909	< 1e-4	XP_001945297.1|PREDICTED: hypothetical protein LOC100160101 [Acyrthosiphon pisum]
Unigene13130	1.6892	< 1e-4	corticosteroid 11-beta-dehydrogenase isozyme 1 [EC:1.1.1.146]
Unigene12819	1.6341	< 1e-4	XP_001948934.1|PREDICTED: probable cytochrome P450 6a13-like [Acyrthosiphon pisum]
CL1361.Contig3	1.6834	< 1e-4	deoxynucleotidyltransferase terminal-interacting protein 1
CL1197.Contig2	1.3474	< 1e-4	glucuronosyltransferase [EC:2.4.1.17]
Unigene18414	-1.4014	< 1e-4	-
CL1702.Contig2	1.5681	< 1e-4	XP_003247455.1|PREDICTED: hypothetical protein LOC100573430 isoform 1 [Acyrthosiphon pisum]
Unigene7568	1.569	< 1e-4	-
Unigene4505	1.2077	< 1e-4	XP_001948324.2|PREDICTED: hypothetical protein LOC100163944 [Acyrthosiphon pisum]

(Continued)

Table E.2 (cont'd) Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and reference susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, log_2 FC, p-value and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.05 and p-value≤0.001 were thresholds for determining the significance of gene expression differences.

GeneID	log_2 FC (Res/Sus)	p-value	Blast nr
Unigene9859	1.0314	< 1e-4	arginine vasopressin receptor 2
CL2071.Contig2	1.4226	< 1e-4	XP_001947484.2|PREDICTED: probable maltase L-like [Acyrthosiphon pisum]
Unigene5652	1.1551	< 1e-4	XP_001943837.2|PREDICTED: UDP-glucuronosyltransferase 2B15-like [Acyrthosiphon pisum]
Unigene9107	1.1628	< 1e-4	XP_001950950.1|PREDICTED: pancreatic lipase-related protein 2-like [Acyrthosiphon pisum]
Unigene8227	1.3104	< 1e-4	XP_001945911.1|PREDICTED: hypothetical protein LOC100162262 [Acyrthosiphon pisum]
Unigene14320	1.5625	< 1e-4	-
Unigene16132	-1.4009	1.00E-04	XP_003241320.1|PREDICTED: hypothetical protein LOC100571804 [Acyrthosiphon pisum]
Unigene8132	-1.1776	1.00E-04	XP_003240836.1|PREDICTED: elongation of very long chain fatty acids protein 4-like [Acyrthosiphon pisum]
Unigene11922	-1.1796	1.00E-04	NP_001191950.1|transmembrane protein 35 [Acyrthosiphon pisum]
Unigene7591	1.3317	1.00E-04	NP_001232968.1|uncharacterised protein LOC100570671 precursor [Acyrthosiphon pisum]
Unigene11621	-1.3468	2.00E-04	-

(Continued)

Table E.2 (cont'd) Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and reference susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, log_2 FC, p-value and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.05 and p-value≤0.001 were thresholds for determining the significance of gene expression differences.

GeneID	log_2 FC (Res/Sus)	p-value	Blast nr
Unigene9764	1.2268	2.00E-04	NP_001155418.1|cuticular protein-like precursor [Acyrthosiphon pisum]
Unigene14043	1.4451	2.00E-04	-
CL1764.Contig2	-1.1083	2.00E-04	XP_001952605.1|PREDICTED: hypothetical protein LOC100164709 [Acyrthosiphon pisum]
Unigene9164	0.8839	3.00E-04	XP_003242983.1|PREDICTED: hypothetical protein LOC100570813 [Acyrthosiphon pisum]
CL1795.Contig2	0.8942	3.00E-04	AFM78642.1|glutathione S-transferase sigma 1 [Aphis gossypii]
Unigene10626	-0.9855	3.00E-04	-
CL1136.Contig2	1.3622	3.00E-04	XP_003246965.1|PREDICTED: protein catecholamines up-like isoform 2 [Acyrthosiphon pisum]
Unigene12381	1.1084	3.00E-04	XP_001947639.1|PREDICTED: solute carrier family 23 member 2-like isoform 1 [Acyrthosiphon pisum]
Unigene16958	1.4091	3.00E-04	-
Unigene12300	1.2613	3.00E-04	NP_001155594.1|uncharacterised protein LOC100163907 precursor [Acyrthosiphon pisum]
Unigene11277	1.3611	4.00E-04	XP_003246343.1|PREDICTED: hypothetical protein LOC100573276 [Acyrthosiphon pisum]

(Continued)

Table E.2 (cont'd) Top differentially expressed genes (DEGs) among thiamethoxam resistant (F 101, Glen twn S and Carr) and reference susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, $\log_2$ FC, p-value and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.05 and p-value≤0.001 were thresholds for determining the significance of gene expression differences.

GeneID	$\log_2$ FC (Res/Sus)	p-value	Blast nr
Unigene7686	1.2765	4.00E-04	-
Unigene1323	-1.1049	4.00E-04	BAC06460.1|reverse transcriptase [Papilio xuthus]
Unigene5321	1.0388	4.00E-04	XP_001948661.2|PREDICTED: multidrug resistance-associated protein 4-like [Acyrthosiphon pisum]
Unigene10950	-1.168	5.00E-04	XP_003390397.1|PREDICTED: ribosome maturation factor rimM-like [Amphimedon queenslandica]
Unigene10587	-0.8959	5.00E-04	-
Unigene8260	-1.034	5.00E-04	-
Unigene7712	1.389	5.00E-04	-
Unigene5775	-1.0128	5.00E-04	XP_001944317.1|PREDICTED: hypothetical protein LOC100166606 [Acyrthosiphon pisum]
Unigene11370	1.3438	5.00E-04	-
CL218.Contig3	-1.1885	5.00E-04	CBY13234.1|unnamed protein product [Oikopleura dioica]
Unigene11782	1.1022	5.00E-04	XP_001950274.1|PREDICTED: receptor expression-enhancing protein 4-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 reference susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, $\log_2$ FC, p-value and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.05 and p-value≤0.001 were thresholds for determining the significance of gene expression differences.

GeneID	$\log_2$ FC (Res/Sus)	p-value	Blast nr
CL1926.Contig1	1.3544	6.00E-04	XP_001951035.1|PREDICTED: hypothetical protein LOC100168536 [Acyrthosiphon pisum]
Unigene2702	0.8908	6.00E-04	XP_003240219.1|PREDICTED: protein sel-1 homolog 1-like isoform 2 [Acyrthosiphon pisum]
Unigene11373	1.0883	6.00E-04	NP_001156185.1|uncharacterised protein LOC100164299 [Acyrthosiphon pisum]
Unigene7326	1.2306	7.00E-04	XP_003247881.1|PREDICTED: putative ankyrin repeat protein L25-like [Acyrthosiphon pisum]
Unigene21384	-1.0895	7.00E-04	XP_002119208.1|PREDICTED: similar to Uncharacterised protein K02A2.6 [Ciona intestinalis]
Unigene2864	1.0142	8.00E-04	XP_001948937.1|PREDICTED: endoplasmin-like [Acyrthosiphon pisum]
Unigene19062	-1.2568	8.00E-04	-
Unigene1524	-1.3368	8.00E-04	XP_003242262.1|PREDICTED: hypothetical protein LOC100570092 [Acyrthosiphon pisum]
Unigene8930	-1.1431	8.00E-04	XP_001944456.2|PREDICTED: nose resistant to fluoxetine protein 6-like [Acyrthosiphon pisum]
CL739.Contig2	1.2848	9.00E-04	XP_003244699.1|PREDICTED: fructose-1,6-bisphosphatase 1-like isoform 2 [Acyrthosiphon pisum]
Unigene12106	-1.1325	9.00E-04	XP_003493086.1|PREDICTED: alanyl-tRNA synthetase-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 reference susceptible (Sus F 96) *Aphis gossypii* strains. Transcript ID, $\log_2$ FC, p-value and orthologue gene name in the appropriate organism as retrieved from the GenBank are indicated (Blast nr). False discovery rate≤0.05 and p-value≤0.001 were thresholds for determining the significance of gene expression differences.

GeneID	$\log_2$ FC (Res/Sus)	p-value	Blast nr
Unigene4508	0.9361	9.00E-04	XP_001949024.1|PREDICTED: dnaJ homolog subfamily C member 3-like [Acyrthosiphon pisum]
CL1547.Contig1	-1.0517	9.00E-04	-
CL1248.Contig1	1.1502	9.00E-04	XP_001949294.2|PREDICTED: ring canal kelch homolog [Acyrthosiphon pisum]
Unigene12511	1.3063	0.001	XP_001948934.1|PREDICTED: probable cytochrome P450 6a13-like [Acyrthosiphon pisum]
Unigene5190	0.8876	0.001	AEV66509.1|aminopeptidase N 1 [Aphis glycines]

Table E.3 Four differentially expressed transcripts by 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	qRT-PCR fold[a]		
		F 101	Glen twn S	Carr
CL1418	cytochrome P450 6k1-like [Acyrthosiphon pisum]	23.85518555	31.61857289	27.82101852
CL1190	cytochrome P450 6k1-like [Acyrthosiphon pisum]	9.268142804	12.79010477	10.92957044
Unigene10451	Rhopalosiphum padi virus clone RhPV6	-	0.085260928	0.513416077
Unigene10452	Rhopalosiphum padi virus clone RhPV6	0.168513252	0.13612662	0.639917571

[a]Delta Delta C(T) method (2−ΔΔCT) was used for analysis of relative expression.

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