Insights into the defence of honey bees, *Apis mellifera* L., against insecticides

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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April 2015

DECLARATION

This work has not previously been accepted in substance for any degree and is not being currently submitted in candidature for any degree.

Signed .....(Candidate)

Date .....

Statement 1

This thesis is the result if my own investigation, except where otherwise stated. Other sources are acknowledged and bibliography is appended.

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Date .....

DEDICATION

This thesis heartily dedicated to my mom, dad, sister and husband for their constant support and unconditional love.

I love you all.

ABSTRACT

There are some contradictory theories on how tolerant honey bees are of pesticides. Since the honey bee genome has been published (Honey bee Genome Sequencing Consortium, 2006), more is known about their metabolic systems, especially the detoxification pathways for potential xenobiotics. Bioassay and biochemical data from various studies have shown that both P450s and carboxylesterases are responsible for pesticide metabolism in honey bees. Here, those metabolic enzymes that confer primary defence to different classes of insecticides (mainly neonicotinoid, thiacloprid) in honey bee were validated. Metabolic enzymes are characterised regarding their ability to interact with the insecticide. Synergist bioassay results with PBO and EN 16/5-1 suggest that detoxification mechanism(s) play an important role in protecting honey bees from tau-fluvalinate and thiacloprid toxicity. No binding was found between honey bee esterases and tested insecticides (thiacloprid and tau-fluvalinate), whilst inhibition of P450 activity sensitised the honey bees to these chemicals. Metabolism of tau-fluvalinate and thiacloprid in honey bees is due to P450 activity, but this metabolism may not be the only reason for the relatively benign action of this insecticide on bees. Honey bees are less sensitive to neonicotinoids containing a cyanoimino pharmacophore than to those with a nitroimino group, however the specific enzymes involved in detoxification remain to be characterised. In this work, pre-treatment of honey bees with a sub-lethal dose of thiacloprid induced protection to the same compound immediately following thiacloprid feeding. Transcriptome profiling, using microarrays, identified a number of genes encoding detoxification enzymes that were overexpressed significantly in insecticide-treated bees compared to untreated controls. These included five P450s, CYP6BE1, CYP305D1, CYP6AS5, CYP301A1, CYP315A1 and an esterase CCE8.

The four P450s and cytochrome b5 were functionally expressed in *E. coli* and their ability to metabolise thiacloprid examined by LC-MS analysis. There was no obvious metabolism of thiacloprid, thus their role in the metabolism and disposition of thiacloprid is still unclear.

Race-based and caste-based bioassay studies were carried out to investigate the differential sensitivity. Honey bee caste-based synergism studies revealed that nurse bees (younger) may tolerate thiacloprid toxicity more than forager bees (older) by means of esterase-based metabolism/sequestration. In addition to the metabolic differences, race-based studies also suggested that target-site insensitivity of nAChRs may enhance thiacloprid tolerance in honey bees. However, screening of nAChRs of *A. m. caucasica* and *A. m. buckfast* did not identify polymorphism variants except several splice variants of subunits. As the integrity of the sample material was compromised this mechanism has not been confirmed.

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LIST OF ABBREVIATIONS

7-EC	7-ethoxycoumarin
ACh	acetylcholine
AChE	acetylcholinesterase
ALA	5-Aminolevulinic acid
ala-ala-ala-pNA	ala-ala-ala para-nitrophenylacetate
bp	base pair
BSA	bovine serum albumin
cDNA	complimentary DNA
CE	carboxylesterase
CHAPS	3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate
CPR	cytochrome P450 reductase
cRNA	complimentary RNA
C_ts	threshold cycle
CYP	cytochrome P450 protein
Cyt *b*5	cytochrome *b*5
dATP	deoxyadenosine triphosphate
DEF	S,S,S,-tributylphosphophorotrithioate
Defra	Department for Environment, Food and Rural Affairs
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
EST	expressed sequence tag
GABA	gamma-aminobutyric acid
gDNA	genomic DNA
GO	gene ontology
GPCR	G-protein-coupled receptor
GST	glutathione-*S* transferase
HPLC	High Performance Liquid Chromatography
IC_{50}	concentration that confers 50% inhibition

IPTG	isopropy-1-thio-beta-D-galactopyranoside
IRAC	Insecticide Resistance Action Committee
LB	Luria Bertani
LC-MS/MS	liquid chromatography tandem mass spectrometry
LD_5	lethal dose to kill 5% of population
LD_{25}	lethal dose to kill 25% of population
LD_{50}	lethal dose to kill 50% of population
MDP	methylenedioxyphenyl
mOD	mili optical density
mRNA	messenger RNA
nAChR	nicotinic acetylcholine receptor
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
ºC	degree celcius
OD	optical density
P450	cytochrome P450
PBO	piperonyl butoxide
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PTU	phenylthiourea
qRT-PCR	Quantitative real time PCR
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecylsulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SOC	super optimal culture
SRM	selected-reaction-monitoring
TAE	Tris-acetate EDTA electrophoresis buffer
TB	Terrific Broth
TBE	Tris borate EDTA electrophoresis buffer
TMBZ	3,3′,5,5′-tetramethylbenzidine
TSE	Tris-acetate sucrose EDTA buffer
U	units
UV	ultraviolet
V	volts

v/v	volume per volume
v:v	ratio volume to volume
w/v	weight per volume
WHO	World Health Organisation
x *g*	relative centrifugal force to gravity
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
β-ME	β-Mercaptoethanol

1. GENERAL INTRODUCTION

1.1 Honey Bees

Honey bees belong to the family Apidae in the order Hymenoptera within the class Insecta. They are represented in the genus *Apis*. According to Ruttner (1988) four species of honey bees are recognised; *Apis cerana*, *Apis dorsata*, *Apis florea*, and *Apis mellifera*. However, recent studies increased Ruttner's list by adding new species: *Apis andreniformis*, *Apis koschevnikovi*, *Apis laboriosa*, *Apis nigrocincta*, *Apis nuluensis*, *Apis binghami* and *Apis breviligula* (Michener, 2000).

This study focuses exclusively on *Apis mellifera* which shows wide geographical distribution being found in Europe, the Middle East, Asia, and Africa. This wide distribution has resulted in the classification of four different lineages based on morphological and genetic properties, M (West and North Europe), C (South and East Europe), O (East and Central Asia) and A (Africa) (Garnery *et al*., 1993; Arias and Sheppard, 1996; Whitfield *et al*., 2006).

Among these races, 10 originate in Europe. The present study was carried out utilising 4 European races *A. mellifera carnica, A. mellifera caucasica, A. mellifera ligustica* and *A. mellifera buckfast*.

1.1.1 Biology and life cycle of honey bee

Apis mellifera lives in large social clusters called "colonies" which consists of three different castes with a designated specific task for each: a queen bee (fertile female), workers (sterile females) and drones (males).

The life cycle in the colony begins with the eggs laid by the queen. During mating activity, the queen bee gathers sperm cells from drones (whose only task is to mate with the queen in the colony), she then manages colony development through fertilization control by transferring or not transferring sperm to eggs. This reproductive strategy called haplodiploidy results in haploid males from unfertilized eggs while diploid females are produced from fertilized eggs (Charlesworth, 2003; Heimpel and de Boer, 2008). Emergence of a new adult from the laid egg takes around 21 days: 0-3 days for the egg stage, 4-10 days for the larval stage and 11 – up to 24 days for the pupal stage. The length of the pupal stage differs for each cast. While development of the queen from egg to its emergence as an adult is completed within 16 days, workers require 21 days and drones 24 days to emerge as an adult (Figure 1.1). The reason for this difference is linked to the final morphological development of the adult in sealed cells (pupal stage).

Worker bees are the main task force of the colony. The responsibility of the worker is defined by age. The young workers (from adult emergence, adult day 1 to adult day 21), so called “nurse bees” are responsible for cleaning the hive, feeding the larvae, comb building, and similar ‘in hive’ tasks. Older workers, so called “forager bees” (from adult day 22 to adult day 42) are responsible for nectar collecting, orientation flights, hive ventilation and guarding the colony (Figure 1.2) (Winston, 1987).

This text box is where the unedited thesis included the following third party copyrighted material:

Meyer, M. (1996-2015) The Pollinator Partnership. Online source: http://pollinator.org/beeissues.htm

Figure 1.1 Illustration of honey bee larval and adult life cycle (by Marguerite Meyer).

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Waller, G.D. (1980) 'Honey Bee Life History'. Beekeeping in the United States Agriculture Handbook 335: 24 – 29

Figure 1.2 Activity chart of a honey bee through the life cycle (adapted from Waller, 1980).

1.1.2 Economic importance of honey bees and concerns on negative impacts of pesticides

Honey bees are commercially important beneficial organisms worldwide. Their pollination service has been estimated to be worth £120-200 million and their honey production contributes £10-30 million annually to the UK economy (Defra, 2008). Their contribution to human diet and human health are also very important. For example; laboratory and clinical studies have demonstrated that the antibacterial property of honey could be used as a treatment to control bacterial growth in human health problems (Jeffrey and Echazarreta, 1996).

Food demand increases in parallel with rising global population. In this case pollinators, especially honey bees, have an economically and agriculturally important role in crop production as well as a positive effect on biological diversity (Herrera and Pellmyr, 2002). For example, today one third of global food crops are pollinator-dependent, essentially honey bee pollination service-dependent (e.g. berries, nuts, plums, cherries, kiwi, melon) (Klein *et al*., 2007; Aizen *et al*., 2008). However, a variety of factors represent significant threats to apiculture including disease, parasites and unintended insecticide exposure (Southwick and Southwick, 1992). Chemical control is currently an indispensable input for global agriculture but pesticides are suspected by many to be involved in the disappearance of honey bees since the first report of colony collapse disorder in 2006 (Le Conte *et al*., 2010).

The current concern regarding the adverse effects of neonicotinoids on honey bee health is resulting in many studies that investigate the effects of this class of compounds on economically important pollinators (Godfray *et al*., 2014). Due to their positive impacts in crop protection including low toxicity to mammals, birds

and fish, systemic control of plant-sucking insect pests (aphids, whiteflies, thrips, some coleopteran and lepidopteran) and selective toxicity, the neonicotinoids have rapidly become the preferred new generation of synthetic insecticides (Tomizawa and Casida, 2003). It is therefore vital to understand the effects of neonicotinoids on honey bees to gain advantages from these innovative chemicals with regards to controlling pest species safely and effectively (Atkins, 1992). There are various methods to apply neonicotinoids associated with multiple agricultural purposes. Seed treatment and soil applications are mostly preferred because they protect young plants and are long-lasting due to their systemic effect (Elbert *et al*., 2008). Multiple routes of pesticide exposure are therefore possible for honey bees including both contact (visiting the plants during foraging activity) and oral routes (consuming the infected food source) (Krupke *et al*., 2012). There is evidence that sublethal exposure of neonicotinoids may cause disorientation in foraging activity and impairment on olfactory memory and learning activity (Henry *et al*., 2012; Yang *et al*., 2008; Decourtye *et al*., 2005). However, the question of whether the sublethal doses, received by pollinators in the field, leads to significant impairment in individual and colony performance is a topic of active research and considerable controversy (Godfray *et al*., 2014).

1.2 Pyrethroids

Pyrethroids are synthetic insecticides which have been designed by replicating the structure of the naturally occurring insecticide compounds, pyrethrins. Pyrethrins are neurotoxicant compounds that occurred naturally in the metabolism of the chrysanthemum plants (pyrethrum daises; *Chrysanthemum spp*.) (Elliott, 1995). They affect the insects' nerve system by acting on insect sodium channels present in nerve membrane, by altering the "open and close" sequence/rhythm and prolonging the

open phase, which results in paralysis and death due to extended stimulation (Soderlund and Bloomquist, 1989; Naumann, 1990; Soderlund *et al*., 2002). In comparison with pyrethrins pyrethroids are both more stable and more active against a range of insect species and also have other desirable properties such as relatively low mammalian toxicity, rapid and broad-spectrum activity, photo-stability and low bioaccumulation potential. As a result this class of insecticide has enjoyed increasing market share since its commercial development in 1960s (Casida *et al*., 1983; Casida and Quistad, 1998). In this study, two of the members of the pyrtehroid class of insecticides, tau-fluvalinate and alpha cypermethrin, have been used.

Tau-fluvalinate is a pyrethroid insecticide and acaricide used against several pest species on fruits, vegetables, ornamentals and many other crops. In relation to this PhD study it has also been used to control the honey bee parasitic mite *Varroa destructor* as a hive acaricide due to its relatively low toxicity to honey bees (Roberts and Hutson, 1999; Johnson *et al*., 2006).

Alpha cypermethrin is a non-systemic pyrethroid insecticide used against several pest species especially active on Lepidoptera family members on wide range of agricultural crops. Additionally, it is also used for vector control in public and animal health (Roberts and Hutson, 1999).

1.3 Neonicotinoids

Neonicotinoids are synthetic neurotoxic insecticides which have structural similarities and a similar mode of action, with naturally occurring nicotine (and nicotinoids, synthetic insecticides modelled against nicotine with an improved

insecticidal activity) but are both more potent and safe (Tomizawa and Casida, 2003).

Neonicotinoids are systemic compounds that once absorbed to the plant are, translocated through the vascular tissues to roots, leaves, flowers and stem, which makes them excellent in sucking-pests control. They affect the central nervous system of insects, by binding agonistically to the nAChR, and results in persistent excitation and ultimately death (Jeschke and Nauen, 2008). Different to nicotinoids which are mostly protonated and bind to an anionic subsite of the mammalian nAChR, neonicotinoids are negatively charged (have a nitro or cyano pharmacophore) and binds to a possible unique cationic subsite of insect nAChRs, which makes them selective to insects (Tomizawa and Casida, 2003; Tomizawa and Casida, 2004) and as a result display generally low toxic to mammals, birds and fish.

Neonicotinoids can be divided into two main groups, the cyano-substituted compounds (thiacloprid and acetamiprid) and the nitro-substituted compounds (imidacloprid, clothianidin, thiamethoxam, dinotefuran, nitenpyram).

In this PhD study, two cyano-substituted compounds, thiacloprid and acetamiprid, and one nitro-substituted compounds, imidacloprid, have been used.

Thiacloprid is a novel broad-spectrum neonicotinoid insecticide with high efficacy against sucking (sap-feeding) and biting insects. Uses of thiacloprid globally, vary from top fruits to cotton and vegetables. Thiacloprid has a favourable environmental profile (short half-life in soil, good safety margins for avians, fishes and many beneficial insects) and low acute toxicity to vertebrates (Elbert *et al*., 2000).

Acetamiprid acts on a broad range of pests (Hemiptera, Thysanoptera and Lepidoptera). It shows low toxicity to mammals and also has a good environmental profile (Roberts, 1999).

Imidacloprid is the first representative of the neonicotinoids, synthesized in 1985 (Tomizawa and Casida, 2003). As a systemic and broad spectrum insecticide it offers effective control of sucking (sap-feeding) insects and some species of biting insects (Nauen *et al*., 1998).

1.4 Insecticide Synergists

Compounds that are non-toxic at the concentration used but enhance the toxicity of an insecticide by inhibiting the enzymes responsible for metabolic detoxification are known as synergists (Metcalf, 1967; Matsumura, 1985). Use of synergists may vary depending on the susceptibility of the target insect pest. If used against susceptible populations, synergists allow the insecticide dosage that confers mortality to be reduced, however, if the target is a resistant (metabolic) population, then synergist usage can restore the susceptibility for the partnering insecticide. Furthermore, as employed in this PhD study synergists are also important tools for investigating the molecular basis of resistance. By using different synergists it is possible to identify and characterise the mechanisms that are responsible for resistance.

In this PhD study, two methylenedioxyphenyl (MDP) synergists (a kind gift from Endura SpA, Italy); piperonyl butoxide (PBO) and EN 16/5-1 (an analogue of PBO) were used.

PBO is synthesised from naturally occurring safrole which supplies the MDP moiety (Wachs, 1947). This chemical moiety provides the oxidase inhibitory character of the

compound. This property has been used in laboratory studies to characterise the resistance mechanisms conferred by oxidases (Forgash *et al*., 1962; Matthews and Casida, 1970; Philippou *et al*., 2011). However later findings suggests that PBO also has the capability of inhibiting resistance-associated esterases in pest insects (e.g. *Helicoverpa armigera*, *Bemicia tabaci*) (Young *et al*., 2005; Young *et al*., 2006).

In contrast the analogue of PBO EN16/5-1 has only limited ability to inhibit oxidases but retains the ability to inhibit esterases. As a result synergism studies benefit from these properties by allowin this synergist to be used in parallel with PBO to characterise resistance mechanisms mediated by P450s and esterases (Moores *et al*., 2009).

1.5 Insecticide Resistance

"Sustainable agriculture" refers to practices and systems that result in decreased environmental damage use less natural resources and contribute to the production of sufficient and good quality food to feed an increasing world population. Chemical control of pests is currently an essential component of many crop production programmes and is likely to remain so for the foreseeable future. However, beside all the irresistible offerings for society and farmers (such as food security and food safety, good yield, product quality), it should be recognised that pesticides also carry some risks, and these should be managed to obtain the most benefits. This is acknowledged in the current discussions on the role of the insecticide on honey bee loses and/or a significant risk of development of resistance in some important insect pests. These two issues may seem different to each other, but since both beneficial and pest species in this instance are insects, it is not suprising to see that tools designed to kill insect pests may have a potential to harm benefical insects – such as

honey bees too. *Vice versa*, mechanisms avaliable for an insect pest to develop resistance against insecticides is also avaliable for honey bees to adapt themselves to a toxic compound present in the environment.

IRAC (Insecticide Resistance Action Committee) defines resistance as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species". Another definition by the WHO (World Health Organization) is "the inherited ability of a strain or organism to survive doses of a toxicant that would kill the majority of individuals" (Zlotkin, 1999).

Resistance is a result of intensive, frequent and multiple uses of insecticides with the same mode of action. Some insect strains have become so resistant to a specific insecticide that they can survive exposure to virtually any dose (Scott, 1990). Whalon *et al*. (2008) reported that more than 500 insect species have now become insensitive to at least one insecticide and some sources state that this number has reached 1000 species since 1945 (Miller, 2004).

In order to be effective, an insecticide has to reach its target–site and any barriers to the intact insecticide molecule reaching the target–site may cause resistance to that compound (Nauen, 2007).

Four main mechanisms of resistance have been described 1) biochemical changes in the metabolism of xenobiotics (metabolic resistance), 2) mutations which modify the target site protein (target-site resistance), 3) reduced penetration of insecticide through the insect cuticle and 4) behavioural modification to avoid insecticide exposure (Denholm and Devine, 2001; Li *et al*., 2007).

Most resistant insect populations have one major resistance (defence) mechanism against synthetic chemicals or plant compounds. Similarly, honey bees have also the ability to tolerate toxic compounds by adapting their metabolism by utilising the same defence mechanisms.

Therefore, extrapolations from thoroughly investigated insecticide resistance mechanisims may also guide the research on defining honey bee defence mechanisms against toxic compounds.

1.5.1 Behavioural resistance

Sparks *et al*. (1989) explained behavioural resistance as "the behavioural mechanisms evolved that reduce an insect's exposure to toxic compounds or that allow an insect to survive in what would otherwise be a toxic and fatal environment". It is reported in the same study that the housefly, *Musca domestica* Linnaeus exhibits behavioural resistance to malathion and, although it is not easy to determine behavioural traits in the laboratory, more than 30 insect species with behavioural resistance to insecticides have been described. This mechanism is perhaps the most poorly understood and least well studied of the four resistance pathways.

This kind of avoidance behaviour has been reported in honey bees against imidacloprid-contaminated food resulting in reduced foraging activity. This repellency/antifeedant effect reduced the risk of imidacloprid exposure (Mayer and Lunden, 1997; Kirchner, 1999; Maus *et al*., 2003; Blacquière *et al*., 2012).

1.5.2 Reduced penetration

Reduced penetration, which is the result of a modification to the insect cuticle to prevent insecticide absorption, was first described in the early 1960s (Forgash, 1962; Fine, 1963). It has been shown to function as a resistance mechanism in several reports involving reduced penetration of organophosphates, carbamates (Ku and Bishop, 1967; Price, 1991; Siegfried and Scott, 1991) pyrethroids (Farnham, 1973; Gunning *et al*., 1991) and more recently a neonicotinoid, imidacloprid (Puinean *et al*., 2010). On its own, reduced penetration usually confers limited insensitivity, but it is often found together with other resistance mechanisms.

1.5.3 Metabolic resistance

After penetration, an insecticide may be metabolised by detoxification pathways into a non-toxic molecule or a form that can be eliminated rapidly from the insect body (Fukuto, 1990). These reactions often occur faster in resistant strains due to enhanced levels of detoxification enzymes such as esterases, cytochrome P450s and glutathione *S*-transferases (GST) (Hemingway and Ranson, 2000; Hemingway *et al*., 2004) (Figure 1.3).

Esterases

Esterases are enzymes that hydrolyse/sequester ester bonds and are an important mechanism to detoxify insecticides (that contain an ester bond) such as organophosphates, carbamates and pyrethroids (Devonshire and Moores, 1982; Cahill *et al*., 1995; Hemingway and Karunaratne, 1998; Wheelock *et al*., 2005). Esterase-based resistance can occur by either/both sequestration and/or increased hydrolysis. Sequestration usually occurs when there are increased quantities of an esterase present in resistant individuals, whilst increased hydrolysis results from

changes occurring in the active site of an enzyme, enabling it to hydrolyse insecticidal esters more effectively (Devonshire and Moores, 1982; Oakeshott *et al*., 2005; Wheelock *et al*., 2005). In *Lucilia cuprina* organophosphate resistant acetylcholinesterase may be protected by carboxylesterase E3 via phosphorylation (Chen *et al*., 2001).

Cytochrome P450s

Cytochrome P450s are haem proteins (Omura and Sato, 1964), involved in many biosynthetic pathways including the oxidation of xenobiotics (e.g. insecticides) (Nelson *et al*., 1996). Xenobiotic oxidation was first reported in the endoplasmic reticulum of rabbit liver (Axelrod, 1955; Brodie *et al*., 1955). It was subsequently found that the pigment from pig and rat liver when saturated with carbon dioxide had a discernible peak of absorbance at a wavelength of 450 nm (cited by Omura and Sato, 1964). P450s follow a general detoxification pathway as represented below:

$$\text{Xenobiotic (X)} + \text{NADPH} + H^+ + O_2 \rightarrow \text{XO} + NADP^+ + H_2O$$

In this reaction one atom of molecular oxygen is incorporated into a xenobiotic (X) and a second atom of oxygen is reduced to produce a molecule of water. The resulting XO molecule may be less toxic and/or more easily excreted. This catalytic activity requires two electrons. This electron transportation is usually provided by NADPH via cytochrome P450 reductase and cytochrome b5 (Feyereisen, 1999; Paine *et al*., 2005; Murataliev *et al*., 2008). Cytochrome P450s can be divided into several clades according to their similarity and evolutionary pathways. In insects 4 clades of P450s have been described CYP2, CYP3, CYP4 and mitochondrial-specific CYPs. It is reported that P450s from the CYP3, CYP4 and the mitochondrial CYP clades are most frequently involved in conferring insecticide resistance (Claudianos

et al., 2006; Feyereisen, 2006). Enhanced expression of specific P450s confers resistance to insecticides in many crop pest species with two examples CYP6CY3 in *Myzus persicae* (Bass *et al*., 2013) and CYP6CM1 in *Bemicia tabaci* (Karunker *et al*., 2008).

Esterases and P450s which act directly on the insecticidal molecule are considered "phase 1" metabolic enzymes.

Glutathione S-transferases

Another class of enzymes, the glutathione *S*-transferases (GSTs), are usually considered "phase 2" enzymes because they act on primary or secondary metabolites of the insecticide rather than the intact insecticide itself. They catalyse the conjugation of glutathione to the electrophilic centers of xenobiotics which produces formation of a water-soluble product. Additionally, GSTs are also involved in insecticide binding and sequestration (Li *et al*., 2007). There are three groups of GSTs: cytosolic, microsomal and mitochondrial (Lumjuan *et al*., 2007). The Delta and Epsilon classes of the cytosolic group are specific to insects and may be related to GST-caused insecticide resistance (Lumjuan *et al*., 2005, Strode *et al*., 2008). High levels of GST activity have been recorded in some resistant insects such as houseflies (Motoyama and Dauterman, 1975; Ottea and Plapp, 1984) and resistance development mediated by enhanced GST activity has been confirmed (Fournier *et al*., 1987; Zhu *et al*., 2007).

This text box is where the unedited thesis included the following third party copyrighted material:

Kranthi, K.R. (2005) 'Insecticide resistance monitoring, mechanisms and management manual'. Central Institute for Cotton Research (Indian Council of Cotton Research), Nagpur

Figure 1.3 Schematic diagram of detoxification pathways whereby insecticides are converted to less lipophilic metabolites through phase 1 and phase 2 metabolic reactions. (adapted from Kranthi, 2005).

1.5.4 Target-site resistance

Target site resistance is usually the consequence of mutations in genes encoding insecticide target proteins, for example, the voltage-gated sodium channel which is the target for pyrethroid insecticides; the γ-aminobutyric acid (GABA) receptor, the target for cyclodienes etc; acetylcholinesterase (AChE) the target for organophosphates and carbamates and the nicotinic acetylcholine receptor (nAChR) the target for neonicotinoids, which is considered further below (Williamson *et al*., 1996; ffrench-Constant *et al*., 1993; Walsh *et al*., 2001; Tomizawa and Casida, 2003; Bass *et al*., 2011).

Acetylcholine (ACh) is a neurotransmitter which is contained in synaptic vesicles of presynaptic axons. The arrival of a nerve impulse leads to release of acetylcholine into the synaptic cleft, it then diffuses into the postsynaptic membrane where it binds

to specific receptors, the nAChRs, which are ligand-gated ion channels causing membrane electrical depolarization and propagation of the nerve impulse. nAChRs are members of the cys-loop ligand-gated ion channel superfamily and are composed of five subunits arranged around a central pore (Sine and Engel, 2006). Each subunit shares a similar structure, composed of three main regions, 1) a large hydrophilic N-terminal region that protrudes into the synaptic cleft and has the ligand binding site, 2) four hydrophobic transmembrane regions M1-M4 of which M2 is thought to line the channel pore and 3) a hydrophilic segment between M3 and M4 that is exposed to the cytoplasm of the cell and carries phosphorylation sites important in regulation and desensitisation of the receptor (Unwin, 1989; Wagner *et al*., 1991; Miles *et al*., 1994; Hucho *et al*., 1996). There are two kinds of subtypes of vertebrate nAChR; the muscular subtype (*Torpedo*) which consists of $\alpha_2\beta\gamma\delta$ in embryonic muscle and $\alpha_2\beta\delta\varepsilon$ in adult muscle and the neuronal subtype which has greater subunit diversity being composed of α2-α10 and β2-β4 subunits expressed in vertebrate brain and ganglia (Arias, 2000; Millar, 2003). nAChR subunits have been identified from many insects and classified as α or β according to the presence or absence respectively of two cysteine residues that form a disulphide loop (Kao *et al*., 1984); however, little is known about subunit diversity *in vivo*.

1.6 Sensitivity of honey bees to pesticides

There are some contradictory theories on how tolerant bees are to pesticides. Since the honey bee genome was sequenced (Honey bee Genome Sequencing Consortium, 2006), more is known about their metabolic systems, especially the detoxification pathways of potential xenobiotics. The honey bee genome contains a smaller number of genes encoding detoxification enzymes than the published genomes of *Drosophila melanogaster* and *Anopheles gambiae* with only 46 P450 genes, 24 carboxylesterases

and 10 glutathione *S*-transferases present in the honey bee, compared to 85 P450 genes, 35 carboxylesterases and 38 glutathione *S*-transferases for *D. melanogaster* and 106 P450 genes, 51 carboxylesterases and 31 glutathione *S*-transferases for *A. gambiae*. It has been suggested that this reduction in detoxification diversity may make honey bees more susceptible to xenobiotics than other insects (Claudianos *et al*., 2006). However, a lower number of detoxification genes does not necessarily correspond to lower detoxification activity (Hardstone and Scott, 2010). Bioassay and biochemical data from various studies have shown that both P450s and carboxylesterases are responsible for pesticide metabolism in honey bees. For example, studies using P450 inhibitors indicated that P450s have an important role in detoxification (Yu *et al*., 1984; Pilling *et al*., 1995; Suchail *et al*., 2003). In line with other insects, P450 genes in the honey bee genome group into four clades, CYP2, CYP3, CYP4 and mitochondrial CYPs. P450s in the CYP3 and CYP4 clades have been commonly associated with insecticide resistance in a range of insect species (Feyereisen, 2005). In particular the CYP3 clade includes the CYP6 and CYP9 families that have been shown to participate in detoxification of a range of xenobiotics including pesticides. It has been reported that P450s of the CYP9Q family are responsible for tau-fluvalinate detoxification in the honey bee midgut (Mao *et al*., 2011). Although the total CYP gene complement is lower in honey bees compared to other insects, the CYP 6 family contains 43% of all honey bee P450s.

In terms of the target-site of neonicotinoid insecticides the nAChR, *A. mellifera* has 11 nAChR subunit genes (Amelα1-9 and Amelβ1-2), a similar number to *D. melanogaster* (10 subunit genes) and *A. gambiae* (10 subunit genes) (Satelle *et al*., 2005; Jones *et al*., 2005; Jones *et al*., 2006). Although these insects have a smaller number of subunit genes than birds and mammals (17 subunit genes) (Millar *et al*.,

2003) subunit diversity can be increased by alternative splicing (Stetefeld and Ruegg, 2005) and mRNA A-to-I editing (Seeburg, 2002). Jones *et al*. (2006) have shown, using RT-PCR that Amelα4 has two alternative splice forms for exon 4 which are differentially expressed during the honey bee life cycle. Moreover, there are two variants for Amelα3 (long and short variants) which have TM3-TM4 intracellular loops that differ in length by 13 amino acid residues. mRNA A-to-I editing may also serve to increase subunit diversity (Jones *et al*., 2007).

1.7 Background of research

Following the latest incidents of honey bee losses, the role of insecticides has been widely vocalised in public discussion platforms. It is sometimes believed that honey bees are defenceless organisms that will be killed by all insecticides. However, an extensive number of studies found honey bees to be no more sensitive to numerous insecticides than other insect species, but their metabolic capacity may be limited. Since the honey bee genome has been published, it is known that detoxification genes are relatively lower in number than other insects but other studies have shown that certain genes play an important role in defence mechanisms of the honey bees against specific insecticides (in-hive). This PhD study aims to demonstrate, honey bees may have intrinsic mechanisms that provide protection against certain insecticide classes.

1.8 Aims and Objectives

The overall aim of this project is to characterise the molecular and biochemical mechanisms of defence against pesticides in the honeybee.

This may be sub-divided into two main objectives:

1) Determination of the metabolic enzymes conferring the primary defence to select ‘less toxic’ insecticides, with a focus on esterases and P450s:

    a. Esterases – use the ‘interference assay’ (Khot *et al*., 2008) to determine the interactions between esterases and insecticides, to determine if bee esterase has the capability to inhibit/bind toxicants conferring defence mechanism.

    b. P450s – identify defence P450(s) by microarray analyses following exposure to sub-lethal concentrations of insecticides, to determine if the upregulation of genes encoding metabolic enzymes protects honey bees by detoxifying that insecticide Any candidate genes will be heterologously expressed and the interaction between the P450 and insecticide characterised by functional assays.

    c. Synergism studies - investigate phase 1 metabolic enzyme(s) responsible for defence by bioassaying bees with PBO and EN16/5-1.

2) Investigation of the sensitivity of different honey bee races/castes to neonicotinoid insecticides. If differential sensitivity in bee races/castes is found then the underlying molecular mechanisms will be explored by:

    a. Sequencing nAChR subunits to examine if qualitative changes (mutations) in the target-site are associated with insensitivity.

b. Transcriptome profiling of different bee races using microarrays to examine if qualitative changes in gene expression are associated with insensitivity (such as genes encoding P450s and nAChR subunits).

c. Synergism studies and biochemical analyses to assess the metabolic defence of different bee races/castes

2. GENERAL MATERIALS AND METHODS

2.1 Oligonucleotide Primers

Oligonucleotide primers were designed using Primer3web version 4.0.0 (http://primer3.ut.ee/) and custom synthesised by Sigma-Aldrich. They were dissolved in nuclease-free water to a final stock concentration of 100 μM and used to prepare working concentrations of 10 μM for PCR and sequencing and 5 μM for qRT-PCR.

2.2 Plasmid Vectors

The strataClone PCR cloning vector pSC-A-amp/kan (Agilent Technologies) was used for standard cloning. For expression of P450s, the pCW-ori+ vector was used and for the expression of P450 reductase (from *Anopheles gambiae*), the vector pACYC184 was used; both were obtained from Liverpool School of Tropical Medicine.

2.3 Bacterial Strain

The *Eschericia coli* strain JM109 was obtained from Sigma-Aldrich.

2.4 Honey Bee Strains

This study was conducted with four different honey bee races (Table 2.1): *Apis mellifera ligustica*, *Apis mellifera caucasica* and *Apis mellifera buckfast* which were kindly supplied by Prof H. Vasfi Gencer (Ankara University Agricultural Faculty, Turkey). Hives were maintained and checked regularly by beekeepers before bee collections to ensure they were disease and pest free. Bees were placed in RNAlater/on dry ice and transported from Ankara University to Rothamsted

Research. All studies with bee races from Turkey were carried out at Rothamsted Research with the exception of *in vivo* assays.

Apis mellifera carnica was provided by the AgroEcology Department, Rothamsted Research. All Rothamsted Research hives were treated with Fumidil (an antibiotic to treat Nosema disease) in September, and treated with Apiguard (to reduce Varroa mite) in August and September by the beekeeper. The colonies were checked weekly between April and September, given more boxes/space as required, honey taken off for extraction during the summer, and fed sugar syrup in the Autumn to maintain survival.

Table 2.1 Origins of *Apis mellifera* races.

Bee Race	Origin	Subspecies
Apis mellifera carnica	Rothamsted Research, UK	Western
Apis mellifera ligustica	Ankara University, Turkey	South-west of Europe
Apis mellifera caucasica	Ankara University, Turkey	Middle East
Apis mellifera buckfast	Ankara University, Turkey	Cross of several subspecies*

*****Buckfast bees are a bee 'strain' containing heritage from several *A. mellifera* subspecies that was developed by Brother Adam at Buckfast Abbey (Bee keeping at Buckfast Abbey, 2014).

2.5 Insect Bioassays

2.5.1 Full dose contact toxicity bioassays

Worker honey bees were transferred to plastic cages after anesthetising with CO_2 (ten honey bees per cage, at least three replicates per insecticide dosage). For each cage a reservoir of 50% sucrose in water was available for *ad lib* feeding. Prior to treatment bees were anesthetised by exposure to low levels of CO_2 and then placed in a circular

plastic container which allowed release of CO_2 from the bottom to keep the bees inactive during treatment. Each bee was topically dosed with either insecticide in acetone or acetone alone applied to the dorsal thorax using a Burkard microapplicator (Burkard, Rickmansworth, UK). All treated bees were maintained at 25^{o}C in the dark. All bioassays were scored after 24 h and if any bees were not walking or flying they were counted as dead.

2.5.2 Full dose oral response bioassays

For oral toxicity tests, technical grade thiacloprid (Sigma Aldrich) was firstly dissolved in acetone and then combined with sucrose syrup (50%) in water. Worker bees were exposed to a range of thiacloprid concentrations while control groups were exposed to acetone combined with sucrose syrup for 24 hours through oral feeding; the amount of treated diet consumed by each cage was measured by weight. All treated bees were maintained at 25^{o}C in the dark. All bioassays were scored after 24 h and if any bees were not walking or flying they were counted as dead.

2.5.3 Statistical Analysis

The data for full dose contact and oral bioassays were analysed using a 4 parameter non-linear fit (Grafit 3.0, Leatherbarrow). The curves and LD_{50} values were generated using 4 parameter logistic equation given below, where a is the maximum y range, and s is a slope factor:

$Y= a/1+(x/IC_{50})s + background$

When the curve is fitted and the equation resolved, then values of y can be entered for values of x (i.e. if y= 5% mortality, what is the concentration (x)).

2.6 Biochemical Methods

2.6.1 Total protein assay (Bradford protein assay)

Protein measurements were carried out using the Bradford Protein Assay (Bradford, 1976) with bovine serum albumin used as a standard. 10 µL replicates of bee homogenate were mixed with 200 µl Bradford Reagent (Sigma-Aldrich) in a separate microplate (NUNC, maxisorb). After 20 minutes incubation at room temperature, readings were taken at 595 nm using a SpectraMax 250 microplate reader (Molecular Devices, Menlo Park, CA).

2.7 Molecular Methods

2.7.1 Standard polymerase chain reaction (PCR) protocol

Each PCR reaction (total volume 20 µL) was carried out in 0.2 mL tubes and consisted of: 10 µL DreamTaq Green PCR Master Mix (2X) (Thermo Scientific), 7 µL nuclease-free water, 1 µL of each forward and reverse primers (10 µM) and 1 µL of cDNA or gDNA template. PCR was performed in a thermal cycler (G-storm) using the following conditions: 2 minutes at 94°C (initial denaturation) then 35-40 cycles of 30 seconds at 94ºC (denaturation), 30 seconds at the specific annealing temperature and 1.20 minutes at 72ºC (extension) with a final extension step at 72°C for 5 minutes. When required, the primary PCR products were used as templates for a second nested PCR reaction.

2.7.2 Gel electrophoresis

DNA (PCR products, digestion products) and RNA were examined by electrophoresis. 1% agarose gels were prepared with the addition of ethidium bromide (Sigma-Aldrich) in 1X TAE (Tris Acetate EDTA) buffer (Appendix 9.1.1) for digestion products or a 1X TBE (Tris Borate EDTA) buffer (Fisher Scientific)

(Appendix 9.1.1) for PCR products and RNA. For 1% agarose gel, 0.5 mg agarose in 50 mL TBE or TAE was mixed in a flask and melted in a microwave. When the solution was cooled down to handling temperature, 5 μL of 10 mg/mL ethidium bromide was added. This was transferred to a casting system and left to set at room temperature. When DreamTaq Green (which contains a loading dye) (Thermo Fisher Scientific) was not used as the master mix, samples were mixed with 1/10 volume of 6X loading dye (Thermo Fisher Scientific). Samples were run in the same buffer as the gel at a voltage of 100V for approximately 45 minutes with GeneRuler 100 bp or 1 kb ladder (Thermo Fisher Scientific) run as a DNA standard. The gels were visualised on an UV transilluminator.

2.7.3 Purification of gel and PCR products

To purify gel and PCR products the Wizard® SV Gel and PCR Clean-up System (Promega) was used. The desired band was excised from the agarose gel with a sterilized, sharp blade under a blue light transilluminator and placed in a pre-weighed 1.5 mL Eppendorf tube. Membrane binding solution (1 μL per 1 mg gel slice) was added to the gel slice which was then melted at 50-65°C on a heat block (with vortexing at regular intervals). For PCR product preparation, the membrane binding solution was mixed with an equal amount of PCR product. After the preparation step, samples were loaded into an SV Minicolumn system and centrifuged at 13,000 rpm for 1 minute on a desktop centrifuge to bind the DNA to the filter column. Two washing steps were then carried out with membrane wash solution using 700 μL for the first step and 500 μL for the second step. The washes were removed by spinning at 13,000 rpm for 1 minute and 5 minutes, respectively with the flow through discarded after each step. DNA was eluted from the filter column with 30 μL of nuclease-free water by spinning the column at 16,000 × *g* for 1 minute.

2.7.4 PCR cloning

PCR products were cloned using StrataClone PCR cloning kit (Agilent Technologies) for cloning *Taq* DNA polymerase-amplified PCR products. Cloning steps were performed according to the manufacturer's protocol.

Ligation

PCR products were ligated into the pSC-A-amp/kan cloning vector with 2 µL PCR product (5-50 ng), 3 µL of StrataClone Cloning Buffer and 1 µL StrataClone vector mix added to a 1.5 mL Eppendorf tube and incubated at room temperature for 5 minutes before being placed on ice.

Transformation

After the ligation step, 1 µL of cloning reaction was transformed into StrataClone SoloPack competent cells which carry the *lacZ*ΔM15 mutation (allowing blue/white selection to take place (in conjunction with X-gal)). The ligation/cell mixture was incubated on ice for 20 minutes followed by a heat-shock step at 42°C for 45 seconds then immediately transferred to ice for 2 minutes. After adding 250 µL of warm LB medium (Appendix 9.1.1), samples were incubated for 1 hour at 37°C with shaking (200 rpm). 100 µL of the transformation mix was plated on LB/ampicillin (50 mg/mL) agar plates with 40 µL of 2% X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) and incubated overnight at 37°C. White colonies were selected and analysed by single colony PCR using the plasmid specific primers T3 and T7 or M13F/R that flank the cloning site and using the standard PCR protocol (see above) before they were screened on a 1% agarose gel. A single colony containing the insert was picked and grown in 5 mL LB/ampicillin media overnight at 37°C with shaking.

Plasmid DNA minipreps

Plasmid DNA was extracted from overnight 5 mL cultures using GeneJET Plasmid Miniprep kit (Thermo Scientific) following the manufacturer's protocol. Cells were pelleted by centrifugation (4,000 rpm, 4 minutes, 4°C) and the supernatant was removed. The pellet was resuspended in 250 µL of resuspension solution and transferred to a 1.5 mL microcentrifuge tube before 250 µL of lysis solution was added. After mixing by inverting the tube, 350 µL of neutralization solution was added and the tube mixed again by inversion. After spinning for 5 minutes at 13,000 rpm to remove cell debris and chromosomal DNA, the supernatant was loaded onto the GeneJet spin column and centrifuged for 1 minute at 13,000 rpm. The column was then washed twice with 500 µL of the wash solution with centrifugation for 1 minute at 13,000 rpm between each wash. The remainder of the wash solution was removed by centrifugation as above. The column was placed in a clean 1.5 mL microcentrifuge tube and 50 µL of the elution buffer was added. The column was incubated at room temperature for 2 minutes before it was centrifuged for 2 minutes as above to elute the plasmid DNA. The quantity and quality of this was then checked with a NanoDrop 1000 spectrophotometer.

2.7.5 Extraction of total RNA

RNA was extracted from a pooled sample of four honey bees using the Bioline Isolate II RNA Mini Kit (Bioline Reagents) following the product manual with minor modification. Bees were ground to a fine powder in liquid nitrogen using a pestle and mortar. To ~30 mg of powdered sample, 350 µL of lysis buffer RLY and 3.5 µL of β-ME was added and mixed by vortexing before centrifugation at 16,000 x *g* for 10 minutes. The cell - lysis supernatant was then applied to Isolate II filter and centrifuged at 11,000 x *g* for 1 minute to clean the lysate. The filter was then

discarded and the clean lysate was mixed with 350 µL of 70% ethanol and directly transferred to an Isolate II RNA mini column and centrifuged at 11,000 x *g* for 30 seconds. The column was desalted by adding 350 µL of membrane desalting buffer and then dried by centrifugation at 11,000 x *g* for 1 minute. DNase I reaction mixture was prepared as described in the protocol, and 95 µL of this was added to the centre of the membrane before it was incubated at room temperature for 15 minutes. The column was washed by adding 200 µL of wash buffer RWI, 600 µL of wash buffer RW2 then 250 µL of wash buffer RW2. The column was centrifuged at 11,000 x *g* for 30 seconds between the first 2 washing steps then for 2 minutes after the 3rd washing step to dry the column. The column was then placed in a 1.5 mL microcentrifuge tube and RNA was eluted with 30 µL RNase-free water (centrifugation at 11,000 x *g* for 1 minute). RNA quantity and quality were checked with a NanoDrop 1000 spectrophotometer.

2.7.6 cDNA synthesis

First strand cDNA was synthesised from total RNA using Superscript™ III Reverse Transcriptase (Invitrogen). To 4 µg of RNA, 1 µL random primers (50 ng/µL), 1 µL dNTP mix (10mM) and nuclease-free water (up to 13 µL) were added and the reaction was incubated at 65ºC for 5 minutes, and then on ice for 1 minute. To this, 4 µL 5X First-Strand buffer, 1 µL 0.1M DTT, 1 µL RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, 40 units/µL) and 1µL SuperScript™ III RT were added and incubated at 25ºC for 5 minutes and then terminated at 50ºC for 60 minutes. Finally the reaction was stopped by incubation at 70ºC for 15 minutes and samples chilled on ice.

2.7.7 Quantitative real time PCR (qRT-PCR)

Microarray results were validated by qRT-PCR for selected genes. Primers were designed to amplify products of 90–150 bp in length. All the primer pairs tested had efficiency between 90 and 110%. Each PCR reaction consisted of 4 µL of cDNA (10 ng), 5 µL of SensiMix SYBR Kit (Bioline Reagents) and 0.5 µl of each forward and reverse primer (0.25 µM). The experiment was performed on a Rotor-Gene 6000 (Corbett Research) using the following cycling conditions: 10 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds and 72°C for 20 seconds. A final melt-curve step was included post-PCR (ramping from 72°C to 95°C by 1°C every 5 seconds) to confirm the absence of any non-specific amplification. The efficiency of PCR for each primer pair was assessed using a serial dilution from 100 ng to 0.01 ng of cDNA. Each qRT-PCR experiment consisted of at least three independent biological replicates with two technical replicates for each. Data were analysed according to the ΔΔCT method (Pfaffl 2001), using the geometric mean of three selected housekeeping genes (ef1 (elongation factor-1), tbp (tubulin) and actin) for normalisation according to the strategy described previously (Vandesompele *et al*., 2002).

2.7.8 Microarray analysis

Using the official honey bee coding sequence gene set release 1 (~10,000 genes), and a manually curated file comprising genes encoding detoxification enzymes, an expression array of 15744 elements was designed using Agilent Technologie's eArray online software (https://earray.chem.agilent.com/earray/). A SurePrint HD (8x15k) expression array was designed using the Agilent eArray platform. The base composition and best probe methodologies were selected to design sense orientation

60-mer probes with a 3′ bias. The OGS CDS 1 was used as the reference transcriptome to eliminate potential probe sequences that would have significant cross-hybridization with targets other than the one of interest. In the case of detoxification genes (P450s, GSTs and CEs) three alternate probes for each EST were designed.

Prior to microarray experiments the quality and quantity of RNA was checked using a nanodrop spectrophotometer and by running an aliquot on a 1.5% agarose gel. For the latter, RNA was mixed with 1x loading buffer (95% formamide; 0.025% xylene cyanol; 0.025% bromophenol blue; 18 mM EDTA; 0.025% SDS), heated for 5 minutes at 65°C and briefly chilled on ice prior to loading. Two micrograms of each RNA was used to generate labelled cRNA, which was hybridised to arrays and these were washed and scanned as described in the Agilent Quick Amp Labeling Protocol (Version 5.7). The microarray experiments consisted of four/five biological replicates and for each of these, hybridisations were completed in which the Cy3 and Cy5 labels were swapped between samples to avoid any dye effects.

Microarrays were scanned at the University College of London with an Agilent G2565CA scanner and fluorescence intensities of individual spots were obtained using the Agilent Feature Extraction software with default Agilent parameters. Data were analysed using GeneSpring GX software and using the t-test against zero function. Genes were considered differentially expressed if they had a p value of <0.05 and a fold change (up or down) of greater than 1.5. A less stringent approach was used in this study in the selection of the fold-change cut-off than in previous studies where a fold change value (FC>2) is commonly used. In this thesis a FC >1.5 was used as a relatively small number of genes were identified as differentially

expressed and we wished to capture even more subtle changes in gene expression associated with insecticide exposure. It is important to stress that the expression of all candidate genes, even those which showed an change in expression <2-fold were experimentally validated by qRT-PCR.

3. CHARACTERISING THE BIOLOGICAL AND BIOCHEMICAL RESPONSE OF HONEY BEE PHASE 1 DETOXIFICATION ENZYMES

3.1 Introduction

As for other insects, honey bees have several superfamilies of enzymes that have the capacity to detoxify xenobiotics including pesticides. These include esterases, P450s and glutathione *S*-transferases.

Esterases and P450s as phase 1 metabolic enzymes (i.e. act directly upon the intact insecticide) have been reported to metabolise a variety of insecticides such as organophosphates, carbamates, pyrethroids and neonicotinoids (Devonshire *et al*., 1998; Stok *et al*., 2004; Feyereisen, 2005).

Esterases contribute to metabolism through hydrolysis and/or sequestration whilst P450s catalyse oxidation of xenobiotics in the presence of electron donors (CPR and cyt *b5*) (Oppenoorth, 1985; Feyereisen, 1999). P450 and esterase involvement of pyrethroid metabolism in honey bees has been demonstrated by synergistic interactions e.g. the toxicity of deltamethrin was enhanced when bees were treated with a mixture of the P450 inhibitor fungicide prochloraz and deltamethrin (Colin and Belzunces, 1992). P450 and esterase associated metabolism of lambda-cyhalothrin was also screened in the presence of prochloraz and the observation of a metabolite of esterase hydrolysis (Pilling and Jepson, 1993; Pilling *et al*., 1995). Further, Johnson *et al*. (2006) tested the tolerance of honey bees to pyrethroids in the absence and presence of 'specific' detoxification enzyme inhibitors and the involvement of three main detoxification enzymes in pyrethroid metabolism was reported as mainly due to P450s, followed by esterases, with GSTs having little effect on detoxification.

Similar synergistic interaction studies were assayed on bees using neonicotinoids. Triflumizole (14α-demethylase inhibitor fungicide) enhanced the toxicity of acetamiprid and thiacloprid (cyano substituted) in laboratory studies. Additionally, PBO synergism of thiacloprid was found to be much higher than synergism of imidacloprid (nitro-substituted), suggesting that P450s are a significantly more important mechanism for cyano-substituted neonicotinoids compared to nitro-substituted neonicotinoids (Iwasa *et al*., 2004).

The role of esterase (E4) in insecticide detoxification in *Myzus persicae* was characterised by Devonshire (1977) as the organophosphate resistance-associated isozyme which offers a broad spectrum resistance in aphids by both ester hydrolysis and sequestration (Devonshire, 1977; Devonshire *et al*., 1982). The inhibition of E4 by PBO, which can be demonstrated by *in vivo* bioassays, is not easily observed with simple *in vitro* spectrophotometric assays using model substrates such as esters of 1-naphthol. However, by using the interference assay reported by Khot *et al*. (2008), esterase inhibition and/or binding can be observed *in vitro* (Bingham *et al*., 2008).

Piperonyl butoxide (PBO) has been used as a synergist to increase the efficacy of insecticides for many years (Casida, 1970; Wilkinson *et al*., 1984). Since its inhibition of both phase 1 metabolic enzymes (esterases and P450s) was reported, it has also been used to control resistant pests in the laboratory and field, and, in combination with its analogue EN 16/5-1, as a discriminating tool to characterise metabolic resistance (Gunning *et al*., 1998; Moores *et al*., 1998; Young *et al*., 2005; Young *et al*., 2006; Moores *et al*., 2009).

Despite tau-fluvalinate and thiacloprid having considerably lower toxicity to honey bees than many other pyrethroids and neonicotinoids, it has been reported that P450 inhibition following exposure to PBO may enhance their toxicity to honey bees (Johnson *et al*., 2006; Iwasa *et al*., 2004). However, since it has been shown that PBO can inhibit both phase 1 detoxification enzymes, PBO synergism alone is not sufficient to indicate P450 detoxification (Moores *et al*., 2009).

This study was conducted to test binding of semi-purified esterase from honey bee with two pyrethroids (tau-fluvalinate and α-cypermethrin) and two neonicotinoids (imidacloprid and thiacloprid). Resistance-associated esterase E4 from *Myzus persicae* was used as a control enzyme for binding studies to demonstrate the 'blockade' of an esterase by insecticide or synergist. Also, *in vitro* experiments were carried out to measure the potency of synergists as inhibitors of oxidase activity of honey bees. Metabolic metabolism of two relatively less toxic insecticides, tau-fluvalinate and thiacloprid, was characterised by differential synergist studies using PBO in combination with its analogue EN 16/5-1 (Moores *et al*., 2009). Further, semi-purified esterase from honey bee was tested for the ability to bind to both synergists.

3.2 Materials and Methods

3.2.1 Insects

Worker bees were collected from the entrance of the hive using a rechargeable vacuum collector and kept in a bee housing cage (Bioquip, California). They were then distributed to plastic containers for assays following anaesthetic (CO_2) for no more than 2 minutes. Drones and dead bees were removed from the containers. Two separate honey bee strains from different locations were collected: *A. mellifera*

carnica was tested for tau-fluvalinate studies while *A. mellifera buckfast* was utilized for thiacloprid studies (see section 2.4, Chapter 2).

3.2.3 Insect bioassays

3.2.3.1 Full dose contact toxicity bioassay

To obtain a suitable discriminating dose of insecticide to combine with synergists, full dose response assays were carried out using a range of thiacloprid concentrations and tau-fluvalinate concentrations (see section 2.5.1, Chapter 2).

Full dose-response bioassay results of *Apis mellifera* carnica against tau-fluvalinate were analysed by probit analysis using the statistical programme PC Polo Plus (LeOra, Software, Berkeley, USA). Polo Plus programme calculated the concentrations required to kill 50% of the population, 95% confidence intervals (CI 95%), slopes with standard errors (SE), chi-square (X^2) and degrees of freedom (df).

3.2.3.2 Discriminating dose response bioassays

To investigate synergism activity, bioassays were carried out using a discriminating dose of thiacloprid or tau-fluvalinate obtained from the full dose response bioassays as described above. Each bee was dosed topically with either 1 µl of 1% PBO in acetone, 1 µl of 1% EN 16/5-1 in acetone or 1 µl acetone alone and then allowed to rest for 2 hours to provide time for transport of the compound into the insect system. Afterwards 1 µl of 0.5 µg (LD_{25}) thiacloprid or 10 g L^{-1} tau-fluvalinate (5 µg per bee) tau-fluvalinate in acetone or acetone alone as control was applied topically. All treated bees were stored and scored as described in section 2.5.1, Chapter 2.

3.2.4 Enzyme assays

3.2.4.1 Esterase purification

Non-specific esterase was purified from honey bees using the method developed by Devonshire (1977), with some modification. 70 honey bees without heads were homogenised in 20 mL 0.02 M phosphate buffer (pH 7.0) and centrifuged at 10,000 x *g* for 5 minutes. Supernatant was passed through a Sephadex G-25 column (Amersham Biosciences) and proteins eluted with 0.02 M Tris/HCl buffer pH 8.5. Protein fractions were further purified by ion exchange chromatography using a DEAE Sepharose Fast Flow column and eluted with a linear 0-0.35 M NaCl gradient in 500 mL of 0.02 M Tris/HCl (pH 8.5). Fractions were tested for esterase activity using 1-naphthyl acetate and those containing the highest levels were pooled and concentrated by centrifugal filters (Amicon Ultra, 30K).

3.2.4.2 Esterase inhibition (Esterase interference assay)

To examine the interactions of purified honey bee esterase/esterase E4 with PBO and insecticides, an esterase 'interference assay' was carried out as described by Khot *et al*. (2008). Briefly, stock solutions of synergist and insecticide (1% in acetone) were pre-incubated with purified esterase for 1 hour at 4°C. Aliquots (15 µL) of esterase, esterase+PBO, esterase+insecticides were incubated for 1 hour with 60 µL of serial dilutions of azamethiphos in 0.02 M phosphate buffer, pH 7.0, in separate wells of a NUNC microplate. Aliquots (25 µL) of housefly head homogenate (source of AChE) were added to each well and incubated for 15 minutes at room temperature. Esterase in acetone and buffer only served as the positive and negative controls respectively. AChE activity was measured at 405 nm using a Tmax kinetic microplate reader (Molecular Devices, Menlo Park, CA). Readings were taken automatically for 10

minutes at 10 seconds intervals. The rate (mOD min^{-1}) was calculated by the integrated software Softmax Pro 5.2. Concentrations to inhibit 50% of the enzyme activity (IC_{50}) were calculated using Grafit 3.0 (Leatherbarrow, Erithacus Software). All treatments were performed in triplicate.

3.2.4.3 Microsomal preparation

After collection using a vacuum aspirator, bees were anesthetised with ether. Because of suspected P450 inhibitor 'xanthommatin' activity, which was detected within eye pigment of housefly (Danielson and Fogleman, 1994), heads were removed using a scalpel and samples were kept on ice during experiments. To prepare microsomes, 50 worker bees were homogenised in sodium phosphate buffer, pH 7.6, 0.1 M with protease inhibitors (1 mM EDTA, 1 mM DTT, 1 mM PTU and 1 mM PMSF) using a motorised homogeniser. Samples were centrifuged at 35000 rpm at 4ºC for 1 hour. The supernatant was discarded and the pellet resuspended in 2 ml sodium phosphate buffer containing protease inhibitors.

3.2.4.4 Fluorometric kinetic oxidase assay

O-deethylation inhibition was measured according to the protocol as described by de Sousa *et al*. (1995). Fifty µl of resuspended microsomal pellet were added to the wells of a 96-well microplate (Perkin Elmer, white). Subsequently, 80 µL of 0.5 mM 7-ethoxycoumarin in sodium phosphate pH 7.8, 0.1 M was added and the plate incubated for 5 minutes at 30ºC, followed by 10 µl of 9.6 mM NADPH in sodium phosphate pH 7.8. The plate was read using 370 nm excitation and 460 nm emmision for 1 hour at 30 seconds intervals using a fluorometer (SpectraMax Gemini XPS, Molecular Devices, Menlo Park, CA).

3.2.4.5 Intact abdomen oxidase inhibition

After collection using a vacuum aspirator adult worker honey bees were anesthetised with ether. *O*-deethylation of 7-EC was measured in intact honey bee abdomens according to Ullrich and Weber (1972), following the method of de Sousa *et al*. (1995) with some modifications. Five bee abdomens were placed in a 2 mL Eppendorf containing either 0.1 M phosphate buffer, pH 7.6 alone, or buffer containing 10 mM PBO or buffer containing 10 mM EN 16/5-1. After 10 minutes, 7-EC was added to give a final concentration of 0.4 mM and incubated with constant shaking at 30ºC for 1 hour. An aliquot (100 µL) was taken from the assay mix and the reaction stopped by the addition of a mixture of 0.1 mM glycine buffer, pH 10.4 / ethanol (1:1; v:v). Fluorescence was measured using 390 nm excitation and 450 nm emission.

3.2.4.6 Protease activity towards ala-ala-ala-pNA

Protease activity was checked against the substrate ala-ala-ala-pNA. To prepare the homogenate different body regions of honey bee (head, thorax and abdomen) were separated with a fine scalpel. Each body part was homogenised in 500 µL of 0.05 M Tris buffer pH 7.5 and 50 µL of homogenate was added to a microplate (NUNC, maxisorb). Following addition of 100 µL of 10 mM substrate in 0.05 M Tris/HCl buffer or 100 µL buffer only for controls the plate was read at 405 nm overnight at 10 minutes intervals using a Thermomax microplate reader (Molecular Devices, Menlo Park, CA).

3.3 Results

3.3.1 Insecticide bioassays

The result of the full dose response bioassay of tested honey bee colony was characterised for thiacloprid and is presented in Figure 3.1 where LD_{50} is 1.86 µg (1µl/bee). A discriminating dose (0.5 µg) was derived from this toxicity data plot and was applied after pretreatment with either PBO or EN 16/5-1 on the honey bee colony. It has been well-documented in the literature that EN 16/5-1 has a reduced capacity to inhibit insect P450 activity. This has been used experimentally to characterise oxidative resistance in *M. persicae* against imidacloprid, and in *M. aeneus* against pyrethroids (Moores *et al*., 2009; Philippou *et al*., 2010) In both cases, these findings have been confirmed by subsequent molecular investigations (Puinean *et al*., 2010; Zimmer and Nauen, 2011).

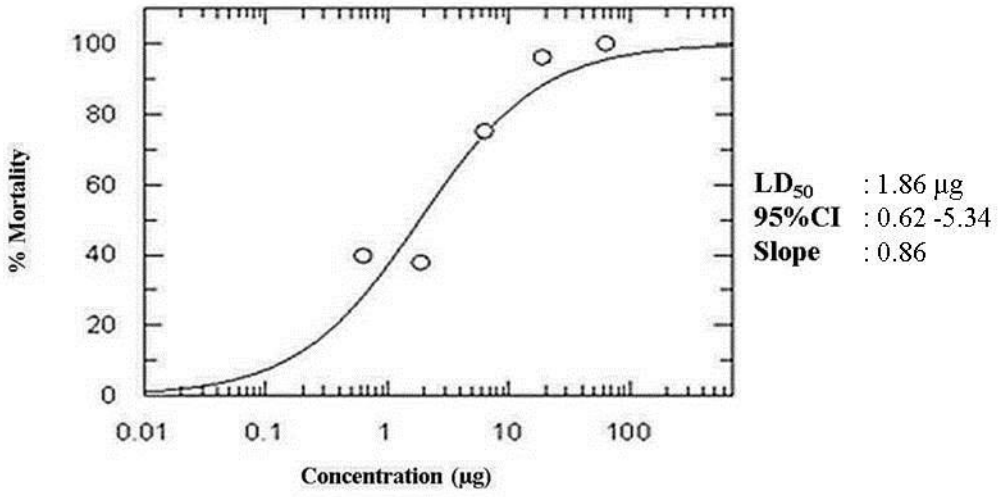

Figure 3.1 Full dose response bioassay results for thiacloprid on *Apis mellifera buckfast* population.

The effect of a 2 hours pre-treatment with either PBO (1%) or EN 16/5-1 (1%) on worker honey bees prior to exposure to a discriminating dose of thiacloprid is presented in Figure 3.2. In the experiment all control groups (insecticide, PBO or EN 16/5-1 alone) show no mortality while pretreatment with PBO resulted in 78.3% mortality and pre-treatment with EN 16/5-1 resulted in 12.78% mortality.

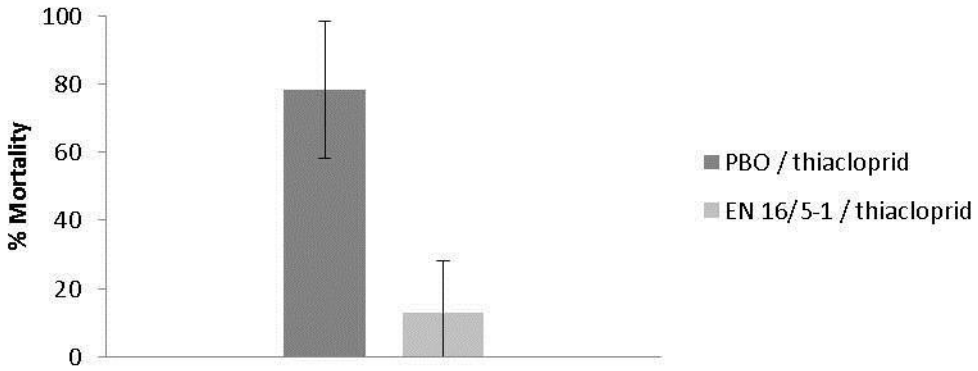

Figure 3.2 Synergistic effect of PBO and EN 16/5-1 mixed with a discriminating dose of thiacloprid on *Apis mellifera buckfast* population (Error bars represent standard error of mean).

Results from the probit analysis of the full dose-response bioassays of the honey bee colony using tau-fluvalinate are presented in Table 3.1. The LD_{10} (5.5 µg) relates to a concentration of 11 g L^{-1}; the LD_{25} (9.8 µg) to 19.6 g L^{-1}; and the LD_{50} (18.5 µg) to a concentration of 37 g L^{-1}.

Table 3.1 Full dose-response bioassay results of *Apis mellifera carnica* against tau-fluvalinate.

LD_{10} [a]	95% CI [b]	LD_{25} [c]	95% CI [b]	LD_{50} [d]	95% CI [b]	Slope	df [e]	X^2 [f]
5.5	2.8 – 8.2	9.8	6.3 – 13.6	18.5	13.3 – 27.4	1.86 ± 0.41	18	33.7

[a] LD_{10} = lethal dose of tau-fluvalinate to kill 10% of the population (µg/bee)

[b] CI = confidence limits

[c] LD_{25} = lethal dose of tau-fluvalinate to kill 25% of the population (µg/bee)

[d] LD_{50} = lethal doseof tau-fluvalinate to kill 50% of the population (µg/bee)

[e] df = degrees of freedom

[f] X^2= chi-square

Based on the results of the full dose bioassays (Table 3.1), a concentration of 10 g L^{-1} tau-fluvalinate (5 µg per bee) was chosen as the discriminating concentration for the evaluation of synergism by PBO and EN 16/5-1, to give a response of approximately 10 – 20% mortality.

The addition of PBO increased the mortality conferred by tau-fluvalinate to around 90%, whilst that of the PBO analogue, EN 16/5-1, increased the mortality from around 20% to just above 30% (Figure 3.3). Although both synergists significantly increased mortality above that of tau-fluvalinate alone ($P < 0.05$), the mortality given by the EN 16/5-1 + tau-fluvalinate mixture was not greater than the sum of their separate mortalities. In contrast, addition of PBO resulted in a significant increase in mortality ($P < 0.05$), and this mortality was greater than the sum of the individual mortalities.

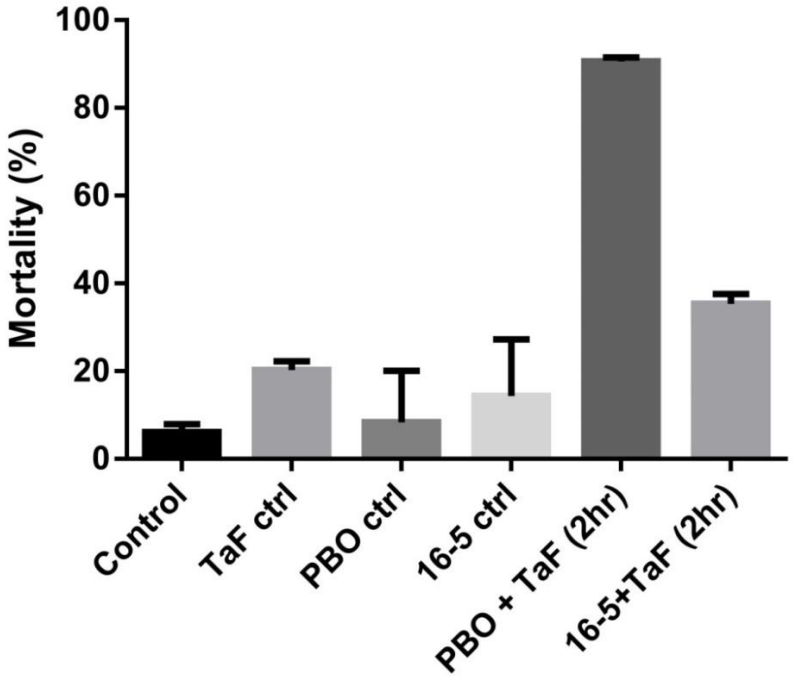

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Figure 3.3 Synergistic effect of PBO and EN 16/5-1 mixed with a discriminating dose of tau-fluvalinate on *Apis mellifera carnica* population (Error bars represent standard error of mean).

3.3.2 Esterase interferance assay

The interference assay detects the AChE activity remaining following esterase blockade on azamethiphos. If tested insecticide or synergist binds to the esterase, the esterase cannot bind to azamethiphos and as a result AChE activity is present (Figure 3.4).

The reduced protection of AChE towards azamethiphos provided by the purified honey bee esterase following pre-incubation with PBO, EN 16/5-1 and tau-fluvalinate is shown in Figure 3.5. This shows that PBO (final concentration 0.01%) does inhibit, or block, the bee esterase resulting in a loss of sequestration of the azamethiphos. However, results with tau-fluvalinate show that the bee esterase does not bind to tau-fluvalinate. Since the bee esterase does not bind to tau-fluvalinate, it can be surmised that although PBO inhibits bee esterase, this will not result in higher

sensitisation. However, it does show that caution would have to be used if PBO were used in conjunction with insecticides that are sequestered by bee esterase, as this protection would be reduced. The influence of tau-fluvalinate on esterase E4 was detected by the same method. In contrast E4 showed binding with tau-fluvalinate after 1 hour incubation period (Figure 3.6). Considering the recent structure-activity characterisation between E4 and PBO, E4 is the most suitable candidate esterase as a control to validate the method for the determination of the esterase binding (Philippou *et al*., 2012).

Figure 3.4 The interaction between the insecticide, target site, synergist/tau-fluvalinate and esterase enzymes, used as the basis for the 'esterase interference assay' (Khot, 2009). In this assay esterase activity is detected indirectly by measuring AChE activity. A negative symbol denotes an inhibitory action. *measured by activity on a model substrate (ATChI). (Reproduced from Khot, 2009).

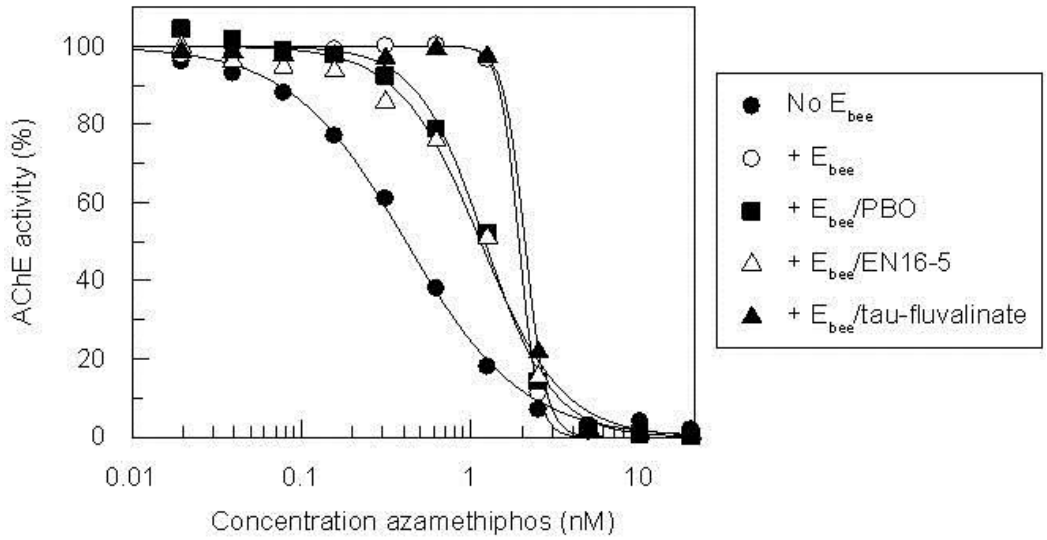


Figure 3.5 Binding of honey bee esterases with PBO, EN 16/5-1 and tau-fluvalinate.

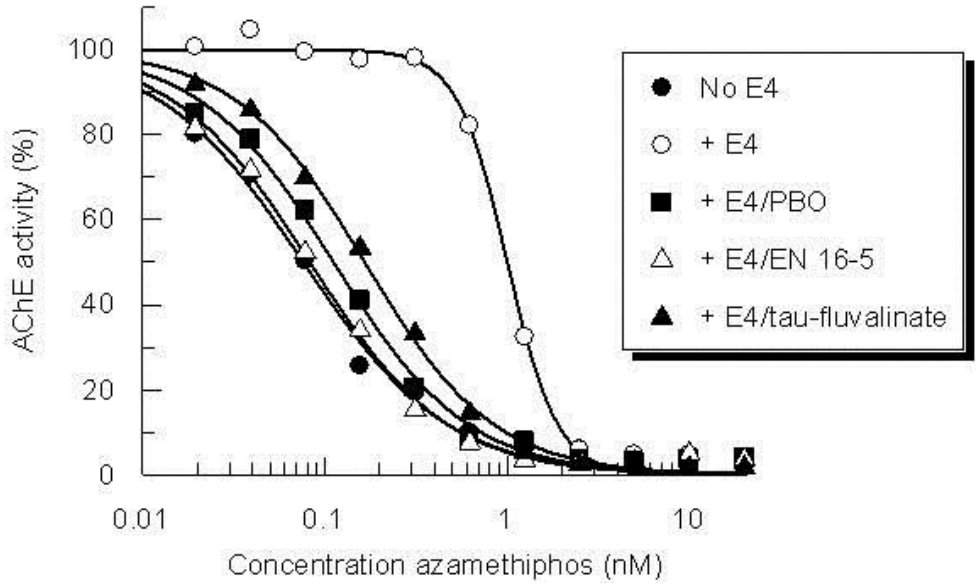


Figure 3.6 Binding of esterase E4 with PBO, EN 16/5-1 and tau-fluvalinate.

The impact of honey bee esterase on neonicotinoids was determined by the interference assay. After 1 hour incubation of bee esterase with each neonicotinoid, no sequestration was observed. Purified honey bee esterase failed to bind to α-cypermethrin, imidacloprid and thiacloprid (Figure 3.7).

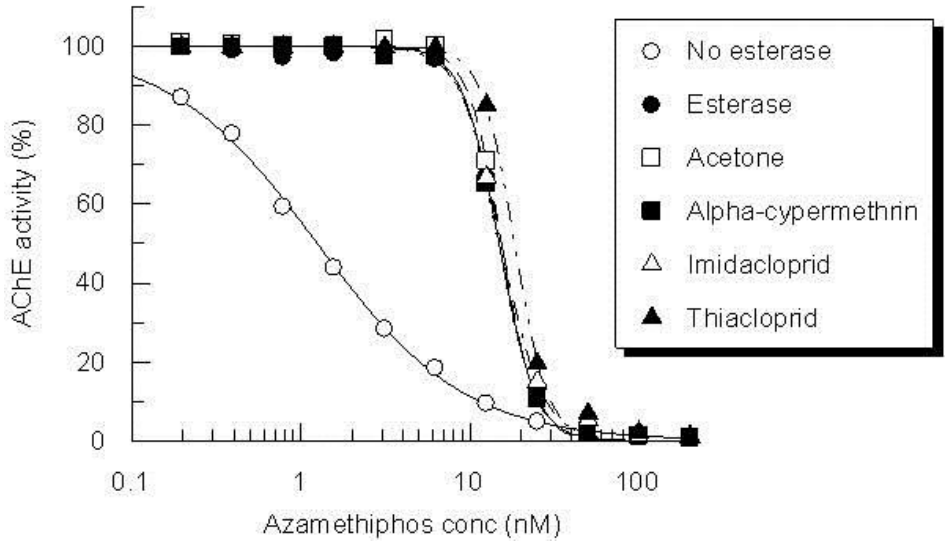


Figure 3.7 Binding of honey bee esterases to α-cypermethrin, imidacloprid and thiacloprid.

3.3.3 Oxidase assays

Although measurement of honey bee oxidase activity has been reported (Kezic *et al*., 1992) microsomal preparations from honey bee homogenate failed to demonstrate any *O*-deethylation activity (Figure 3.8), perhaps due to strong protease activity (as reported in aphids) (Philippou *et al*., 2010).

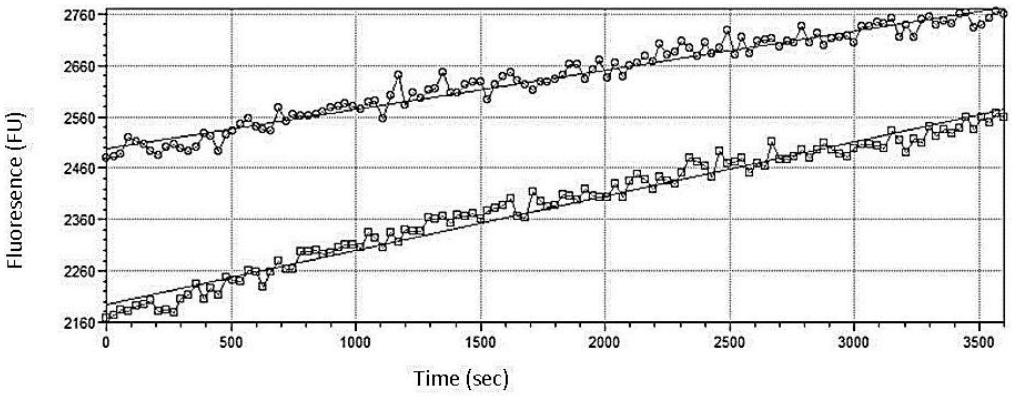


Figure 3.8 *O*-deethylation activity of bee microsomal homogenate (homogenate+substrate (hollow circles) and honey bee microsomal homogenate-substrate (hollow squares)).

However, a method using honey bee intact abdomen bioassay was utilized to measure *O*-deethylation inhibition by PBO and EN 16/5-1. The ability of PBO and EN 16/5-1 to inhibit honey bee oxidase activity was compared using 7-EC as substrate (Table 3.2). The activity after inhibition by EN 16/5-1 is greater than that found after inhibition by PBO. This is a strong indication that the honey bee oxidases that are capable of *O*-deethylation, in common with those found in many insect pest species, are more susceptible to inhibition by a molecule containing an intact MDP ring.

Table 3.2 Inhibition of honey bee *O*-deethylation activity by PBO and EN 16/5-1 (Values are means of triplicates).

Treatment	*O*-deethylation ($fmol^{-1}$ bee^{-1} h^{-1})	±SE	% Activity remaining	±SE
Acetone	174	23.5	100	13.5
PBO	87.8	4.3	50.3	7.2
EN 16/5-1	135	17.8	77.4	10.2

3.3.4 Protease activity measurement

The substrate ala-ala-ala-pNA offers an indirect measurement possibility for protease activity by the proteolytic cleavage of ala-ala-ala resulting in free pNA and so allowing the measurement of esterase activity. Because of the suspected high protease activity while measuring the oxidase activity this experiment has been conducted to exhibit proteolytic activity of tested honey bees. All the insect body parts examined showed varying protease activity against the substrate ala-ala-ala-pNA (Table 3.3). These proteases may affect the microsomal extract and cause the failure of oxidase activity measurement. There were several protease inhibitors included in the microsome preparations, but this does not negate the possibility of

protease inhibition of P450 activity. It has been reported that trypsin-like, chymotrypsin-like enzymes and the total caseinolytic activity within the honey bee midgut was age dependent (nurse bees had the highest activity) (Grogan and Hunt, 1980; Moritz and Crailsheim, 1987). When the functional role of nurse bees is considered, the proteolytic activity in the midgut is probably employed to digest rich proteins like pollen (Crailsheim, 1990). Therefore the variation of the thorax and abdomen activity against the substrate ala-ala-ala-pNA may be explained by the age-correlated differences of tested bees. For the future, P450 activity measurement studies using microsomes may be possible if the proteases responsible for P450 inhibition are identified and nullified by bespoke proteolytic inhibitors.

Table 3.3 Protease activity of head, thorax and abdomen with the presence and absence of ala-ala-ala-pNA.

	with substrate			without substrate		
Body parts	**mean**	**n**	**SEM**	**mean**	**n**	**SEM**
Head	5.14	3	0.14	-0.16	3	0.1
Thorax	20.8	3	4.96	-0.07	3	0.02
Abdomen	13.27	3	2.47	0.08	3	0.08

3.4 Discussion

Prior work has documented the involvement of three detoxification enzyme families in honey bee 'defence' against pyrethroids and their contribution to metabolism of tau-fluvalinate in the presence and absence of several enzyme inhibitors by calculating the synergism ratios (LD_{50} pyrethroid without inhibitor/LD_{50} pyrethroid with inhibitor). Johnson *et al*. (2006), for example, reports that tau-fluvalinate toxicity was enhanced with PBO and DEF synergism (980-fold and 4.8-fold synergistic ratio respectively).

Similar detoxification studies are also published with neonicotonids, e.g. the investigations on enzyme inhibitors by Iwasa *et al*. (2004), which highlights that whilst PBO enhances thiacloprid toxicity, there was no significant effect on imidacloprid toxicity.

In this chapter, the contribution of esterases and P450s to tau-fluvalinate and thiacloprid metabolism was assayed in honey bees. Synergist bioassay results with PBO and EN 16/5-1 suggest that detoxification mechanism(s) play an important role in protecting honey bees from tau-fluvalinate and thiacloprid toxicity. P450s were found to contribute the most protection to tau-fluvalinate, which confirms data from Johnson *et al*. (2006) and to thiacloprid confirming the results of Iwasa *et al*. (2004). Bioassays with collected samples from two different honey bee colonies showed different results for synergist alone mortality (i.e. the controls). This variation in results may be linked to the differential exposure to xenobiotics, age differences of tested bees and seasonal variation in different honeybee colonies (Terriere, 1984 and Meled *et al*., 1998).

However, in the case of tau-fluvalinate it is seen that whilst the resistance-associated esterase from *Myzus persicae* is capable of binding to tau-fluvalinate and prevent it interacting with azamethiphos, the esterases in honey bees are seemingly unable to bind to the pyrethroid. Thiacloprid and imidacloprid were also assessed for interactions with honey bees' esterases, and also found to remain free of binding. Regardless, tau-fluvalinate remains an insecticide that can be considered relatively less toxic. Both the lack of esterase interaction and the relatively low toxicity of the molecule could possibly be due to the presence of an aromatic moiety in the acid part of the molecule. This could both prevent access to the active site of the esterase and

also lead to steric hindrance within the sodium channel of the honey bee. Alternatively, it may be that the action of honey bee esterases is limited to organophosphate insecticides. Further investigations would be needed to confirm this hypothesis.

Although there is evidence to measure oxidase activity in previous studies (Kezic *et al*., 1992) characterisation of oxidase activity could not be determined by the usual ECOD assay in preliminary experiments due to strong protease activity. An alternative method using intact bee abdomens was utilised. However, intrinsic activity was found to be very variable according to the abdomen tested. The action of PBO upon the oxidases resulted in sensitisation of the honey bees to insecticides, confirming the protection provided by this enzyme system.

As a future work it may be interesting to monitor how honey bee microsomes respond to the absence and presence of CYP3A4 (human P450) for which oxidase activity measurement is possible by using model substrate 7-EC. By that way it would be possible to build a preliminary understanding on whether honey bee microsomes have protease activity that inhibits P450 activity.

4. INDUCTION OF NEONICOTINOID INSENSITIVITY IN HONEY BEES AND GLOBAL TRANSCRIPTOME PROFILING

4.1 Introduction

The work described in this chapter aimed to establish which (if any) molecular defence mechanism(s) in bees can affect their sensitivity to neonicotinoids. It is known that in humans and other vertebrates neonicotinoids are metabolised by cytochrome P450 monooxygenases, heme proteins that contribute to insecticide detoxification metabolism in many organisms (Casida, 2011). Indeed, neonicotinoid resistance in a range of crop pests is reported to be most commonly mediated by P450 detoxification (Suchail *et al*., 2003). Although honey bees have a reduced number of P450 genes (46 P450s) compared to other insect genomes, it is not known whether this results in lower neonicotinoid detoxification. To better understand the detoxification function and response against xenobiotics of specific P450s in the honey bee, gene expression induction has been used as a method in studies to identify P450s that are induced following exposure to xenobiotics. Phenobarbital, which is a well-known general inducer of P450s, failed to enhance the expression of honey bee P450s (Johnson *et al*., 2012). In contrast, Kezic *et al*., (1992) reported that benzo(a)pyrene monooxidase activity was induced after exposure to benzo-(a)-pyrene, tau-fluvalinate and cymiazole. Moreover the CYP9Q family of P450s was found to be associated with tau-fluvalinate metabolism with CYP9Q1 and CYP9Q2 transcript levels being higher after exposure to tau-fluvalinate and cypermethrin and CYP9Q3 transcript levels being induced approximately 1.5-fold by tau-fluvalinate (Mao *et al*., 2011). Taken together these findings demonstrate that using xenobiotics as inducing factors might increase oxidative activity and lead to the identification of the specific P450s involved in detoxification of neonicotinoids in the honey bee.

Neonicotinoids include two groups of chemicals, the cyano-substituted (thiacloprid and acetamiprid) and the nitro-substituted (imidacloprid, clothianidin, thiamethoxam, dinotefuran, nitenpyram). Laboratory bioassays have demonstrated that cyano-substituted neonicotinoids have been shown to be orders of magnitude less acutely toxic to honey bees than nitro-substituted compounds (Iwasa *et al*., 2004). Insecticide bioassays of honey bees using inhibitors of detoxification enzymes has provided strong indications that the differential toxicity observed between the two groups of neonicotinoids is due to increased metabolism of cyano-substituted neonicotinoids, rather than intrinsic differences in their affinity for the nAChR (Iwasa *et al*., 2004). The use of synergists has suggested that the difference observed may be mediated by metabolic activity as pretreatment of honey bees with piperonyl butoxide (inhibitor of P450s and esterases) and other chemically distinct P450 inhibitors was shown to dramatically increase the toxicity of thiacloprid and acetamiprid, whereas no significant differences were observed between bioassays with imidacloprid alone and those pretreated with these inhibitors (Iwasa *et al*., 2004). These findings provided the rationale to use a cyano-substituted neonicotinoid (thiacloprid) as the P450 inducer in this PhD study.

The aims of this study were to use an induction strategy, in combination with a range of biological, biochemical and genomic approaches to determine: 1) do honey bees have the ability to mount a molecular defence (via gene induction) to a neonicotinoid (thiacloprid) after initial exposure to a sub lethal dose that results in a measurable alteration in phenotype to subsequent exposure? 2) What are the specific detoxification genes, particularly members of the P450 superfamily, induced by exposure to a neonicotinoid (thiacloprid)?

4.2 Materials and Methods

4.2.1 Insects

Studies in this chapter were carried out with two different honey bee strains: 1) For induction experiment 1, worker bees of *A. mellifera buckfast* were used which were collected from the entrance to a hive, 2) for induction experiment 2, newly emerged worker bees of *A. mellifera carnica* were used which were collected as sealed brood (see section 2.4, Chapter 2).

4.2.2 Toxicity bioassays

4.2.2.1 Contact toxicity tests

For contact toxicity tests separate samples were subjected to a range of thiacloprid concentrations by topical application. Each bee was topically dosed (1µL) with either thiacloprid in acetone or acetone alone applied to the dorsal thorax using a microapplicator (see section 2.5.1, Chapter 2).

4.2.2.2 Oral toxicity tests

Worker bees (~10) were transferred to plastic cages and then subjected to a range of thiacloprid concentrations for 24 hours through oral feeding (see section 2.5.2, Chapter 2).

4.2.3 Sampling for transcriptome profiling

To investigate thiacloprid as a potential P450 inducer, two unconnected induction experiments were carried out with different sampling methodologies/strategies employed

4.2.3.1 Induction experiment 1 - Sampling after topical application

Worker bees of *Apis mellifera buckfast* were dosed topically on the dorsal thorax region with 1 µL containing sub lethal dose of (LD_{25}) thiacloprid/acetone. At 1 h, 3 h and 10 h after topical application, treated and control bees (live bees) were stored in RNA*later*™ (Sigma-Aldrich) according to the manufacturer's instruction and transported from Ankara University, Turkey to Rothamsted Research, UK for microarray studies.

4.2.3.2 Induction experiment 2 – Sampling after oral exposure and post-exposure bioassay assessment of changes in phenotype

Thiacloprid toxicity was assayed *in vivo* after exposure to a sub-lethal concentration of thiacloprid, to check for measurable alteration in phenotype. A factorial set of 16 treatments was tested in two repeat experiments. Bees (9–15 per cage = one replicate, 72 cages in total) were fed either a sub-lethal dose (LD_5) of thiacloprid (dissolved in acetone and then sugar solution) or acetone in sugar solution (controls) for 24 h, as detailed above (16 and 20 cages for each in experiments 1 and 2, respectively). At each of 0 h, 48 h, 96 h and 144 h a diagnostic dose of thiacloprid (LD_{50}) in acetone was then topically applied to a subset of both the treated and control bees (two cages each in experiment 1, three in experiment 2); the remaining cages (two per feed treatment per experiment) received a topical application of acetone alone (Figure 4.1). Before and after topical application test/control bees were snap frozen in liquid nitrogen and stored at −80°C for subsequent molecular analyses.

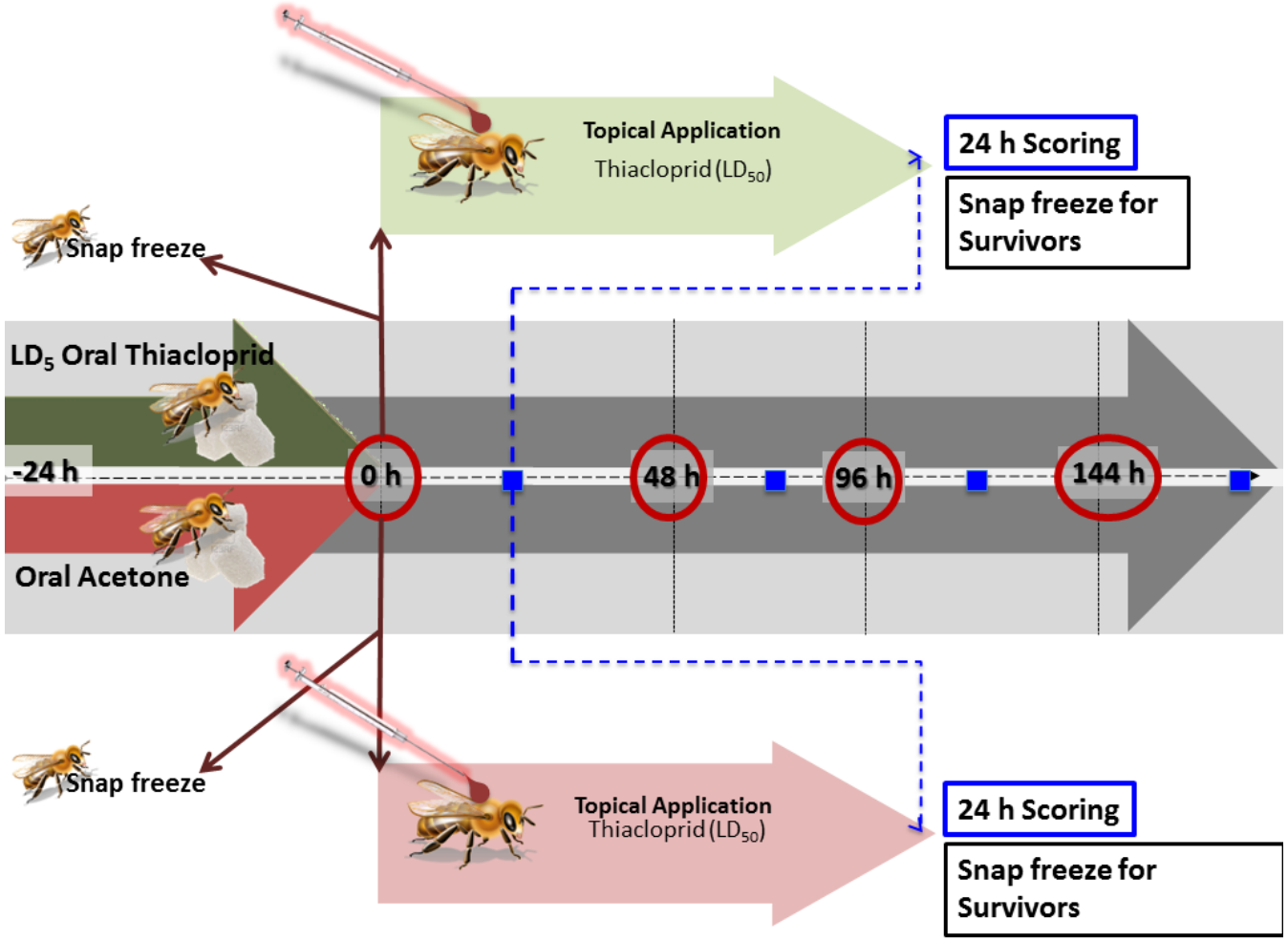


Figure 4.1 General scheme for induction bioassay and sampling.

4.2.4 Statistical analysis

For full dose oral and contact bioassays, the concentration of thiacloprid required to kill 5% of the population (LD_5, oral) and 50% of the population (LD_{50}, contact) were calculated as described in section 2.5.3, Chapter 2.

Data from the induction bioassays were analysed in GenStat (14^{th} edition, VSN International) using logistic regression (i.e. a generalized linear model with binomial error and logit link), allowing for differences between experiments before testing treatment effects and with adjustment for over-dispersion. Statistical analysis for the induction bioassay was carried out by a consultant statistician at Rothamsted Research, Suzanne Clark.

4.2.5 Microarray analysis and GO enrichment analysis

Transcriptome profiling in treated and control groups collected at different time points was assessed to screen gene expression as described at section 2.7.8, Chapter 2.

Functional annotation using Gene Ontology is anapproach to interrogate microarray data to identify common patterns in gene expression changes, in this case relating to biological process, cellular component and molecular functions (Ashburner *et al*., 2000). Enrichment analysis was performed using Fisher's exact test implemented in the Blast2GO software using a false discovery rate (FDR) of < 0.05 to characterize biological properties of significantly differentially expressed genes (Bariami *et al*., 2012). GO enrichment analyses were conducted on GO terms of differentially expressed genes compared to GO terms of all the genes in the microarray. GO term

enrichment analyses were run separately on lists of differentially expressed genes from each time point versus all detected genes.

4.2.6 qRT-PCR analysis

qRT-PCR was used to validate the microarray results for selected genes. All samples are normalized against two housekeeping genes (ef1 (elongation factor-1), and actin) The method is described in section 2.7.7, Chapter 2. Primers used are as in Table 4.1.

Table 4.1 Oligonucleotide primer sequences used in qRT-PCR

Gene	Primer	Sequence (5'-3')	Product size (bp)
CYP4AA1	Forward	TTGCCACATCATTTTCCAGA	115
	Reverse	TCTTGGTCCAGCACTAAAAGG	
CYP6AS3	Forward	TCGAAAGGGACGAGGATATG	129
	Reverse	CCAGTAGGCATCCCATGACT	
CYP305D1	Forward	GGACGTCCTTGGAACGAAT	108
	Reverse	TCGCATCATCCAATTTCGTA	
CYP315A1	Forward	CTGGGTCCCGTTTACAAAGA	101
	Reverse	GGTGTTGACCCTTCAAGTCG	
CYP6AS5	Forward	CAGGCTCTCCCCAATATTCA	120
	Reverse	TCGATGGGCTCATTTTTCTC	
CYP6BE1	Forward	CGAAAGGAACTTGCATAGCC	120
	Reverse	TCTTCGGAAAATCGTTCTGG	
Cytb5	Forward	CAGCGGAAGAAGTAGCGAAA	101
	Reverse	GCCTGGATGTTCGCTTAGAA	
GST1	Forward	AAAAATGCTTGTTATTTTCTGTCTGA	110
	Reverse	TCAAACGCGTCTTCGAGTATC	
FE4	Forward	TCTGCTTGCGCATTCTATTG	106
	Reverse	CTTTACGCGCTTCTTTGTCC	

4.3 Results

4.3.1 Induction experiment 1

4.3.1.1 Toxicity bioassay

Full-dose mortality response curves for contact toxicity bioassays with thiacloprid resulted in the dose for induction (LD_{25}) being calculated as 0.5 µg/bee (derived from fitted curve) (Figure 4.2).

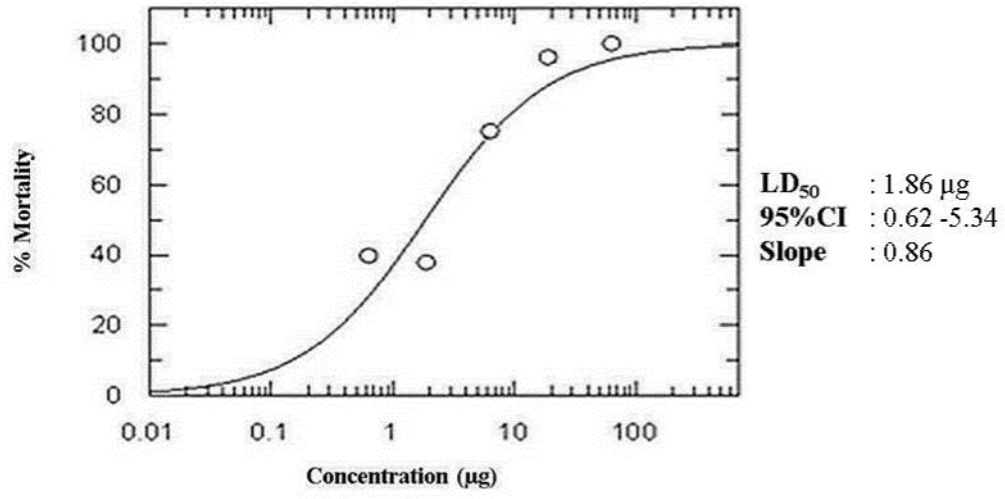


Figure 4.2 Full dose response contact bioassay results for thiacloprid on *Apis mellifera buckfast* population.

4.3.1.2 Transcriptome Profiling and qRT-PCR

Global gene expression in treated and control samples collected at 1h, 3h and 10h time points was assessed by whole transcriptome analysis using microarrays. Microarray analysis after a topical application of thiacloprid (LD_{25}) revealed limited responses in gene induction in treated compared with control bees for all three time points. In the microarray analysis 94 probes (69 up-regulated and 25 down-regulated), 93 probes (23 up-regulated and 70 down-regulated) and 59 probes (6 up-regulated and 53 down-regulated) were identified as significantly differentially expressed between control and treated bees at the 1 h, 3 h, 10 h time points respectively (Appendix 9.2). Only one P450 gene (CYP4AA1) was overexpressed (2.9 fold) after 1 h of thiacloprid treatment, in addition to two other detoxification genes encoding carboxylesterases and a nAChR gene subunit alpha 2. At the 3 hour time point a single P450 gene, CYP6AS3 was down-regulated, as were genes encoding a carboxylesterase and an ABC transporter. At the final 10 hour time point a number of CYP genes were down-regulated along with two carboxylesterase genes and an ABC transporter gene. The overexpression of CYP4AA1 and CYP6AS3 was

confirmed by qRT-PCR as shown in Figure 4.3. In qRT-PCR experiments the up-regulation of CYP4AA1 in the treated group was validated but CYP6AS3 down-regulation was not validated. During quality control of RNA samples prior to microarrays by electrophoresis it was noted that RNA extracted from samples stored in RNA*later*™ showed a degree of smearing indicating a certain amount of RNA degradation had taken place when samples were transported from Turkey to the UK. This finding may, in part, underlie the minimal induction response observed in subsequent microarray experiments.

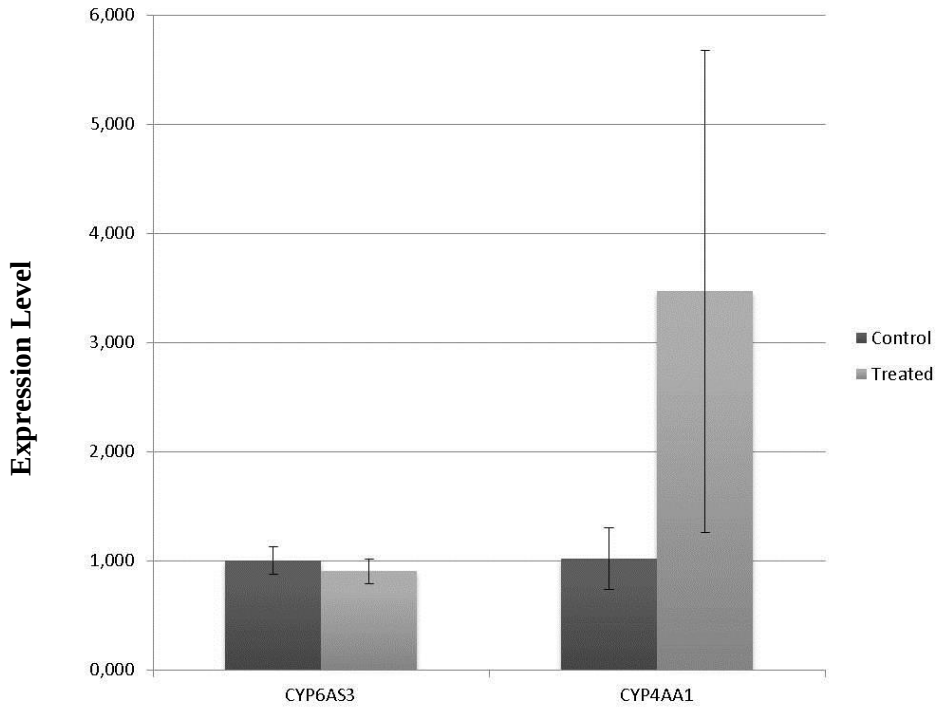


Figure 4.3 Expression levels determined with qRT-PCR for CYP4AA1 and CYP6AS3 using the geometric mean of selected housekeeping genes (ef1 and actin) (Error bars represent 95% confidence limits).

4.3.2 Induction experiment 2

4.3.2.1 Toxicity bioassays

Full-dose mortality response curves for oral and contact toxicity bioassays with thiacloprid resulted in doses for induction (oral LD_5) being calculated as 10 µg/bee (derived from fitted curve) and contact toxicity (LD_{50}) 62 +/-12 µg/bee. For the oral toxicity assays the LD_5 was calculated by assuming average consumption (0.055 mg/ml active) (Figure 4.4 and Figure 4.5).

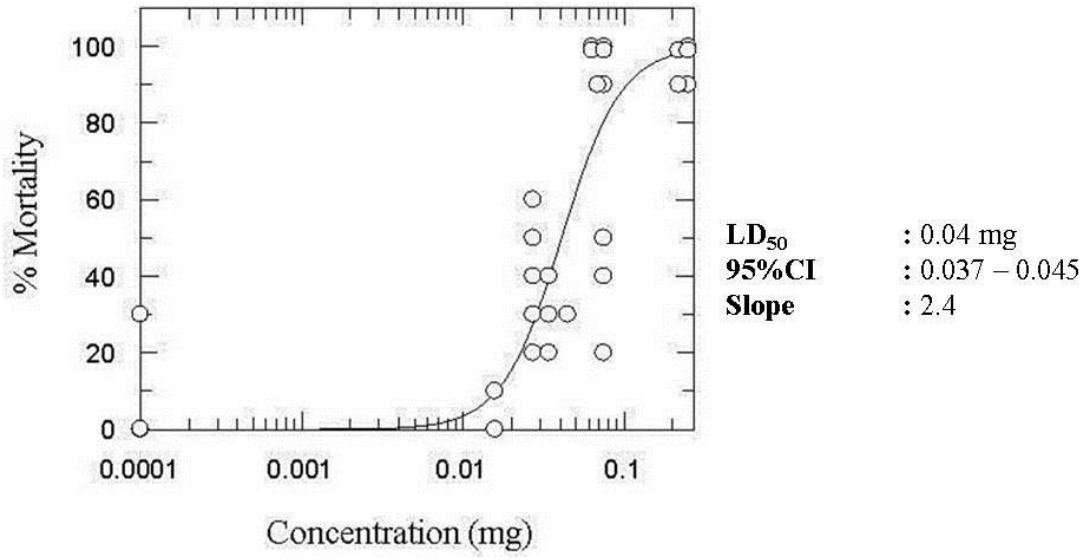


Figure 4.4 Results of analysis on oral toxicity (thiacloprid) data for *A. mellifera carnica*

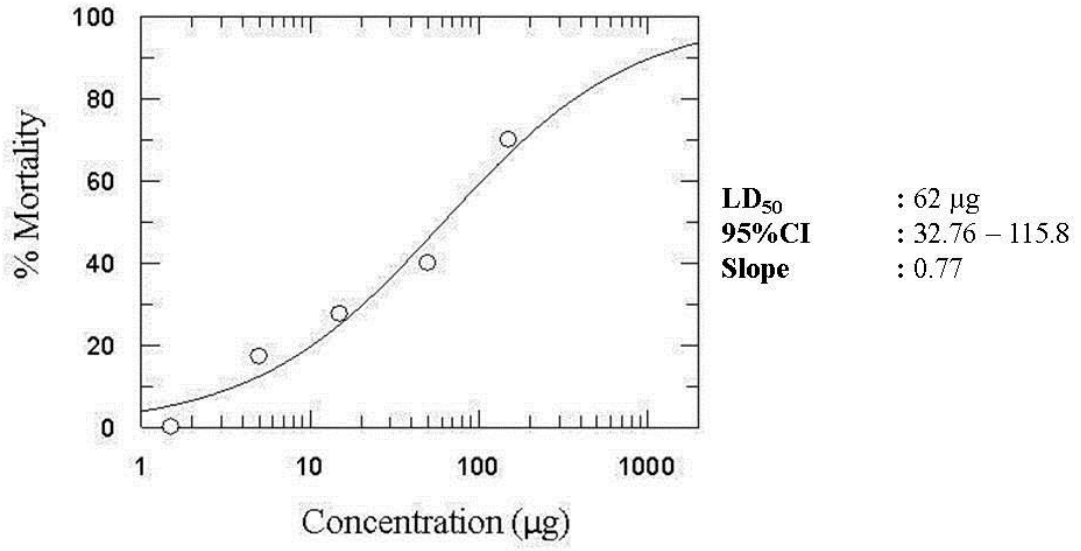


Figure 4.5 Results of analysis on contact toxicity (thiacloprid) data for *A. mellifera carnica*

4.3.2.2 Induction experiment

A second induction experiment was used to investigate if honey bees show a measurable alteration in phenotype to the neonicotinoid thiacloprid after initial exposure to a sub-lethal dose of the same compound. Significant differences were identified in the proportion of mortality resulting from topical application (i.e. whether bees were topically applied acetone or thiacloprid); time (i.e. time after pre-treatment that topical application was made); and with both feed and time parameters combined (i.e. within the group topically applied with thiacloprid, time after pre-treatment). Immediately following a pre-treatment time of 24 h, topical application resulted in a significantly increased tolerance to thiacloprid compared to the controls (t-test, $P = 0.006$). 48 h and 144 h following pre-treatment there were no significant differences between a thiacloprid or acetone pre-treatment (t-test, $P > 0.05$). At 96 h there was a significantly increased sensitivity in the bees pre-treated with thiacloprid (t-test, $P = 0.043$) (Table 4.2, Table 4.3 and Figure 4.6).

Table 4.2 Comparison of mean mortalities (logit scale) for bees fed either thiacloprid or acetone and subsequently topically applied with acetone at various times post-feeding

TIME	0 h	48 h	96 h	144 h
Difference	0.966	0.471	-0.419	0.766
t-statistic	0.628	0.384	-0.403	0.470
***P* value**	0.5327	0.7024	0.6885	0.6403

Table 4.3 Comparison of mean mortalities (logit scale) for bees fed either thiacloprid or acetone and subsequently topically applied with thiacloprid at various times post-feeding

TIME	0 h	48 h	96 h	144 h
Difference	-1.351	-0.628	0.978	0.789
t-statistic	-2.884	-1.199	2.075	1.547
***P* value**	0.0057	0.2360	0.0430	0.1279

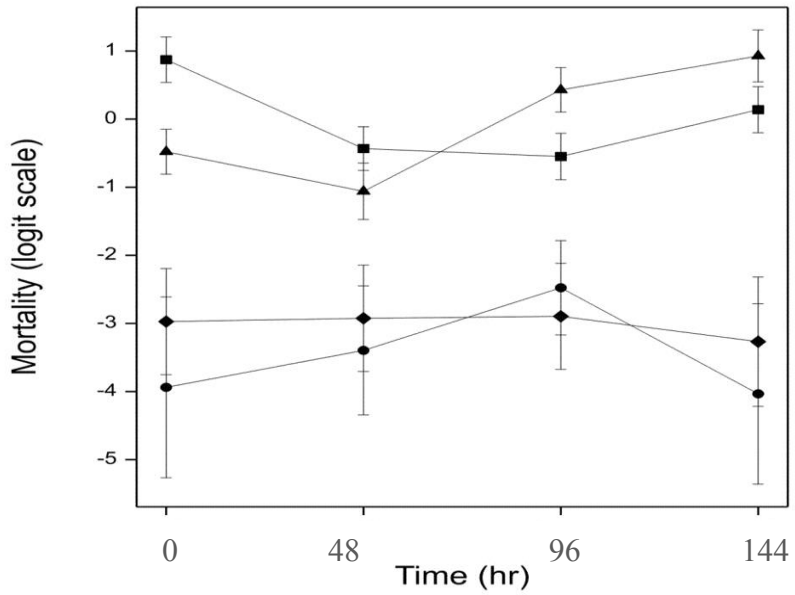


Figure 4.6 Predicted treatment mean mortalities (logit scale; n = 1–3 cages of 9–15 bees) from logistic regression (± SE). Fed acetone, topical acetone (circles); fed thiacloprid, topical acetone (diamonds); fed acetone, topical thiacloprid (squares); fed thiacloprid, topical thiacloprid (triangles). Time = delay following 24 h oral pre-treatment.

4.3.2.3 Transcriptome Profiling

Transcriptome profiling using microarrays was used to compare gene expression in bees fed sucrose-insecticide (treated) and those fed sucrose syrup (control) at each time point. An additional array comparison was conducted comprising bees fed sucrose-insecticide that subsequently survived the 0 h topical bioassay versus the non-treated control from the same time point ('survivor' experiment). In the time course experiment 21 probes (11 up-regulated and 10 down-regulated), 42 probes (21 up-regulated and 21 down-regulated), 27 probes (20 up-regulated and 7 down-regulated) and 13 probes (7 up-regulated and 6 down-regulated) were identified as significantly differentially expressed between control and treated bees at the 0 h, 48 h, 96 h and 144 h time points respectively. In the 'survivor' experiment 96 probes were identified as differentially expressed (57 probes were up-regulated and 39 down-regulated) between treated bees surviving the topical bioassay at 0 h and non-treated controls from the same time point (Appendix 9.3). Gene enrichment analysis based on gene ontology revealed the enrichment of a number of GO-terms in the differentially expressed gene sets of each time point with terms related to stress response ('innate immune response', 'defence response to bacterium', 'response to oxidative stress', 'antioxidant activity'), a common theme observed between the time points. A greater number of GO-terms were enriched in the 'survivor' experiment, with several terms suggestive of enhanced oxidative/P450 activity including 'oxidoreductase activity', 'oxidation-reduction process', 'heme binding' and 'monooxygenase activity' (Figure 4.7, Figure 4.8, Figure 4.9, Figure 4.10, Figure 4.11, Figure 4.12, Figure 4.13, Figure 4.14, Figure 4.15 and Figure 4.16).

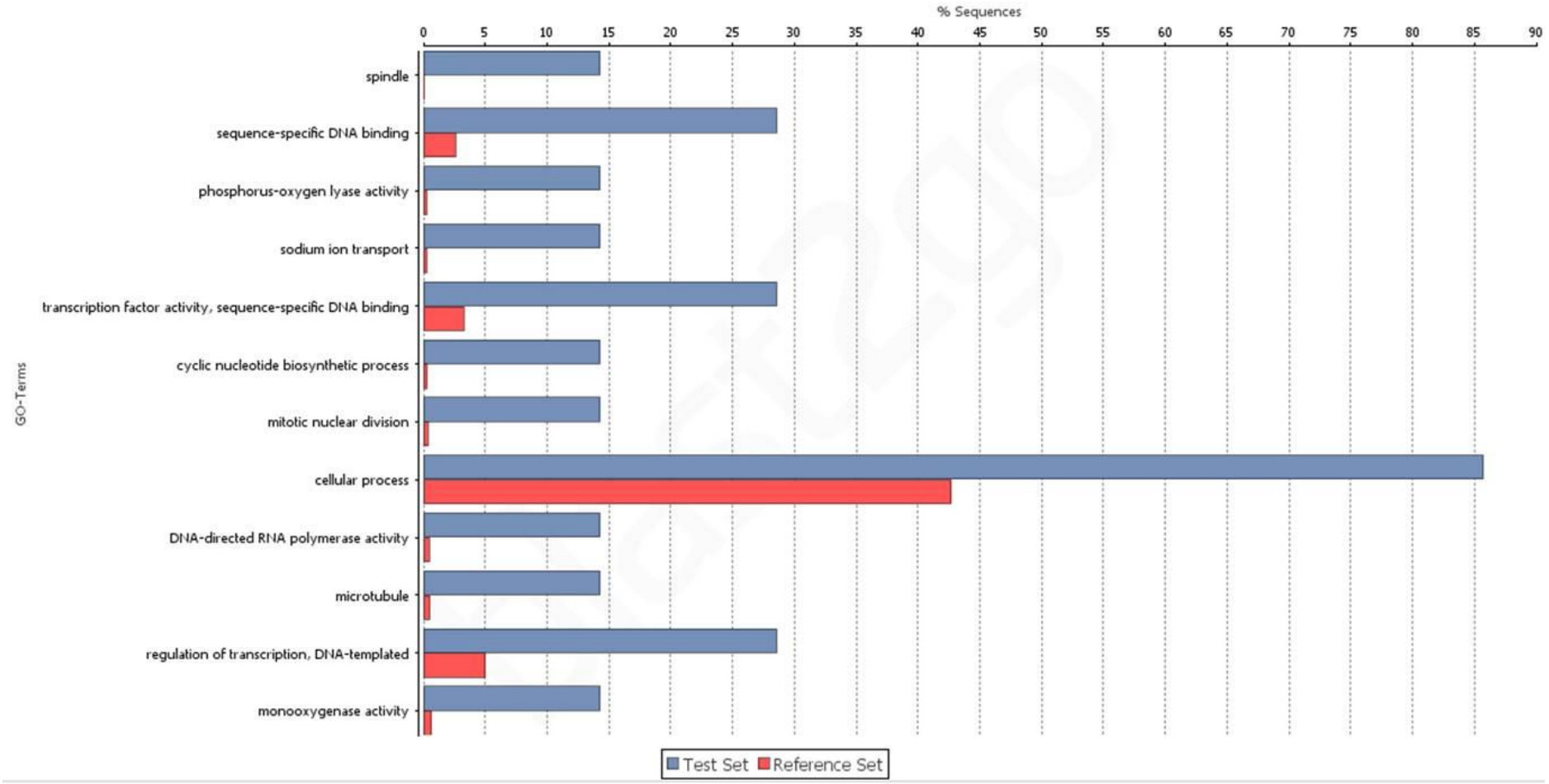


Figure 4.7 Enriched Gene Ontology (GO) terms in genes differentially up-regulated in microarray experiment (0h).

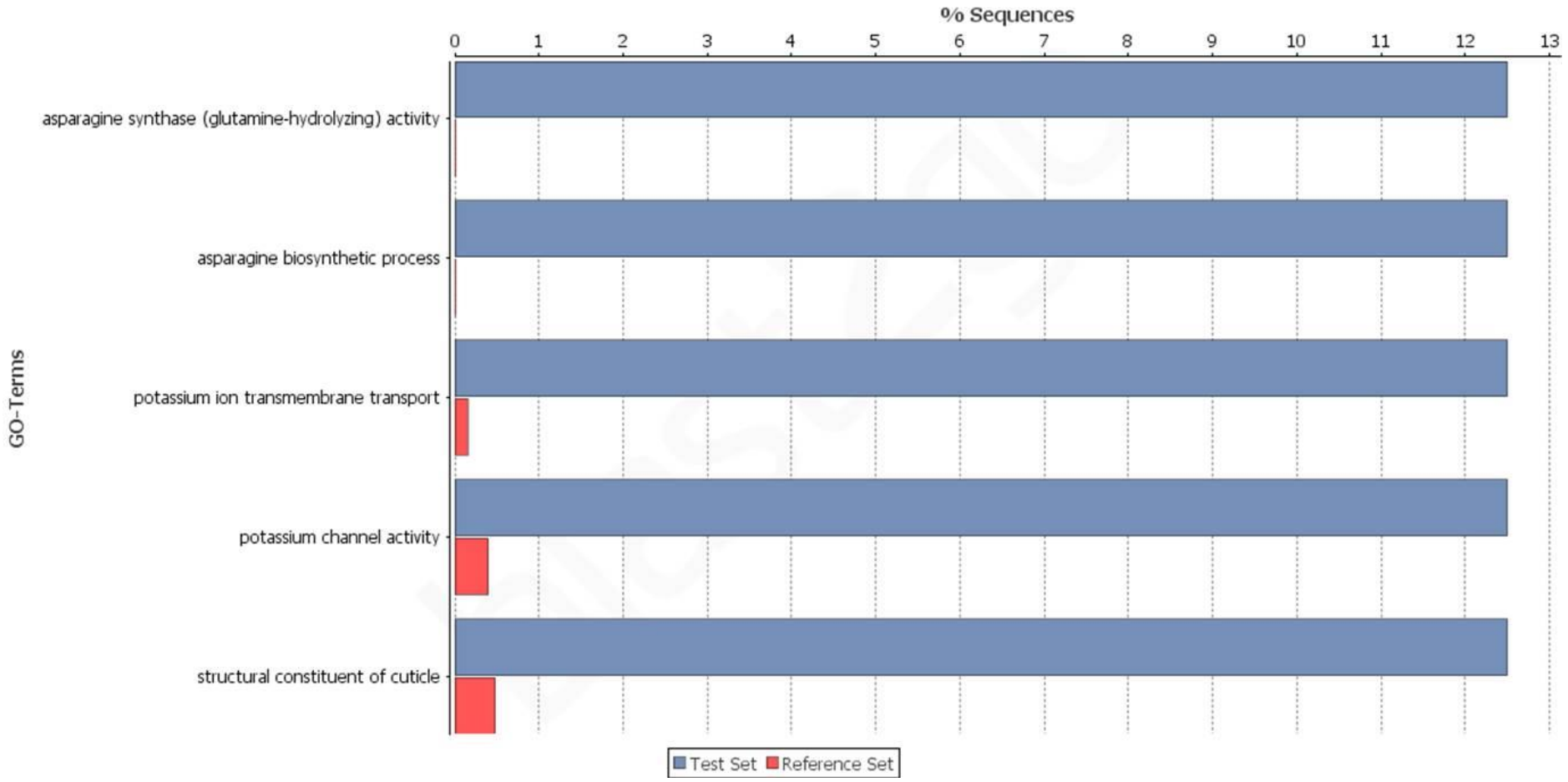


Figure 4.8 Enriched Gene Ontology (GO) terms in genes differentially down-regulated in microarray experiment (0h).

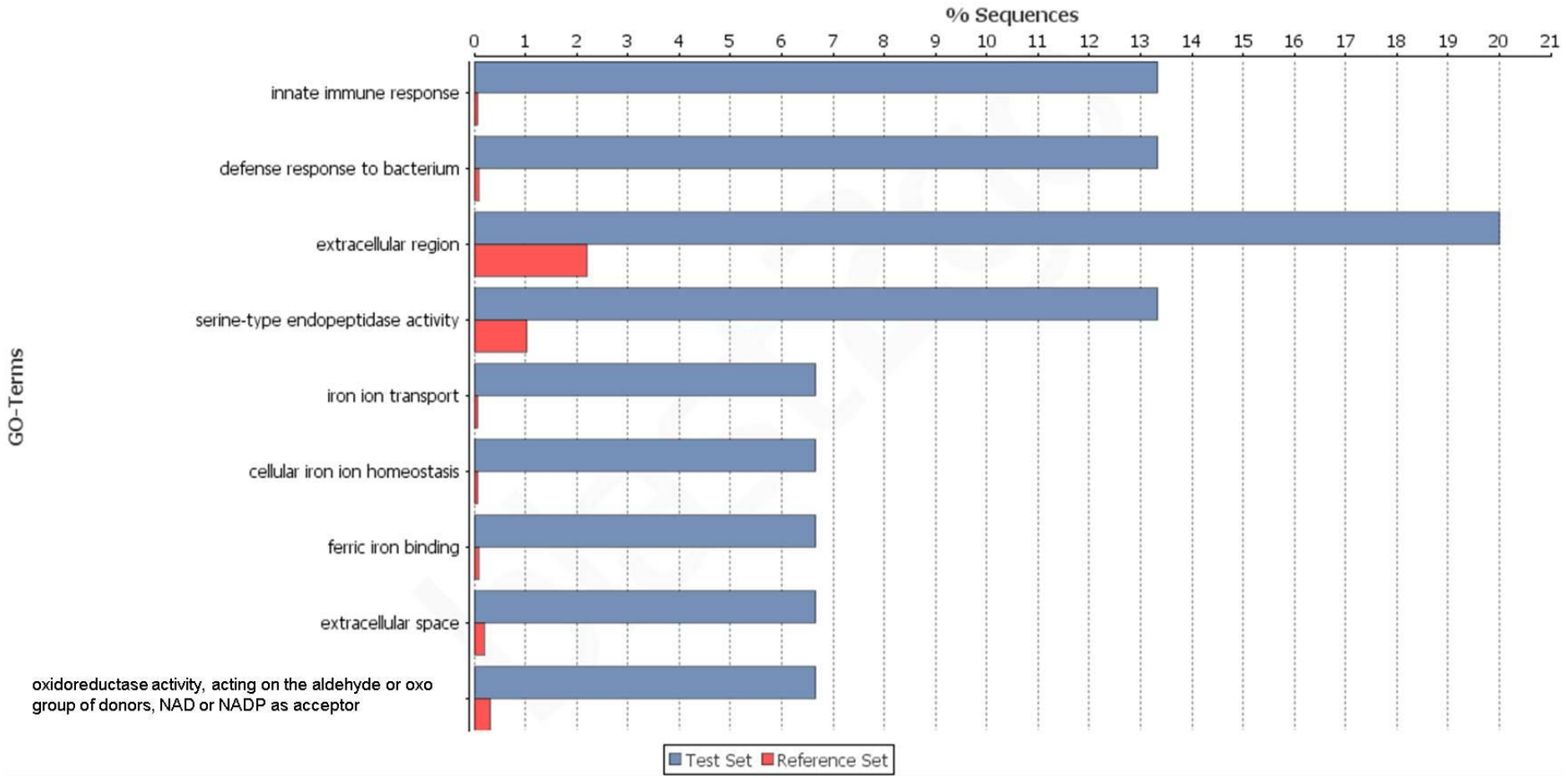


Figure 4.9 Enriched Gene Ontology (GO) terms in genes differentially up-regulated in microarray experiment (48h).

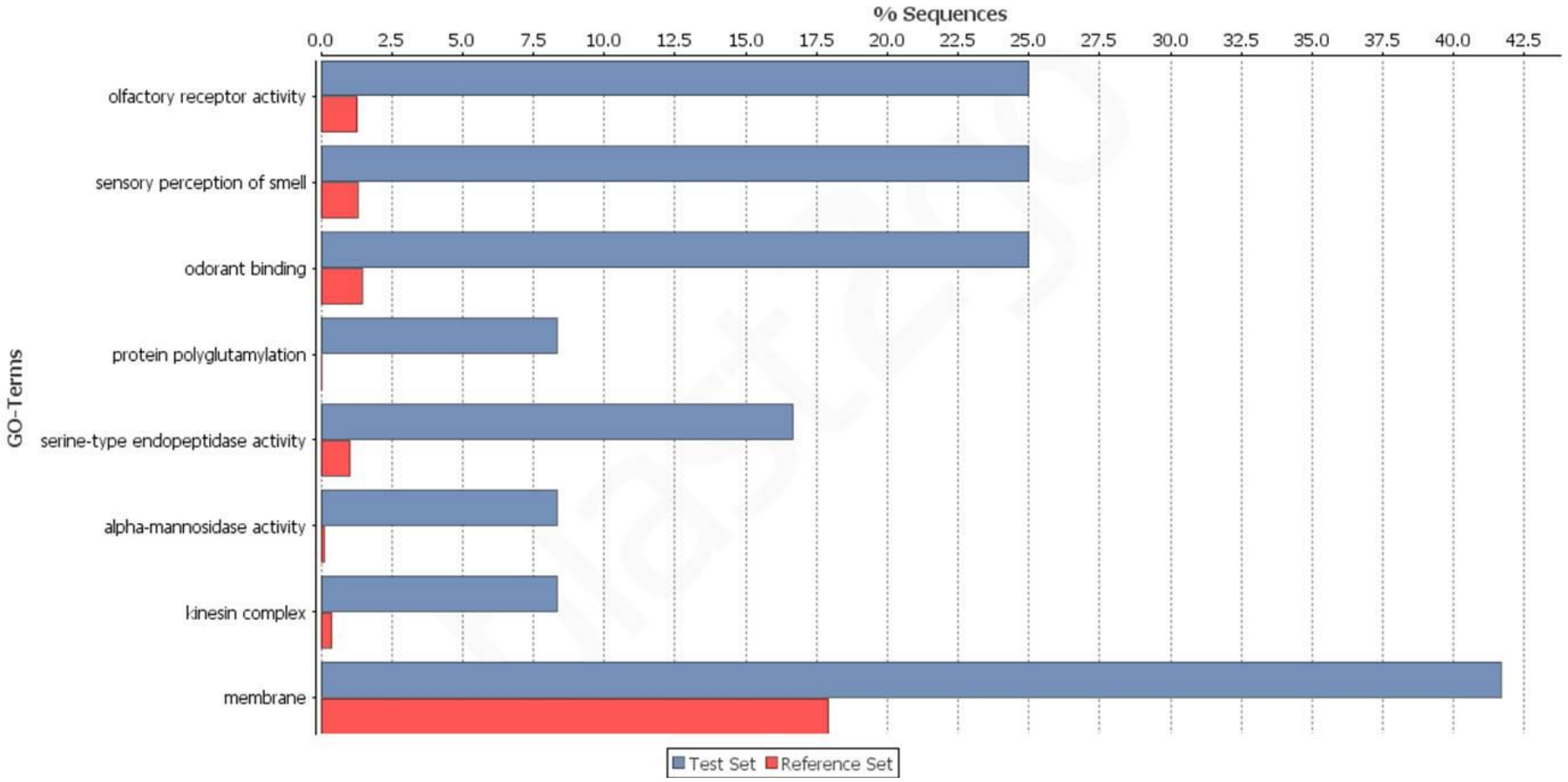


Figure 4.10 Enriched Gene Ontology (GO) terms in genes differentially down-regulated in microarray experiment (48h).

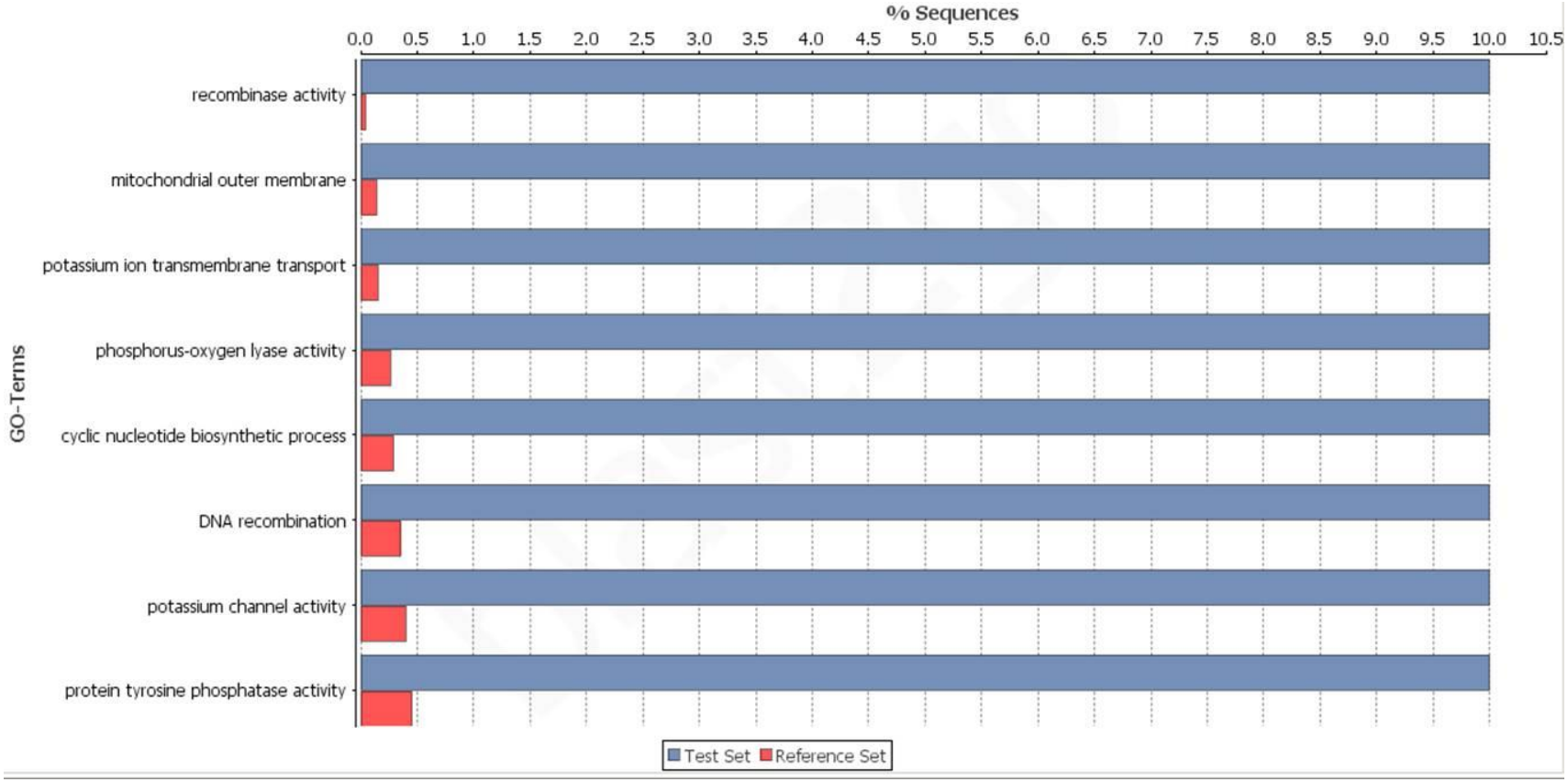


Figure 4.11 Enriched Gene Ontology (GO) terms in genes differentially up-regulated in microarray experiment (96h).

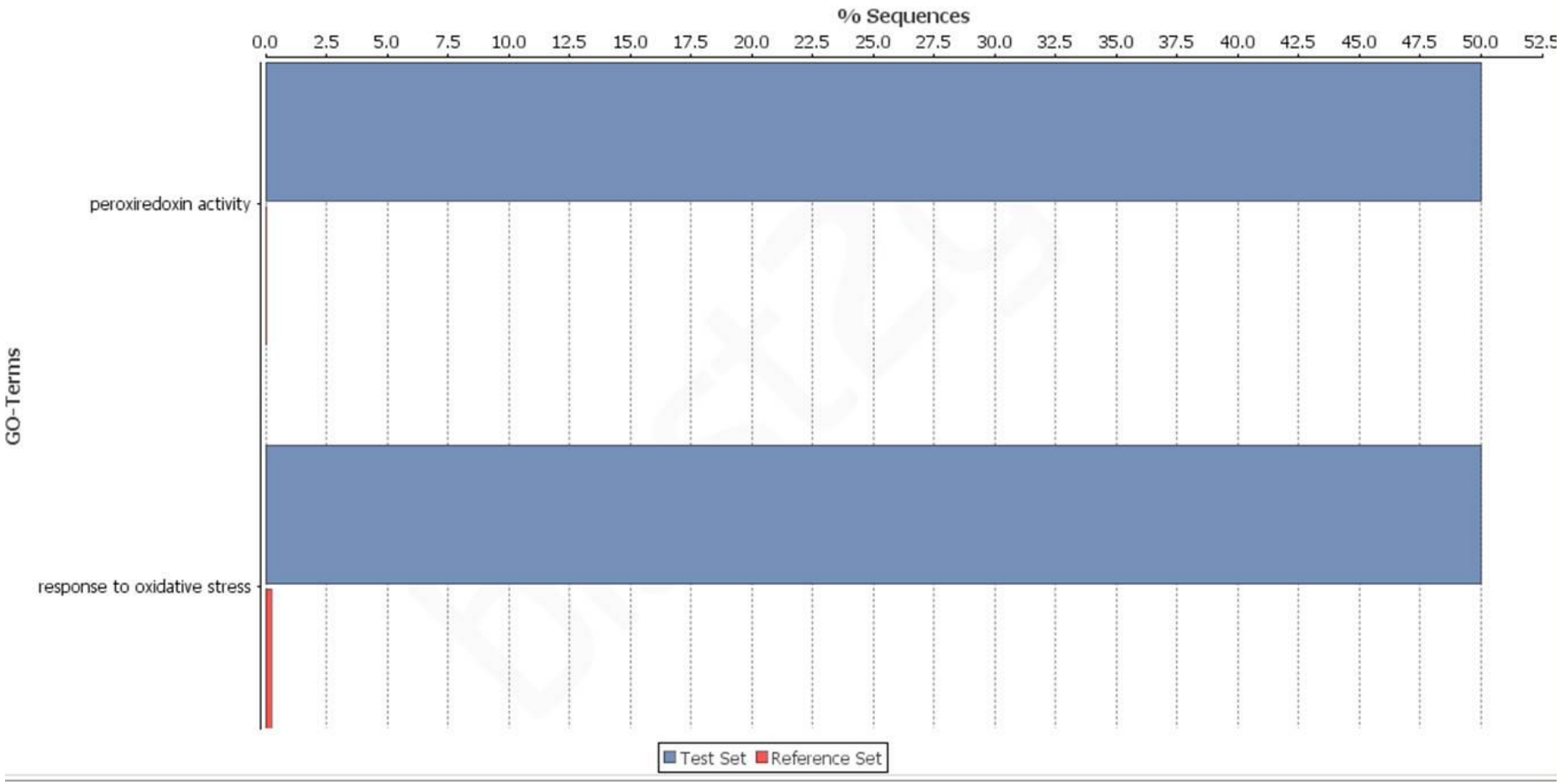


Figure 4.12 Enriched Gene Ontology (GO) terms in genes differentially down-regulated in microarray experiment (96h).

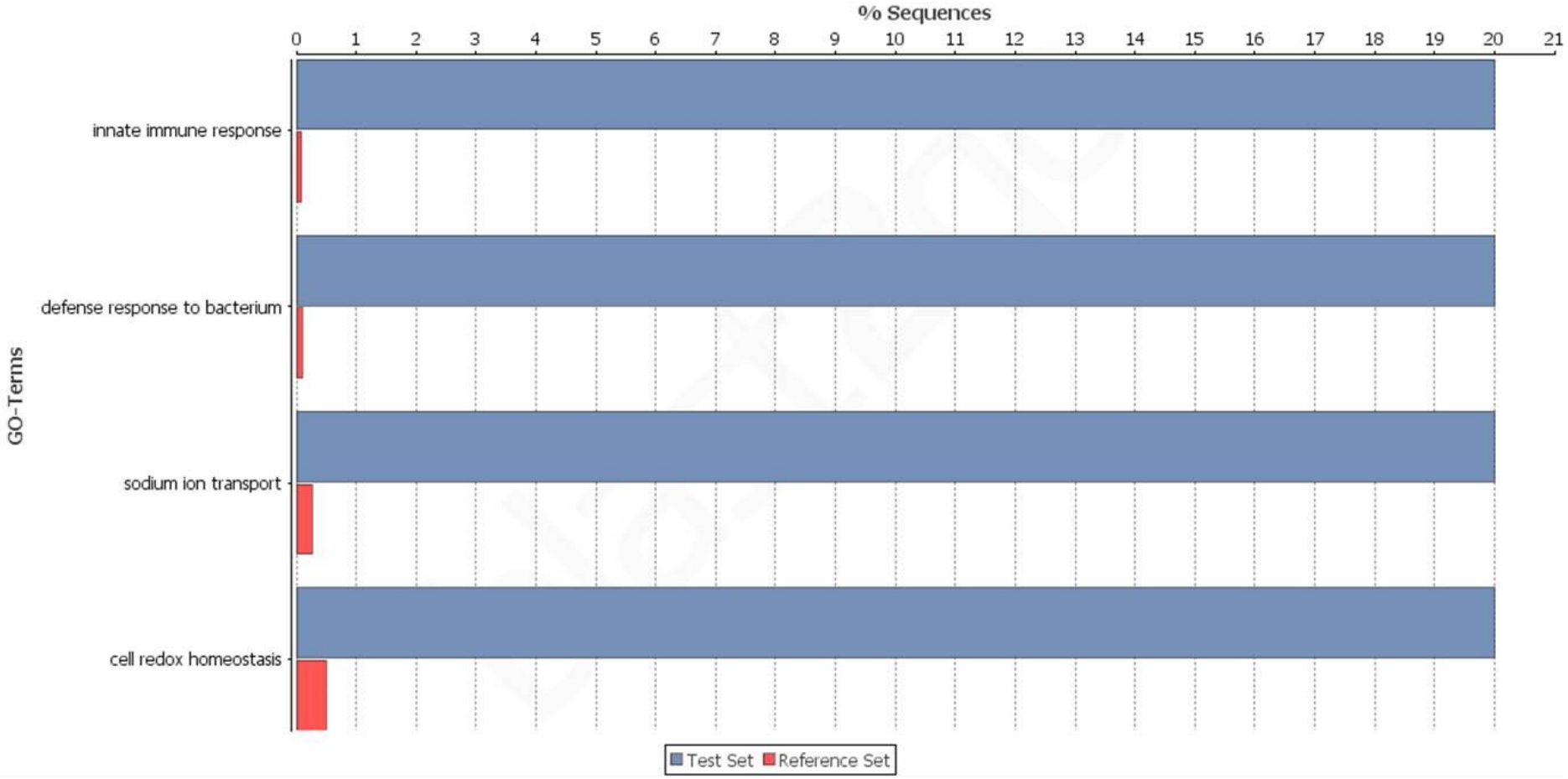


Figure 4.13 Enriched Gene Ontology (GO) terms in genes differentially up-regulated in microarray experiment (144h).

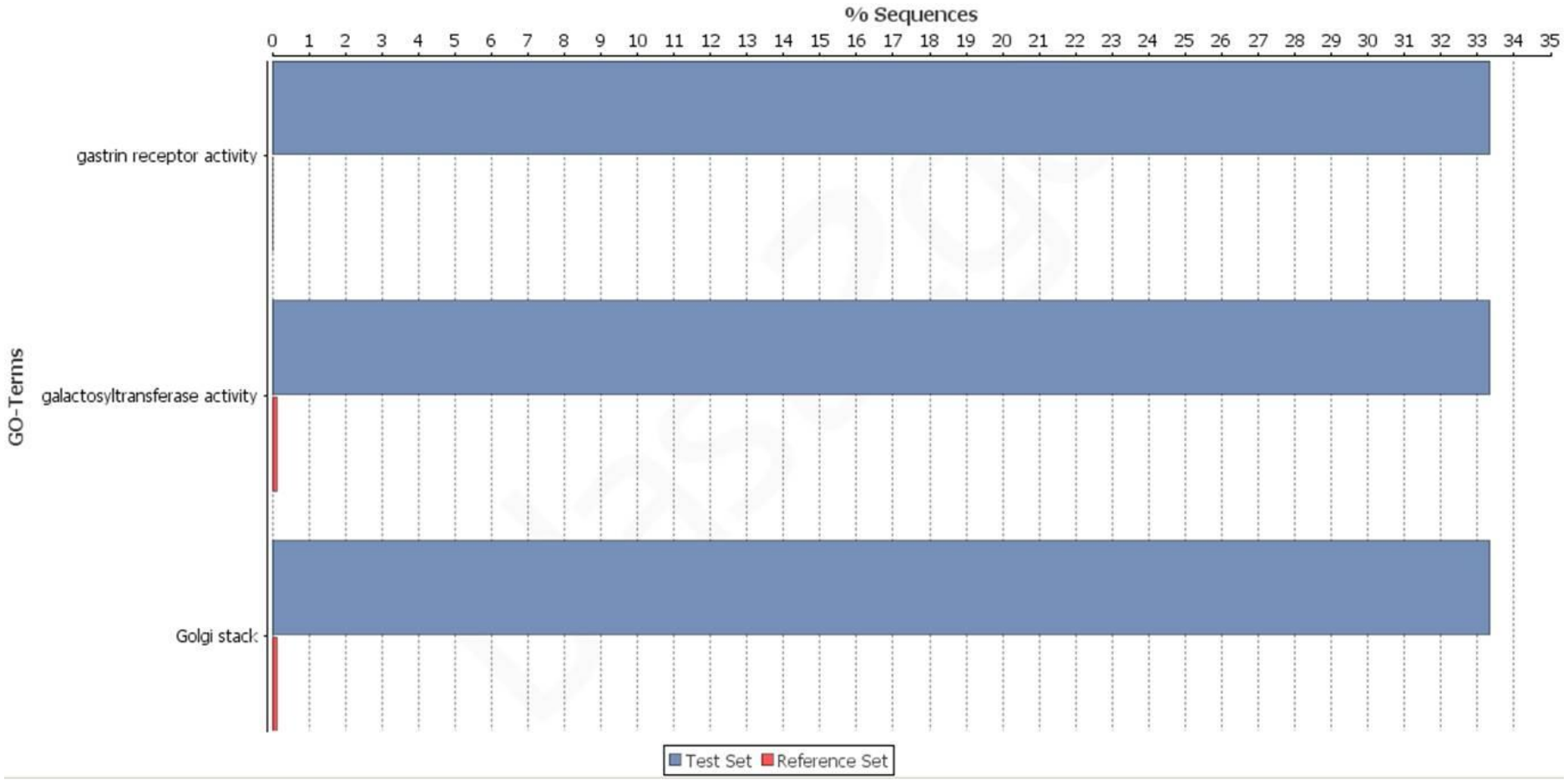
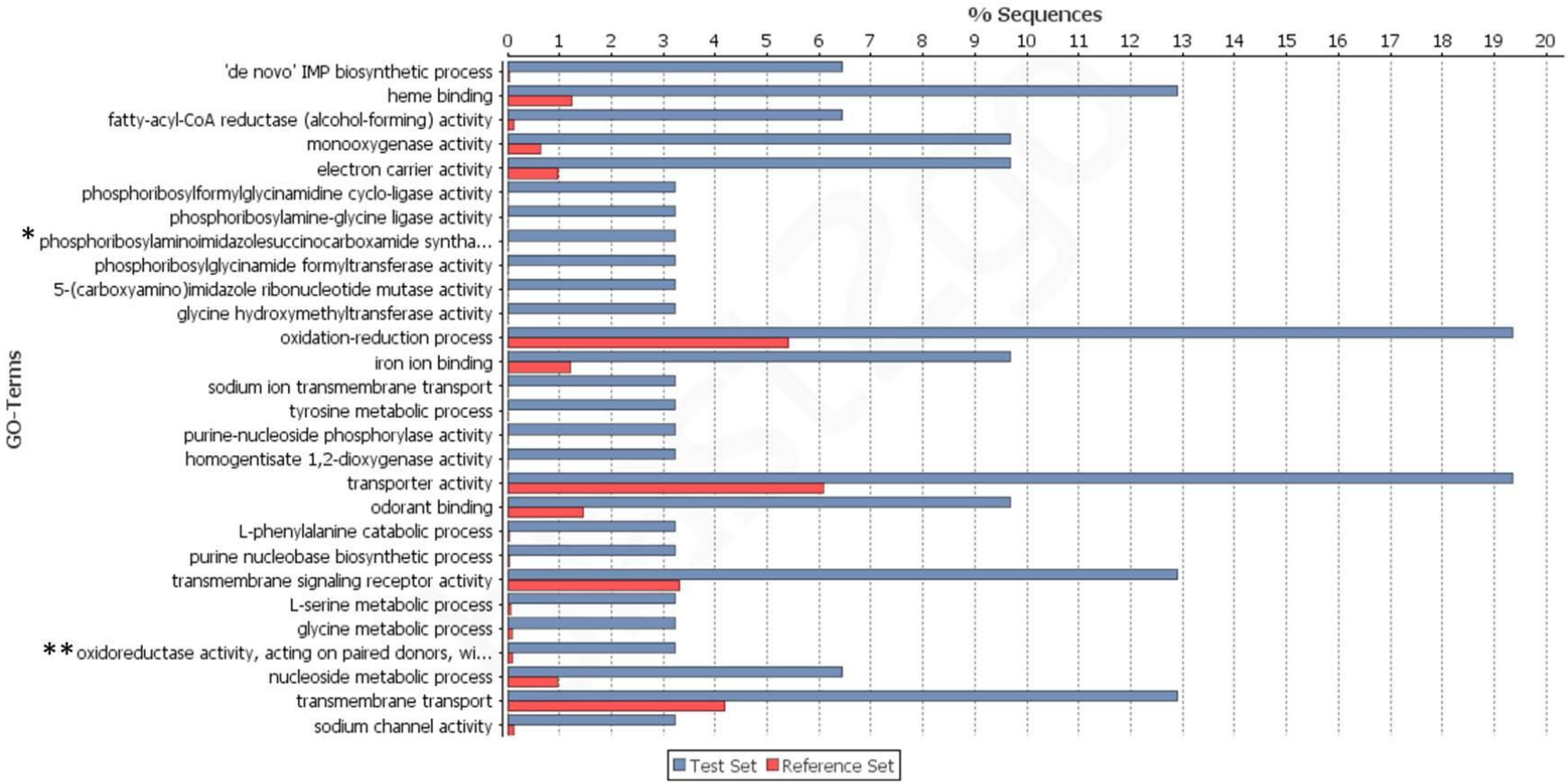


Figure 4.14 Enriched Gene Ontology (GO) terms in genes differentially down-regulated in microarray experiment (144h).



* phosphoribosylaminoimidazolesuccinocarboxamide synthase activity

** oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water

Figure 4.15 Enriched Gene Ontology (GO) terms in genes differentially up-regulated in microarray experiment (survivors).

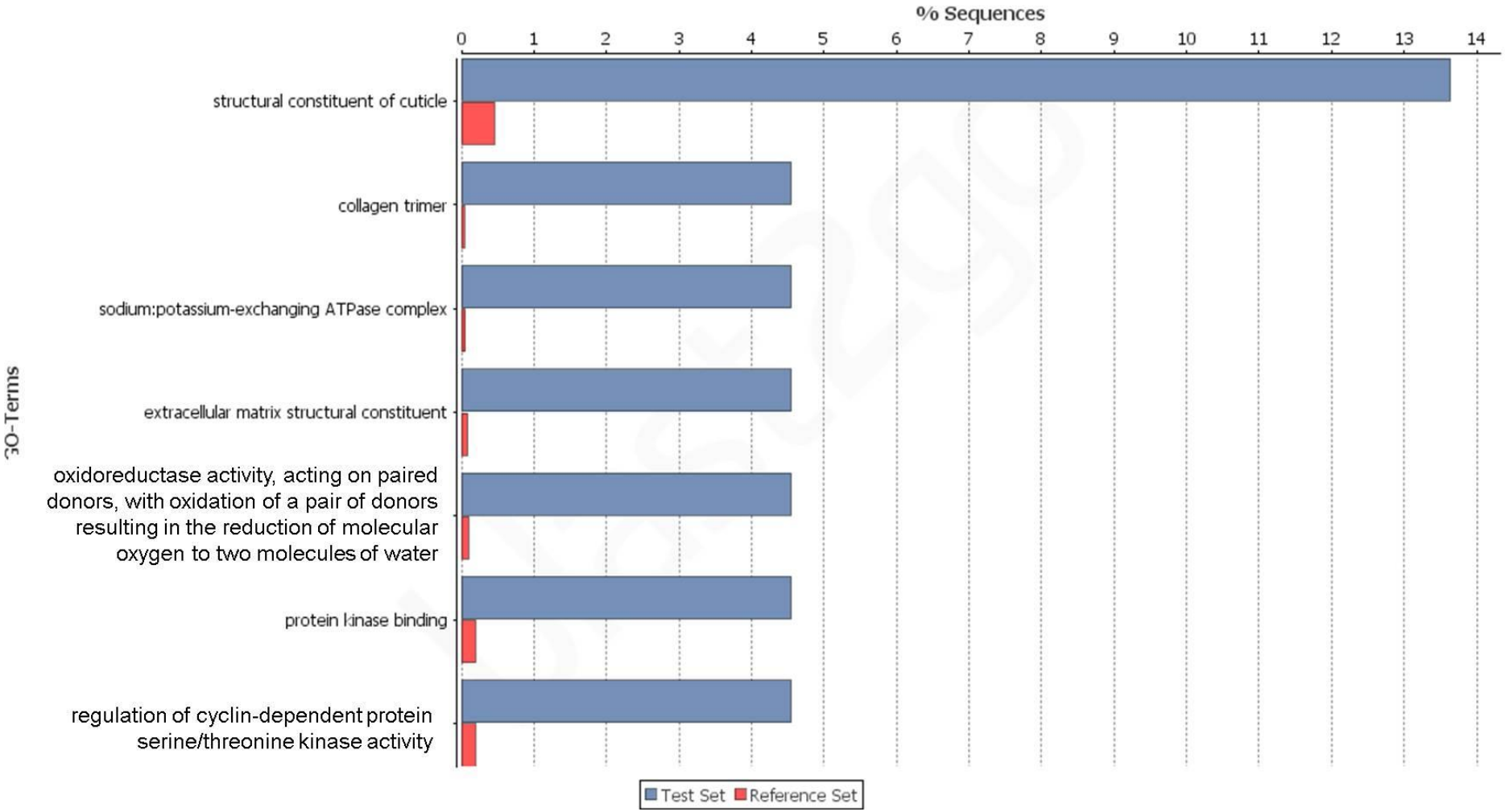


Figure 4.16 Enriched Gene Ontology (GO) terms in genes differentially down-regulated in microarray experiment (survivors).

In relation to the above among the differentially expressed probes were several that correspond to genes with putative roles in insecticide metabolism that are potential candidates to explain the alterations seen in phenotype in treated bees compared to controls. In the 0 h comparison two probes representing the P450 gene CYP315A1 were overexpressed (~1.5-fold) and in the 48 h comparison a single probe representing the gene cytochrome b5 was overexpressed 4.5-fold. At the 96 h time point three probes corresponding to the P450 gene CYP9Q1 and a single probe representing the carboxylesterase gene CCE11 were differentially expressed, however in all cases they were down-regulated (-1.5 to -1.7). At the 144 h time point no probes encoding detoxification enzymes were differentially expressed. The 'survivor comparison' displayed the greatest number of differentially expressed probes encoding detoxification genes with in all cases probes up-regulated. In the case of P450s this included four probes encoding CYP6BE1 (1.9-2.2-fold), four probes encoding the P450 CYP305D1 (1.8-1.9-fold), four probes encoding CYP6AS5 (1.6-1.7-fold) and a single probe encoding CYP301A1 (1.6-fold). For esterases five probes encoding CCE8 were up-regulated 2.1-2.2-fold. In the case of glutathione *S*-transferases a single probe encoding GSTD1 was over expressed 1.9-fold. Finally a single probe representing the gene cytochrome b5 was overexpressed (1.5-fold).

In relation to genes with regulatory roles which might explain the induction of the detoxification genes detailed above a number of probes encoding genes associated with the regulation of transcription/signal transduction were differentially expressed in multiple array comparisons. This included three G-protein-coupled receptor genes (GPCRs) in the survivor comparison (GB18244-RA, GB18304-RA and GB17560-RA), one GPCR related-gene (GB15369-RA) in the 0 h time point and one GPCR in

the 144 h time point (GB18786-RA). Two genes encoding transcription factors (GB15791-RA and GB10501-RA) were identified as differentially expressed in the 0h time point, one in the 48 h time point (GB14951-RA), and two in the 96 h time point (GB18833-RA, GB12301-RA).

The expression levels of seven of the detoxification candidate genes from the microarray experiment were validated by qRT-PCR with excellent concordance seen between fold-changes calculated using the qRT-PCR and array approaches (Table 4.4)

Table 4.4 Fold change in expression of candidate genes in treated groups compared to control groups.

Gene	Fold change (qRT-PCR)	95% confidence limits	Fold change (microarray)
CYP305D1	2.4	0.5	1.8-1.9
CYP315A1	1.8	0.6	1.5-1.6
CYP6AS5	1.4	0.1	1.6-1.7
CYP6BE1	1.7	0.4	1.9-2.2
Cyt *b5*	2	0.2	1.5
GSTD1	1.1	0.3	1.9
CCE8	3.3	0.4	2.1-2.2

The qRT-PCR experiments provided confirmation that six of the seven candidate genes were significantly upregulated in treated bees compared to controls with the exception of GSTD1 which was eliminated as a potential candidate as it showed no significant increase in expression in treated bees.

4.4 Discussion

Cyano-substituted neonicotinoids are reportedly less toxic to bees because of P450 metabolism to less toxic metabolites (Iwasa *et al*., 2004). However, the identification of specific enzymes contributing to thiacloprid metabolism and whether their expression is constitutive or induced upon exposure to neonicotinoids is unknown. A previous study reported no effect on the toxicity of the pyrethroid insecticide tau-fluvalinate to bees fed phenobarbital, xanthotoxin, salicylic acid, or indole-3-carbinol when compared to controls fed sucrose (Johnson *et al*., 2012). Although the effect of tau-fluvalinate feeding on subsequent tau-fluvalinate toxicity was not examined in the previous study, quercetin, a constituent of pollen and honey was found to reduce tau-fluvalinate toxicity (~1.4-fold) (Johnson *et al*., 2012). The main aim of present study was to address these two questions by feeding bees a sublethal dose of thiacloprid and assessing 1) changes in thiacloprid tolerance of honey bees in insect bioassays and 2) changes in gene expression in whole transcriptome microarrays.

In the first approach of induction experiment only a single gene encoding a P450, CYP4AA1, was induced. Although the induction of this CYP was confirmed by qRT-PCR, it is not a strong candidate to metabolise insecticides as it was reported that the function of CYP4AA1 is stearic acid hydroxylation during ecdysteroid hormone production. In addition owing to the absence of a complete coding sequence for CYP4AA1, a three-dimensional model of CYP4G11 (which has close homology to CYP4AA1) was used to demonstrate that the binding pocket of CYP4AA1 is likely to be too small to accommodate neonicotinoid compounds (Hlavica, 2011).

The bioassay time course experiment showed that a measurable reduction in thiacloprid sensitivity could be induced in honey bees after exposure to a sub-lethal

dose of this neonicotinoid for 24 hours. This effect was time dependent with increased tolerance only observed immediately following the 24 h of thiacloprid feeding, with treated bees becoming more susceptible than controls at the later time point (96 h). To our knowledge this is the first report with honey bees of a sub-lethal dose of an insecticide providing a protective effect to subsequent exposure of the same insecticide. Indeed, in a related study on honey bees, no effect on the toxicity of the pyrethroid insecticide tau-fluvalinate was observed in bees fed phenobarbital, xanthotoxin, salicylic acid, indole-3-carbinol compared to controls fed sucrose, although the effect of tau-fluvalinate feeding on subsequent tau-fluvalinate toxicity was not examined (Johnson *et al*., 2012).

One possible explanation for the finding of an induced decrease in sensitivity to thiacloprid in bees is that the sub-lethal exposure activates the transcription of one or more genes encoding detoxification/defence proteins over the 0-48 h time points and that these subsequently return to constitutive levels or lower than constitutive levels at the later time points. To explore this a series of microarray comparisons of global gene expression levels were carried out in treated versus control bees over the time series ('time course experiment') and a second experiment where treated bees surviving the topical bioassay at 0 h were compared with non-treated controls from the same time point ('survivor experiment'). Across all comparisons the number of genes differentially expressed (13-96 probes representing 0.08-0.6% of the 15737 probes on the array), and the fold changes observed (<8-fold), between treated and control bees were low. Nevertheless, the observed changes were subsequently confirmed by qRT-PCR with a number of candidate genes being validated as moderately, but significantly, over-expressed in treated bees. GO-term analysis of these differentially expressed genes revealed enriched ontology terms associated with

a general stress response and also terms relating to P450-mediated detoxification, the latter resulting from the enhanced expression of a number of probes encoding several honey bee P450s/cytochrome b5. P450 genes were only identified in the earlier time points (0 h, 0 h surivors, 48 h) where altered thiacloprid toxicity was observed in bioassays and the only gene related to detoxification observed in two separate array experiments was cytochrome b5, which can act as an electron donor to P450s. Among the CYP genes CYP315A1 was the only P450 identified as overexpressed in the time course experiment (at the 0 h time point) and this is the ortholog of the *Drosophila melanogaster* sad gene encoding the steroid 2-hydroxylase (Claudianos *et al*., 2006). However, in the 'survivor' experiment in which 'treated bees' were fed thiacloprid for 24 h and then survived a subsequent topical application of thiacloprid (LD_{50}) a number of P450s were identified as being over-expressed. This included two members of the CYP3 clade CYP6BE1 and CYP6AS5, whose members have been most commonly involved in detoxification of xenobiotics including pesticides in other insects (Li *et al*., 2007). Two further P450s CYP305D1 and CYP301A1, the latter of which was only represented by a single probe, belong to the CYP2 and mitochondrial clades respectively were also overexpressed. The role of CYP305D1 is yet to be determined but CYP301A1 is thought to be involved in ecdysone regulation during adult cuticle formation (Sztal *et al*., 2012). Beyond detoxification genes several genes involved in the regulation of transcription/signal transduction were also identified as differentially expressed in multiple array comparisons including both transcription factors and a number of G-protein-coupled receptors (GPCRs). It is possible that these genes may play a role in triggering/regulating the enhanced transcription of the CYP/detox genes. In the case of GPCRs recent work has suggested they may be involved in regulating overexpressed P450s observed in

resistant moquitoes, *Culex quinquefasciatus,* and housefly, *Musca domestica,* (Li *et al*., 2014; Li *et al*., 2013). For *C. quinquefasciatus* knockdown of four GPCR genes by RNAi both decreased resistance to permethrin and repressed the expression of four insecticide-resistance related P450 genes (Li *et al*., 2014). It would be interesting to examine the role of these receptors in honey bee gene expression responses to xenobiotics in more detail using a similar approach.

From the overexpressed genes induced in thiacloprid fed bees from this experiment four P450s, CYP6BE1, CYP6AS5, CYP315A1 and CYP305D1, cytochrome b5 and the esterase CCE8 all of which were represented by multiple overexpressed probes in array comparisons and validated by qRT-PCR, were selected for further functional analysis (see Chapter 5).

5. PROTEIN EXPRESSION OF HONEY BEE CYP305D1, CYP315A1, CYP6AS5, CYP6BE1, CYTOCHROME b5 AND CCE8

5.1 Introduction

Expression or production of recombinant enzyme proteins offers the opportunity to conduct structural and enzymatic characterisation in *vitro*. Recently, expression systems have become more commonly used for the characterisation of metabolic insecticide resistance or detoxification enzyme-mediated insecticide tolerance (Feyereisen, 1999; Li *et al*., 2007). Functional characterisation of detoxification enzymes associated with resistance is an important step in demonstrating their causal role in resistance. There are several approaches to express heterologous proteins in different host cells including *Escherica coli* (*E. coli*), yeast, baculovirus infected insect cells and mammalian cells. All have pros and cons for the expression of high amounts of high quality protein, therefore the most appropriate system for each specific study should be selected (Battula *et al*., 1987; Guengerich *et al*., 1993; Waterman *et al*., 1995; Grogan *et al*., 1995; Gonzalez and Korzekwa, 1995).

Expression in *E. coli* is a popular system to express cytochrome P450s because of the simple protocols involved, economy and its ability to provide large amounts of catalytically active P450s (Waterman *et al*., 1995; Pritchard *et al*., 2006). The first successful eukaryotic P450 expressed in *E. coli* was bovine P45017α (Barnes *et al*., 1991). However, the most important limitation of expressing eukaryotic P450 in *E. coli* has been identified as a requirement to modify the N-terminal sequence of P450s which Pritchard *et. al.* (1997) overcame by fusion of the N-terminal sequence of P450 with *E. coli* signal sequence, thus succeeding in expressing unmodified P450s.

It is well known that neonicotinoids can be metabolised by insecticide resistant insect pests as a result of the enhanced expression of specific cytochrome P450s, for example, CYP6CY3 in *Myzus persicae* and CYP6CM1 for *Bemisia tabaci* (Bass *et al*., 2013, Karunker *et al*., 2008). More recently *in vitro* characterisation of eight honey bee P450s of the CYP3 clan revealed that three members of the CYP9Q family have the capacity to metabolise the insecticides tau-fluvalinate and coumaphos (Mao *et al*., 2011). As discussed in the previous chapter several resistance detoxification genes were shown to be overexpressed or associated with metabolism by synergism studies, however, overexpression does not always mean that these genes are functionally responsible in detoxifying insecticides. Thus, clear identification of such metabolism needs further investigation to prove metabolism.

This chapter addresses the following question; do specific candidate detoxification genes which are induced upon exposure to a neonicotinoid (thiacloprid) (see Chapter 4) have the capacity to metabolise these compounds and explain the differential toxicity of different members of the neonicotinoid class?

5.2 Material and Methods

5.2.1 Functional P450 expression

5.2.1.1 Plasmid DNA extraction

The *E. coli* strain JM109 was grown in Luria-Bertani (LB) broth (25 µL of bacteria cells in 5 mL LB) (Appendix 9.1.1) at 37°C with shaking (200 rpm). DNA was isolated from 5 mL bacterial culture using the GeneJET plasmid miniprep kit (Thermo Scientific) following the manufacturer's protocol.

5.2.1.2 Cloning CYPs for expression in Escherichia coli

Honey bee CYP305D1, CYP315A1, CYP6AS5, CYP6BE1 were amplified from cDNA using KAPA high-fidelity DNA Polymerase (Kapa Biosystems) following the product manual and using gene specific primers and the annealing temperatures listed in Table 5.1 in PCR. As a proofreading DNA polymerase was used for amplification, which leaves blunt ended DNA, A-tailing reactions were run before cloning the gel purified or PCR purified PCR products. An adenosine residue was added by incubating the PCR product with 0.25 µL of 5U/µL GoTaq® Flexi DNA Polymerase (Promega), 6 µL of 25 mM $MgCl_2$, 10 µL of 5X GoTaq® Green Reaction Buffer (Promega), 1 µL of 100 mM dATP and nuclease-free water (to 50 µL) at 70°C for 20 minutes. After the product was cleaned, it was ligated into the pSC-A-amp/kan cloning vector (StrataClone PCR Cloning Kit). After transformation, positive colonies (containing an insert of the correct size) were selected by single colony PCR using M13F/R primers. Plasmids were purified using the GeneJET plasmid miniprep kit (Thermo Scientific) and sequenced by Eurofins using M13F/R. Plasmid sequences were verified with the corresponding P450 sequence from the honey bee genome sequence database using the Geneious alignment tool.

For functional P450 expression in *E. coli* the N-terminal coding region of P450 cDNA was modified: the ompA leader sequence (21 amino acid residues) and two linker amino acid residues (alanine-proline) were added to the 5' end of P450s (ompA+2 strategy) (Figure 5.1) (Pritchard *et al*., 1997; Pritchard *et al*., 2006; McLaughlin *et al*., 2008). Two fusion PCR reactions were carried out using high-fidelity DNA polymerase (Kapa Biosystems) according to the manufacturer's instructions. In the first PCR, JM109 genomic DNA was used as template to amplify

a ~100 bp nucleotide fragment (containing the ompA+2 sequence and the first 21 bases of the target P450 gene) preceded by a *Nde*I restriction site respectively, using the primers described in Table 5.2. Primary PCR reactions (total volume 50 µL) consisted of: 10 µL 5X KAPA HiFi Fidelity buffer, 1.5 µL of 10 mM dNTP mix, 1 µL HiFi DNA Polymerase (1 U/µL), 1.5 µL of each forward and reverse primers (10 µM), 33.5 µL nuclease-free water and 2.5 µL of template JM109 DNA. PCR was performed in a thermal cycler using the following conditions: 2 minutes at 95°C (initial denaturation) then 35 cycles of 20 seconds at 98ºC (denaturation), 20 seconds at the specific annealing temperature and 30 seconds at 72ºC (extension) with a final extension step at 72°C for 1 minute. This intermediate PCR product was purified and then fused to the P450 plasmid template in a second PCR reaction using the same forward and CYP specific reverse primers (Table 5.2) to generate the full-length ompA-AP-CYP coding sequence flanked by *Nde*I and *Xba*I restriction sites. Secondary PCR reactions (total volume 50 µL) consisted of: 10 µL 5X KAPA HiFi Fidelity buffer, 1.5 µL of 10 mM dNTP mix, 1 µL HiFi DNA Polymerase (1 U/µL), 1.5 µL of each forward and reverse primers (10 µM), 33 µL nuclease-free water, 0.5 µL of linker PCR product (first PCR product) and 1 µL of CYP plasmid (diluted 1/1000). PCR was performed in a thermal cycler using the following conditions: 2 minutes at 95°C (initial denaturation) then 25 cycles of 20 seconds at 98ºC (denaturation), 20 seconds at the specific annealing temperature and 90 seconds at 72ºC (extension) with a final extension step at 72°C for 2 minute. The final product was digested and ligated into modified pCWOri+ vector via *Xba*I and *Nde*I restriction sites and the final sequences were confirmed by sequencing prior to expression.

This text box is where the unedited thesis included the following third party copyrighted material:

Pritchard, M.P., *et al*. (1997) 'A General strategy for the expression of recombinant human cytochrome P450s in *Escherichia coli* using bacterial signa peptides: expression of CYP4A4, CYP2A6 and CYP2E1'. Arch. Biochem. Biophys. 345: 342-354

Figure 5.1 ompA+2 strategy process map (Reproduced from Pritchard *et al*., 1997). (1) Primers forward primer (F) and a reverse ''linker'' primer (L) were used to amplify the ompA sequence stitched to the gene of interest. (2) Intermediate product was used as a template to fuse bacterial leader sequence to P450 cDNA by employing Primers forward primer (F) and CYP specific reverse primer (R) with restriction site (X).

5.2.1.3 Cloning cytochrome b5 for expression in Escherichia coli

Honey bee cytochrome b5 (b5) was amplified from cDNA and the gene was then cloned into the pSC-A-amp/kan cloning vector and sequenced as described in section 2.7.4, Chapter 2. The annealing temperature and primers used in PCR are listed below in Table 5.2. To simplify the purification of the expressed protein the N-terminal coding region of b5 was modified with six histidine residues (His6) attached to the 5' end of b5. The His6:b5 was amplified from the holding vector using high-fidelity DNA polymerase (Kapa Biosystems) and the following forward primer 5'-GGAATTCCATATG**CACCATCACCATCACCAC**ATGTCGAAAATTTTTACAGCGGA-3' (*Nde*I restriction site underlined and six histidine codons in bold inserted right before start codon) and reverse primer 5'-GAATTCTCTAGATTATGAATACCAAAAATAGTAAAAT-3' (*Xba*I restriction site underlined). The final product was digested and ligated into modified pCWOri+ vector via the *Xba*I and *Nde*I restriction sites and the final sequences were confirmed by sequencing prior to expression.

5.2.1.4 Restriction enzyme digestion

Before ligation of the pCWOri+ vector and ompA-AP-CYP or 6H-b5 to generate full-length ompA-AP-CYP, 6H-b5 and modified pCWOri+ vector, a double digestion was conducted using two restriction enzymes: *Xba*I (Promega) and *Nde*I (Promega). The digestion was performed with 1000 ng plasmid DNA using 2 µL restriction enzyme 10X Buffer D, 0.2 µL 10 µg/µL acetylated BSA, 1 µL of each restriction enzyme, *Xba*I (10 U/µL) and *Nde*I (10 U/µL) and sterile, deonized water (to 20 µL). The reaction was incubated at 37°C for 4 hours. For ompA-AP-CYP305D1 a partial digestion process was carried out as the CYP305D1 sequence has an internal additional *Nde*I site. In this case digestion with *Xba*I was carried out first following the protocol described above and the digested product obtained was

digested with diluted *Nde*I for 5 minutes at 37°C. Digested fragments were analysed by gel electrophoresis and partially digested DNA fragments of the correct size were extracted from an agarose gel.

5.2.1.5 Ligation of ompA-AP-CYP into vector pCWOri+

Each ligation reaction contained 1 µL digested pCWOri+ vector plasmid (pB13), 7 µL digested insert DNA (ompA-AP-CYP or 6H-b5), 1 µL 10X T4 ligase Buffer (Thermo Scientific), 1 µL T4 DNA ligase (Thermo Scientific) and was incubated at 22°C for 1 hour.

Table 5.1 Primers and annealing temperatures used for amplifying honey bee CYPs (All primers are listed in the 5' to 3' direction).

Genes	Forward Primer	Reverse Primer	Annealing Temperature (°C)
CYP305D1	ATGTTTGTTATAATGTTAATAGTGATA	TTATCGTTTTTCAACTAATACATTATA	54
[a]CYP315A1	ATGAATCTTGCGCAAAATATTTTG	CTAATTTCTCTCCATCAATTTTAATT	60, 60[b]
CYP6AS5	ATGGCGAGCAGTTTCGAAATT	TCATATTTTTGTTATTTTCAAATATATTC	60
CYP6BE1	ATGTTTTTAACTACGTGGTTAATA	TTATATTGGCTCAATATTTAGATG	60
Cyt b_5	ATGTCGAAAATTTTTACAGCGG	TTATGAATACCAAAAATAGTAAAATAT	60

[a]Primary PCR product was used as template. For primary PCR the forward primer used was 5'-CTATCACCAGTGTTATCATTGG-3' and the reverse primer was 5'-GGATAAAATATTATTGCATAGAAGGA-3' designed on the published mRNA sequence.

[b]This annealing temperature was used during the second step PCR.

Table 5.2 Primers and annealing temperatures used for fusion PCRs (All primers are listed in the 5' to 3' direction).

CYPs[a]	Reverse Primer[b]	CYP Specific Reverse Primer[c]	Annealing Temperature (°C)[d]
ompA-AP-CYP305D1	ACTATTAACATTATAACAAACA T XXX	GAATTCTCTAGATTATCGTTTTTCAAC TAATACA	60,60
ompA-AP-CYP315A1	AATATTTTGCGCAAGATTCAT XXX	GAATTCTCTAGACTAATTTCTCTCCAT CAATTT	60,60
ompA-AP-CYP6AS5	AATTTCGAAACTGCTCGCCAT XXX	GAATTCTCTAGATCATATTTTTGTTAT TTTCAAATA	60,57
ompA-AP-CYP6BE1	TAACCACGTAGTTAAAAACAT XXX	GAATTCTCTAGATTATATTGGCTCAAT ATTTAGA	60,60

[a]The forward primer used for ompA+2 fusion PCR strategy was always 5'-GGAATTCCATATGAAAAAGACAGCTATCGCG -3' with *the Nde*I restriction site underlined.

[b]Reverse complement of the start of CYPs and XXX represents 5'-CGGAGCGGCCTGCGCTACGGTAGCGAA-3' which corresponds to the reverse complement of proline and alanine codons and the ompa segment sequence.

[c] The region corresponding to the reverse complement of the end of CYPs with *Xba*I restriction site (underlined).

[d]The first temperature was used to anneal the forward and reverse primer to the ompA+2 template during step 1 of the PCR reaction. The second annealing temperature was used during the second step of PCR to attach the ompA+2 segment to the full length CYP using the forward and CYP specific reverse primers.

5.2.1.6 Transformation in JM109 cells

After cells were thawed on ice, 3 μL of ligation reaction were added and mixed gently. Cells were then incubated for 30 minutes on ice before heat shocking at 37°C for 45 seconds in a water bath and transferred immediately to ice for 2 minutes. To the transformation reaction 450 μL super optimal culture (SOC) media was added and samples left to recover at 37°C for 1 hour with shaking (200 rpm). Transformed cells (200 μL) were distributed onto the LB agar plates containing appropriate

antibiotic (50 µg/mL of ampicillin for pb13-CYPs and pb13-b5 or 25 µg/mL of chloramphenicol for pACYC-AgCPR and plates were incubated overnight at 37°C. Next morning colonies were selected and grown in LB media with selective antibiotic overnight at 37°C. If required an aliquot of overnight incubated samples were preserved long term by mixing an equal amount of culture with 50% glycerol. Plasmid DNA minipreps were prepared as described in section 2.7.4, Chapter 2.

5.2.1.7 Coexpression of CYPs and P450 reductase (CPR)

Competent *E. coli* JM109 cells were co-transformed with pCWOri+-CYPs and pACYC-AgCPR (obtained from LSTM) to enable co-expression of each CYP with *Anopheles gambiae* CPR. 0.5 µL of plasmid of each pb13-CYPs and pACYC-AgCPR were transformed in JM109 cells and plated onto LB agar plates containing 50 µg/mL of ampicillin and 25 µg/mL of chloramphenicol. A single colony was picked into 5 ml of LB/ampicillin+chloramphenicol medium and clones grown overnight at 37°C. 4 mL of these overnight cultures were transferred to 200 mL terrific broth (TB) (Appendix 9.1.1) media containing 50 µg/mL of ampicillin and 25 µg/mL of chloramphenicol in 1L flasks and incubated at 30°C with shaking (200 rpm). The OD_{600} of the cultures were monitored by using an Eppendorf spectrophotometer at regular intervals and when they reached 0.7-1.0 (usually in 3.5 hours) 0.5 mM ALA and 1 mM IPTG were added. Cultures were then incubated at 24°C for 23 hours. P450s were quantified by CO difference spectrophotometry.

5.2.1.8 Purification of P450 membranes

Cells were chilled on ice for 10 minutes and then harvested by centrifugation at 2800 x *g* for 20 minutes at 4°C (Eppendorf centrifuge 5810R). Pelleted cells were resuspended in 10 mL of ice-cold 2X TSE (Appendix 9.1.2) and then diluted by adding an equal amount of ice-cold water (10 mL). After addition of 250 µL of 20 mg/mL lysozyme, cultures were stirred gently at 4°C for 1 hour to degrade the cell walls. Spheroplasts were pelleted by centrifugation at 2800 x *g* for 25 minutes at 4°C and the supernatant discarded. Spheroplasts were resuspended in 8 mL of spheroplast resuspension buffer (Appendix 9.1.2) and after addition of aprotinin, leupeptin (final concentration of 1 µg/mL) and PMSF (final concentration of 1 mM) sonicated for 1.5 minutes. This suspension was then centrifuged at 30,000 x *g* for 20 minutes at 4°C (Beckman Coulter Avanti J-30i centrifuge, JA-20 fixed angle rotor) and the supernatant transferred to ultracentrifuge tubes and centrifuged at 49,600 rpm for 1 hour at 4°C (Beckman optima XL-90 ultracentrifuge, ultra-centrifuge rotor type 45Ti). The supernatant was discarded and the red-brown coloured membrane resuspended in ice-cold 1X TSE buffer in a Dounce tissue homogeniser with 8-10 strokes of the pestle. P450 content in membranes was quantified by spectrophotometry (see below).

5.2.1.9 Spectral determination of P450

P450 content was measured using a Varian Cary 300 spectrophotometer. 2 mL of P450 spectrum buffer (Appendix 9.1.4) was mixed with 100 µL of cell culture or 50 µL of P450 membrane in 2 mL eppendorf tubes. A few grains of sodium dithionite was added and mixed by inversion. This mixture was divided equally to a pair of matched quartz glass cuvettes (Hellma). A baseline scan was then run on the spectrophotometer from 500 to 400 nm and the sample cuvette then removed and CO

bubbled through the solution for approximately 45 seconds (about 1 bubble/second) in a fume hood. Both this cuvette and the reference (which was not exposed to CO) were scanned again across the same wavelengths and the size of the peak at 450 nm recorded and compared with that at 490 nm. These values were used to quantify P450 using the P450 extinction coefficient, 0.091 μM^{-1} cm^{-1} within the formula ([ΔA450–ΔA490]/0.091 = nmol of P450 per ml) (Omura and Sato, 1964; Guengerich *et al*., 2009). The protein concentration of each CYP membrane was calculated by performing a Bradford protein assay (Bradford, 1976).

5.2.1.10 Cytochrome - c reductase assay

The cytochrome - c reductase assay was performed using a 96 – well NUNC microplate. Samples were prepared by mixing 150 µL of 0.1 mM cytochrome-c with 2 µL of membranes and the reaction started by adding 150 µL of 0.1 mM NADPH or buffer in the case of negative controls. Reactions were read at 550 nm in a spectrophotometer. The change in optical density (OD) per minute was observed and the slope then calculated**.**

5.2.2 Expression of cytochrome b5

5.2.2.1 Expression and purification of cytochrome b5

Competent *E. coli* JM109 cells were used to express 6H-b5. 0.5 µL of plasmid of pb13-b5 was transformed into JM109 cells as described above and plated onto LB agar plates containing 50 µg/mL of ampicillin. A single colony was picked into 5 mL of LB/ampicillin medium and grown overnight at 37°C. 4 mL of overnight culture was transferred to 500 mL terrific broth (TB) media containing 50 µg/mL of ampicillin in a 1L flask and shaken at 37°C at 200 rpm. The OD_{600} of the culture was monitored using an Eppendorf spectrophotometer at regular intervals and when it

reached 0.8 (usually in 3 hours) 0.5 mM ALA and 1 mM IPTG were added. The cultures were incubated at 30°C for overnight. Expression of b5 was checked and quantified by spectrophotometry: 2 mL of culture was centrifuged and the pellet was resuspended in 2 mL of 0.1 M potassium phosphate at pH 7.5. The suspension was divided into two cuvettes and the baseline recorded from 380 nm to 580 nm. A few grains of sodium dithionite were added into the sample cuvette and the spectrum was checked over the same wavelengths. Additionally, 5 µL of 30% hydrogen peroxide was mixed into the reference cuvette and the spectrum recorded. This addition does not affect the signal if b5 is present. Cells were harvested as for the P450s. Pelleted cells were resuspended in Buffer A (25 mL of buffer for 0.5 L culture) (Appendix 9.1.3) and lysed by sonication for 1 minute on ice. After addition of CHAPS powder (final concentration of 1% (w/v)) samples were stirred gently at 4°C for 1 hour. Samples were then centrifuged at 30,000 x *g* for 30 minutes at 4°C (Beckman Coulter Avanti J-30i centrifuge, JA-20 fixed angle rotor) and the supernatant transferred onto a 5 mL HisTrap affinity column (GE Healthcare) that had been equilibrated with buffer B2 (Appendix 9.1.3) according to the manufacturer's protocol. A laboratory pump was filled with distilled water and connected to the column which was washed with 25 mL of distilled water. The column was then equilibrated with 25 mL of buffer B2 (5 mL/min flow rate). The sample was applied onto the column using a pump and then the column was washed with 50 mL of 0.5% buffer B3 (Appendix 9.1.3) in buffer B2 to elute loosely-bound endogenous protein. b5 was eluted in 25 mL of buffer B3 and purified b5 was loaded onto the PD-10 desalting column (GE Healthcare) that had been equilibrated with buffer C (Appendix 9.1.3) according to the manufacturer's manual: After removing the top cap and column storage solution the sealed end of the column was cut. For column

equilibration 25 mL of buffer C was applied onto the column and allowed to enter the packed bed and the flow-through was discarded. 2.5 mL of sample was applied and allowed to enter the packed bed and the flow-through was discarded. Then the sample was eluted with 3.5 mL of buffer C.

5.2.2.2 Spectral determination of cytochrome b5

Cytochrome b5 concentration was determined using a Varian Cary 300 spectrophotometer. 20 µl of purified b5 or 2 mL of cell pellet was mixed with 2 mL of 0.1M potassium phosphate buffer (pH7.5) and was divided between two cuvettes: 1 mL for the reference cuvette and 1 mL for the sample cuvette. A baseline scan was recorded from 380 nm to 580 nm. A few grains of sodium hydrosulphite were added to the sample cuvette and it was scanned with the reference cuvette. After adding 5 µL hydrogen peroxide into the reference cuvette the difference spectrum was scanned again. The concentration of b5 was calculated by using reduced vs. oxidised spectroscopy (A424-A409), extinction coefficient = 185 cm^{-1} mM^{-1} (Omura and Sato, 1964). Protein concentration was calculated by performing the Bradford protein assay.

5.2.3 Insecticide metabolism

Insecticide (thiacloprid and imidacloprid) metabolism assays of recombinant bee P450s/CPR/b5 standard reactions were carried out using three replicates for each P450 in the presence or absence of NADPH. 10 mM stock solution of thiacloprid and imidacloprid were prepared in DMSO and diluted as 100 µM in phosphate buffer (0.1 M, pH 7.6) before adding the reaction to avoid the precipitation of insecticide. Standard reactions consisted of final concentrations of 10 µM insecticide, 100 µl NADPH regeneration system (Promega) (or buffer alone in the case of minus

NADPH controls), 0.0117 µM cytochrome b5 and 0.1- 0.4 µM P450 membrane. Reactions (200 µl total) were incubated at 30˚C with 1200 rpm shaking for 2 hours and stopped by adding 300 µl of acetonitrile. Samples were then spun at 2000 x *g* for 5 minutes and 250 µl of supernatant was transferred to HPLC vials and stored at -20˚C for LC-MS/MS analysis.

5.2.4 LC-MS analysis

Due to the equipment and expertise availability all LC-MS analyses were performed by Miriam Daniels at Syngenta, Jealott's Hill.

Aliquots of each sample were diluted 50:50 in acetonitrile prior to LC-MS/MS analysis. Separation was achieved using Ultra Performance LC® (ACQUITY UPLC-System; Waters, UK) using an ACQUITY UPLC column (HSS T3, 1.8 µm, 100 x 2.1 mm), with a mobile phase consisting of water (+0.2% formic acid), with a flow rate of 0.6 mL/min. The gradient elution conditions of acetonitrile:water were as follows: 0 min 0:100, 0.5 min 0:100, 3.5 min 95:5, 4.5 min 95:5, 4.6 min 0:100, 5 min 0:100. The mass spectrometer used was a Finnigan TSQ Quantum Discovery (Thermo Scientific, UK) equipped with an Ion Max source operating in positive ion mode. Analytes were detected using selected-reaction-monitoring (SRM) transitions are outlined in Table 5.3. Quantification was achieved using standard calibration curves constructed in 50:50 acetonitrile:water.

Table 5.3 SRM transitions and collision energies.

Analyte	Molecular Weight (Da)	SRM Transition Parent *m\z* > Product *m\z*	Retention Time (min)
Thiacloprid	254	253 > 126 253 > 186	4.83
Imidacloprid	257	256 > 175 256 > 209	4.47

5.3 Results

5.3.1 Heterologous expression of A. mellifera CYP305D1, CYP315A1, CYP6AS5, CYP6BE1, cytochrome b5 and CCE8 in E. coli

Heterologous expression focused on exploring the functional role of the P450s CYP305D1, CYP315A1, CYP6AS5 and CYP6BE1, as well as cytochrome b5 and CCE8 in insecticide detoxification. Isolated cDNAs were amplified for each gene. The expected band sizes for CYP305D1, CYP315A1, CYP6AS5, CYP6BE1, cytochrome b5 and CCE8 were confirmed by gel electrophoresis.

The candidate P450 genes were fused to a bacterial ompA + 2 leader sequence for functional expression in *E.coli*. In order to produce catalytically active P450s the candidate P450s, were co-expressed together with *A. gambiae* CPR in *E. coli* as previously described by McLaughlin *et al.* (2008). Variation was observed in the yield of recombinant protein produced for each P450 (Table 5.4), however, each P450 reduced CO-difference spectra suggesting correctly folded and active enzyme as indicated by significant peaks at 450 with secondary smaller 420 peaks observed (Figure 5.2). Honey bee cytochrome b5 was also successfully expressed and purified from *E. coli* membranes (0.83 g cytochrome b5/L). It generated a characteristic b5 spectra (Guzov *et al*., 1996), with a peak at 420-430 nm observed when reduced with sodium hydrosulphite (Figure 5.3).

Table 5.4 P450 concentration and CPR activity in expressed CYPs.

CYPs	P450 concentration (nmol P450/mg protein)	CPR activity (nmoles cyt c/min/mg protein)
CYP305D1	0.17	8.8
CYP315A1	0.116	6.9
CYP6AS5	0.045	4
CYP6BE1	0.0518	7

In order to express the candidate honey bee esterase CCE8 in *E. coli*, the enzyme was histidine tagged, expressed and purified using a HisTrap affinity column with minor modification as described in Godinho *et al*. (2011). However, no esterase activity could be measured in the resulting preparations. This could be a result of several factors as detailed in previous studies (Fakruddin *et al*., 2013). Firstly, *E. coli* may have limited ability to produce functional insect esterase as it may lack some of the functional machinery of protein production or post-translational modification present in insect cells. Secondly, active enzyme may not have been observed due to protein degradation or it may be that honey bee esterase insertion into the membrane was incorrect. Finally this result may be linked with expressed protein insolubility, however, due to the limited time frame of this PhD, no further assessment could be conducted to confirm these possibilities by SDS page gel electrophoresis or western blot.

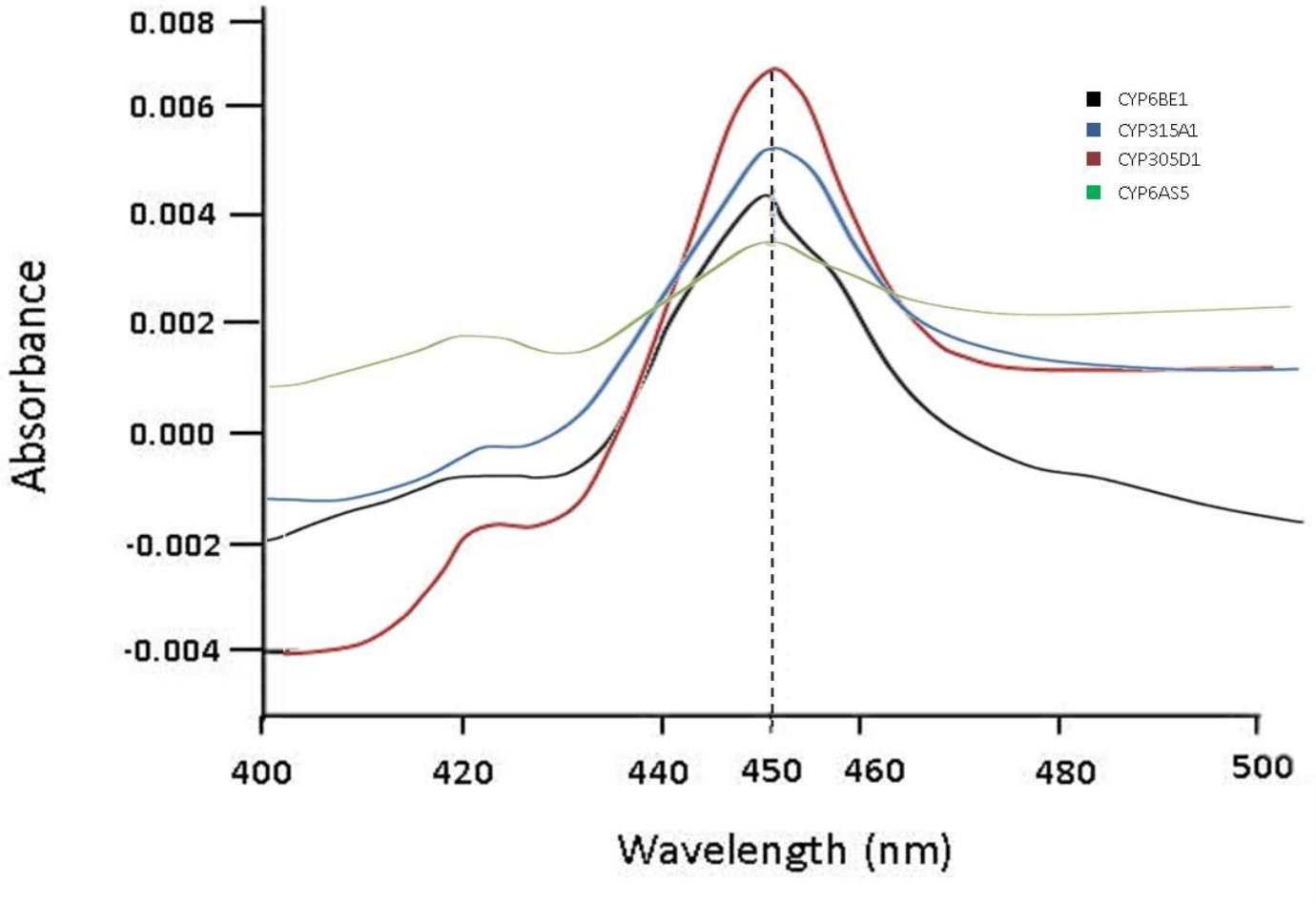


Figure 5.2 CO - difference spectra. Four P450s show an absorbance peak at 450 nm indicating.

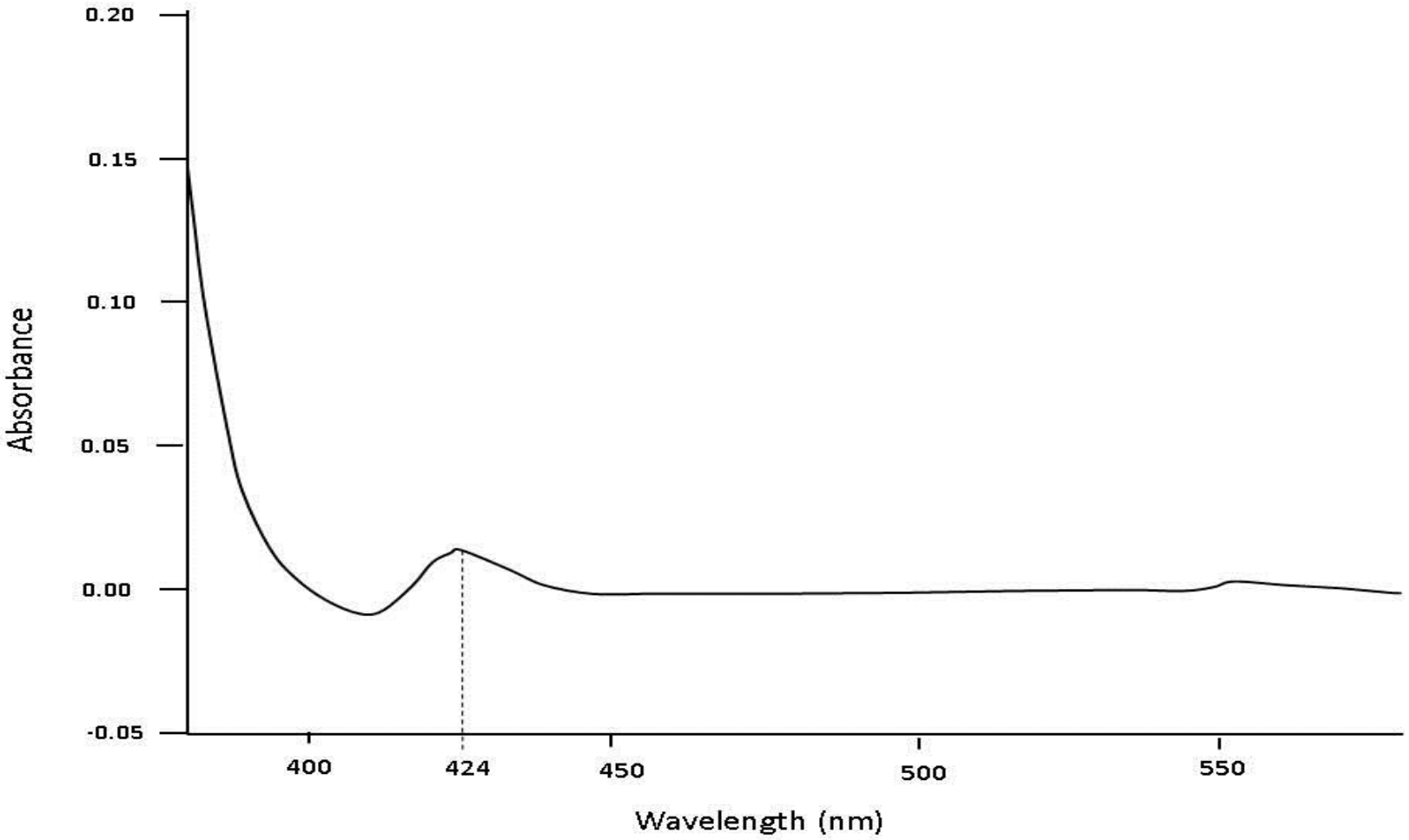


Figure 5.3 Absorbance of reduced CO - difference P450 spectrum. Cyt b5 expression is detected at an absorbance of about 424 nm.

5.3.2 Metabolism Assay

The ability of CYP305D1, CYP315A1, CYP6AS5 and CYP6BE1 (in combination with *A. gambiae* CPR and honey bee cytochrome b5) to detoxify thiacloprid and imidacloprid in the presence and absence of NADPH was examined in insecticide metabolism assays. Figure 5.4 outlines the results of monitoring thiacloprid recovery in the samples using selected-reaction-monitoring (SRM) methods, with quantification against standard calibration curves. NADPH is an obligate cofactor for P450 metabolism as it is required by CPR to deliver electrons to P450 for catalytic activity. Therefore, incubation of substrate was carried out in the presence and absence of NADPH to monitor the NADPH dependant depletion of substrate indicative of P450 metabolism. No significant differences were observed in the thiacloprid recoveries between the +/- NADPH samples for any of the four P450s. Figure 5.5 outlines the results of monitoring imidacloprid recovery in the samples using SRM methods, with quantification against standard calibration curves. Again no significant differences were observed in the IMI recoveries between the +/- NADPH samples.

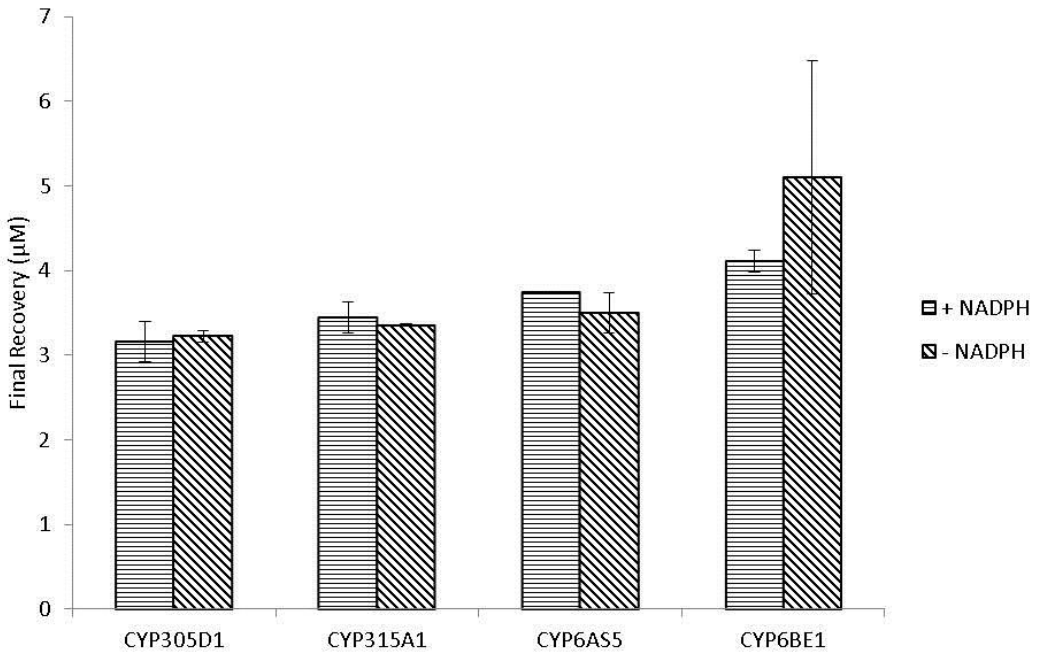


Figure 5.4 Recovery of thiacloprid after a 2 h incubation with the four honeybee P450s. Graph represents mean final recovery (µM) ± SE (n = 3).

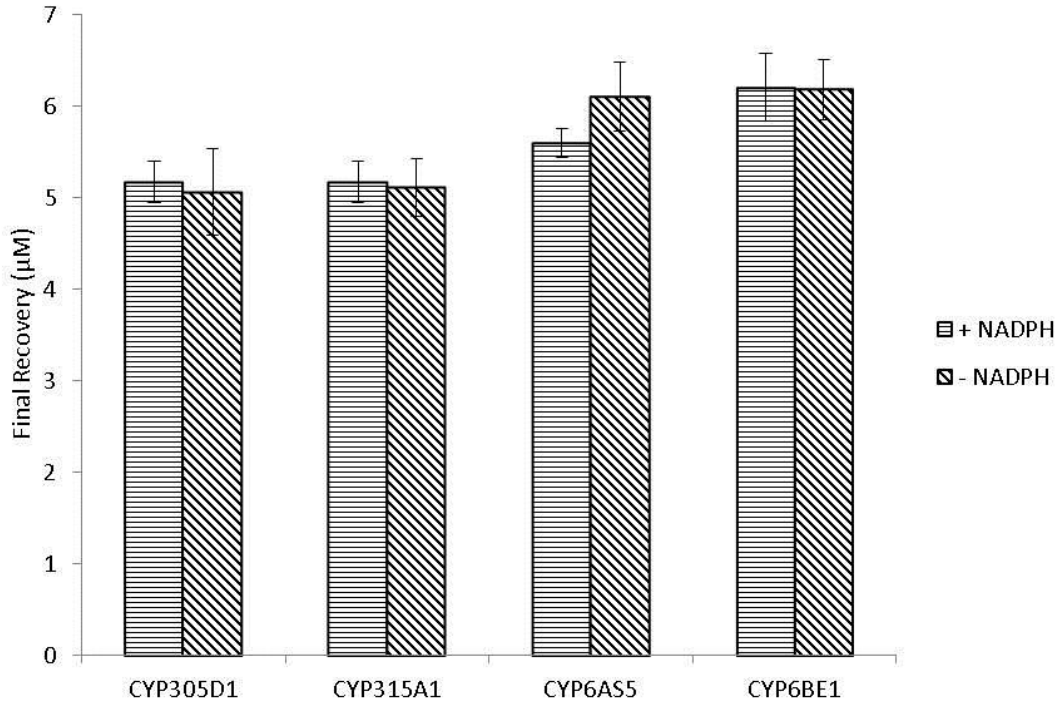


Figure 5.5 Recoveries of imidacloprid after a 2 h incubation with the four honeybee P450 expression systems. Graph represents mean final recovery (µM) ± SE (n = 3).

5.4 Discussion

In order to determine if CYP6BE1, CYP6AS5, CYP315A1 and CYP305D1 were involved in thiacloprid metabolism they were functionally expressed in *E.coli.* Since P450s require electrons from P450 reductase for catalytic activity, the P450s were co-expressed with *A. gambiae* CPR. Honey bee cytochrome b5 was also cloned and expressed and included in metabolism assays as this enzyme has been shown to augment the catalytic activity of insect P450s (Stevenson *et al*., 2011).

In this study the ompA+2 strategy was employed to direct P450s to the inner bacterial membrane. This technique was used previously in human P450 CYP3A4 expression studies and resulted in higher yield in comparison to the 17α hydroxylase NH_2 terminus site modification approach (Pritchard *et al*., 1997). In the case of insect P450s, several *Anapholes gambiae* P450s including CYP6Z2 and CYP6M2 (Mclaughlin *et al*., 2008; Stevenson *et al*., 2011) and the *Bemicia tabaci* P450 CYP6CM1vQ (Karunker *et al*., 2009) have been successfully expressed using the ompA+2 strategy in *E. coli*.

Honey bee P450s (CYP6AS1, CYP6AS3, CYP6AS4, CYP6AS10) have previously been expressed in Sf9 insect cells (Mao *et al*., 2009). However, this is the first report that describes the functional expression of honey bee P450s in *E. coli*. This is significant since there are a number of advantages of using *E. coli* for recombinant protein expression including low cost simplicity and high protein yields, thus recommending this as a useful system for future biochemical characterisation of honey bee P450s.

Although functional P450 was obtained for all CYP genes expressed, incubation of thiacloprid and imidacloprid with recombinant P450 failed to produce evidence for the metabolism of thiacloprid or imidacloprid as assessed by parent compound depletion. There are several possible explanations for this finding. Firstly these P450s may lack the ability to metabolise these insecticides. Secondly, although these P450s were upregulated in response to thiacloprid exposure, it is nevertheless possible that the enzyme responsible for metabolism was not induced, but instead constitutively expressed at levels sufficient to provide protection to honey bees against this compound. In such a scenario our induction strategy would have failed to identify the causal detoxification enzyme that underlies thiacloprid tolerance. Alternatively the lack of metabolism may be due to technical factors. For example, as there are no other substrates for these P450s to act as a positive control, it is not known if the lack of insecticide metabolism was due to lack of substrate recognition or due to experimental factors such as inactive enzyme or suboptimal experimental conditions, as these were not optimised in this study. Due to time constraints the functional activity of P450s were tested only against two well-known fluorescent model substrates (7-Ethoxy-coumarin (7-EC) and 7-Methoxy-4-(tri-fluoromethyl)-coumarin (MFC)). No activity was seen for any of the expressed P450s using these substrates. This result does not indicate that the expressed honey bee P450s were inactive as it is entirely possible that neither 7-EC nor MFC are substrates for the P450s tested. In relation to this finding other studies testing recombinant insect P450 against a range of model substrates have shown that insect P450s can demonstrate very significant differences in their substrate profile for model substrates (Zimmer *et al*., 2014). Therefore, future investigation to identify model fluorogenic substrate is recommended. It is also possible that the P450s examined here are only very weak

metabolisers of imidacloprid and/or thiacloprid, thus metabolism cannot be detected by simply assaying parent compound depletion. A more sensitive approach would be to assay for the appearance of insecticidal metabolites in insecticide/P450 incubations by liquid chromatography tandem mass spectrometry (LC-MS/MS). Such an approach has been used for other neonicotinoid metabolising P450s and identified 5-hydoxy imidacloprid as the primary metabolite produced upon incubation of CYP6CM1 with imidacloprid (Karunker *et al*., 2009). However, this approach requires reference standards of known metabolites which were unavailable to Syngenta (who were in a position to carry out LC-MS/MS analysis) during the course of this PhD. It should also be considered that honey bee P450s were co-expressed with mosquito CPR, which might affect the biotransformation activity of P450. Therefore co-expression of honey bee, P450s with honey bee CPR might produce enhanced activity against insecticides.

Finally a different enzyme system, such as esterases may be responsible for thiacloprid metabolism/sequestration. Although it is reported that P450s play the major role in metabolism of these compounds, the toxicity of the cyano-substituted neonicotinoid acetamiprid was synergised (synergism ration of 2.96) by the inhibitor S,S,S,-tributylphosphophorotrithioate (DEF) suggesting esterases may play a contributory role in detoxification (Iwasa *et al*., 2004). In our 'survivor' array comparison five probes representing the esterase CCE8 were overexpressed and this was confirmed by qRT-PCR. Attempts to functionally express this esterase in *E. coli* resulted in non-functional enzyme (data not shown) so we were unable to confirm or refute the role of this enzyme in thiacloprid metabolism. Although CCE8 expression was not achieved using the *E. coli* system, eukaryote expression systems have been successfully applied to esterases including *Helicoverpa armigera* esterase expression

in baculovirus (Teese *et al*., 2013) and *Tribolium casteneum* esterase expression in methylotrophic yeast *Pichia pastoris* (Delroisse *et al*., 2005). CCE8 expression in baculovirus or yeast expression systems may be worth considering in future.

6. DIFFERENTIAL SENSITIVITY OF DIFFERENT HONEY BEE RACES TO NEONICOTINOIDS

6.1 Introduction

Over the past few years there has been increasing investigation of the effects of insecticides on non-target organisms (especially bee pollinators) (Osborne, 2012). The growing interest and concerns of the general public in relation to reports of recent honey bee declines and the role of pesticides in this has led to response from the scientific community. New data has been generated on the toxicity of pesticides to bees (honey bees, bumble bees and wild bees) and their metabolism (Marletto *et al*., 2003; Mommaerts and Smagghe, 2011). This is especially true for neonicotinoids (Bonmatin *et al*., 2005; Wu *et al*., 2011). The development of comprehensive data sets that evaluate the impact of changing factors (such as bee age, nutritional status and stress factors like temperature affects the colony health) on pesticide sensitivity and physiological condition is critical (Suchail *et al*., 2001). Previous work has demonstrated that a number of factors influence bee sensitivity to pesticides. These include age (the susceptibility of older bees is higher than younger bees), the quality of consumed pollen (the susceptibility of bees fed on protein deficient pollen is higher than bees fed on high protein pollen) and/or the presence of stress factors, such as hive temperature at rearing (lower temperature $<33^{o}C$) develops more susceptible adults (Wahl and Ulm, 1983; Rortais *et al*., 2005; Medrzycki *et al*., 2009).

Another factor that may be relevant in honey bee sensitivity to insecticides is bee subspecies/race. There are only a few studies published on the relationship between honey bee race and toxicity to insecticides. Gromisz and Gromisz (1980) reported

that *A. mellifera* L. (western European bee) was more susceptible than *A. m. caucasica, A. m. ligustica* and *A. m. carnica* to oral exposure to permethrin (synthetic pyrethroid) and phosalone (organophosphate). Another study related to imidacloprid toxicity indicated that the LD_{50} of *A. m. caucasica* is lower than *A. m. mellifera* after contact application, whereas via oral exposure the LD_{50} values were similar for both races (Suchail *et al*., 2000). Although African and European bees showed equal susceptibility to topically applied malathion (organophosphate) and carbofuran (carbamate) (Elzen *et al*., 2003), European bees were slightly more susceptible to methyl parathion (organophosphate) (Danka *et al*., 1986). Despite these findings there are no studies to date on the possible mechanisms underlying race-related differences in sensitivity to insecticides. The identification ofcandidate genes associated with differential sensitivity could be invaluable for directing future development of insecticides/synergists.

6.2 Materials and Methods

6.2.1 Insects

Adult worker honey bees of *Apis mellifera ligustica, Apis mellifera caucasica* and *Apis mellifera buckfast* were obtained from different hives belonging to Ankara University, Agricultural Faculty Campus, Turkey (section 2.4, Chapter 2). Hives were exposed to smoke before collection and nurse bees were shaken from the frames. Forager bees were trapped in front of the hive entrance and were collected into plastic cups following minimal CO_2 exposure. Drones and dead bees were removed from containers.

6.2.2 Toxicity Bioassays

Worker bees were briefly anaesthetised with CO_2 before applying 1 µL of neonicotinoid (thiacloprid or acetamiprid) in acetone (96% AR) (ranging from 1µg to 100 µg) or only acetone for controls to the thorax using a microapplicator. All assays were repeated at least twice, and each treatment contained approximately ten bees (see section 2.5.1, Chapter 2).

Extra sum of squares F-test was applied to compare full dose contact toxicity data between forager and nurse bees of *A. m. ligustica*.

Bioassays combined with synergists (PBO and EN16/5-1) were carried out using a discriminating dose of insecticide obtained from a full dose contact toxicity experiment. Bees were dosed topically with either 1 µl of synergist in acetone or 1 µl of acetone prior to insecticide application (see section 3.2.3.2, Chapter 3).

6.2.3 Haem Peroxidise Assay

Since it was not possible to measure honey bee microsomal oxidase activity (see Chapter 3), the variation of haem content which is directly correlated with the molar amount of oxidases was monitored among the races. Haem peroxidise assays were carried out using the method described by Penilla *et al*. (1998) with some modifications. Ten frozen bees from each race were homogenised individually in 1 mL dH_2O with a motorised homogeniser and centrifuged at 10,000 x *g* for 1 minute. The supernatant was used as the assay source. Ten µl of homogenate and 70 µl of potassium phosphate (90 mM, pH 7.2) were pipetted into the wells of a 96-well microplate (NUNC, maxisorb). Aliquots (200 µl) of 0.2% 3,3′,5,5′-tetramethylbenzidine (TMBZ) in methanol/sodium acetate and 25 µl 3% H_2O_2 were

added. The reaction was incubated for 5 minutes at room temperature. An endpoint reading was taken at 620 nm using a Thermomax microplate reader (Molecular Devices, Menlo Park, CA). The concentration of cytochtome c used in the positive control was calibrated to the standard curve. To create the standard curve several quantities of cytochrome c were used (range between 0.01 μg – 0.2 μg). Results were calculated as μg cytochrome/mg protein for each honey bee. Unpaired t-test was applied to compare the haeme content of the tested races, to determine if the means of the two independent samples were significantly different.

6.2.4 Microarray Analysis and Quantitative Polymerase Chain Reaction (qRT-PCR)

Microarray and qRT-PCR were designed as described previously (section 2.7.8 and section 2.7.7, Chapter 2). All samples are normalized against two housekeeping genes (ef1 (elongation factor-1), and tbp (tubulin)). qRT-PCR was carried out to validate the microarray results for selected genes (Table 6.1).

Table 6.1 Oligonucleotide Primers for qRT-PCR (nAChR alpha 6 subunit)

Alpha 6 F1	TCACTTGGTTCCCCTTTGAC
Alpha 6 R1	CCGATCAGGTACCATTCTCC
Alpha 6 F2	CCGCATCGTTACCTCTTAGC
Alpha 6 R2	AAGCCTGTCCACCACCATAG

6.2.5 Sequencing and Cloning

First strand cDNA was synthesised using random hexamers, SuperScript II reverse transcriptase (Ambion) and total RNA (1 μg) extracted from two separate pooled samples of *A. m.buckfast* and *A. m. caucasia.* cDNA (1μl) was used as template for PCR using PCR Master Mix (Fermentas) and specific primer sets designed on the published nAChR subunit sequences (Jones *et al.*, 2006) (Table 6.2). Temperature cycling conditions were: 94^{o}C for 2 minutes, followed by 40 cycles of 30 seconds at 94^{o}C, 30 seconds at the annealing temperature given in Table 6.3 and 1.20 minutes at 72^{o}C with a final extension step at 72^{o}C for 5 minutes. When required the primary PCR products were used as templates for a second nested PCR reaction. PCR products were analysed by electrophoresis on a TBE gel and then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) before being sequenced by Eurofins.

As required, PCR products were ligated into pSC-A-amp/kan (Agilent) cloning vector. After transformation, positive colonies were selected by PCR using the plasmid's primers T3 (5'-AATTAACCCTCACTAAAGGGAA-3') and T7 (5'-CCTATAGTGAGTCGTATTAC-3'). Plasmids containing the inserts were custom sequenced at Eurofins.

Table 6.2 Oligonucleotide primers for PCR to amplify nAChR subunits

nAChR subunits	Primer Sequence (5' to 3' direction)
Alpha1 F1	GAC GGC CAT TTC CTG TCT T
Alpha1 R1	TGT CGC TCG GCA GGT AG
Alpha1 R2	AGA CCT TCT CCC CGC TGT C
Alpha1 R3	ATC GAG ATC GAG AGC GAG A
Alpha2 F1	TAC TAT TCA AAA TGA TAC TCC AGA CG
Alpha2 R1	AAC ATG GTT TGG GAG AGC AG
Alpha2 R2	AGA GCA GGA TGT TGA TGC AG
Alpha3 F1	ATG ATG AAG AGC CTG GTG G
Alpha3 R1	CCA CTG TCG CTT GGC AG
Alpha3 R2	TGA CCT TCT CGC CAC TGT C
Alpha3 R3	AAT TGA AAT TGA TAG GCT GAC
Alpha4 F1	ATG CCC CCC ATA ATA GGG G
Alpha4 R1	CCC CGC TGT CGC TAG GT
Alpha4 R2	AGA GCG TGA CCT TTT CCC C
Alpha4 R3	TGA TGA GAA TGG AAA TGG AG
Alpha5 F1	ATG TCG CCT TTG GTC CTG TT
Alpha5 R1	CCG ATT CAG ACG GCA CG
Alpha5 R2	CGA AAT CCC GAG GGT GAC
Alpha5 R3	TCA TAG AGA GGA GGG CCG

nAChR subunits	Primer Sequence (5' to 3' direction)
Alpha6 F1	ATG CGC GCA AGT AGT GTA TT
Alpha6 F2	GCA GAG AGT GAT GTG TCA T
Alpha6 R1	TTC TCC CCG GAA TCT GGC
Alpha6 R2	TCC TAA GGT GAG CTT CTC C
Alpha7 F1	ATG GCG GCT ATA GCC CT
Alpha7 R2	AGA GAT AGC TTC TCG CCG G
Alpha7 R3	AGG ATG GTT ACC CCT AGA G
Alpha8 F1	ATG CAA ATA TTG ACG CTT GGT
Alpha8 R1	TTT TTC ACC AGA ATC ACT TGG
Alpha8 R2	TAA TGA TAC TTT TTC ACC AGA
Alpha8 R3	CGT CAA TGA AAG GAG AAT GG
Beta1 F1	GGA CAC CGC CTG AAA CC
Beta1 R1	TGC TGA CCA ACA ACA GGA AC
Beta1 R2	AAC ACG ACC AAC GAG AGG AG
Beta2 F1	TAA ACA TGA AGA ATA TAT TCC CCG
Beta2 F2	ATA TAT TCC CCG TTT TAT TCG TG
Beta2 R1	ATC GCA AGA TGC TTG TCA AC
Beta2 R2	TGT CAA CAT AAG CGA GAA TGC

Table 6.3 Description of PCR reactions and primer squences

	BUCKFAST					CAUCASICA				
Gene	**1st PCR**	**Temp**	**2nd PCR**	**Temp**	**Seq**	**1st PCR**	**Temp**	**2nd PCR**	**Temp**	**Seq**
Alpha 1	F1/R3	58°C	-	-	F1/R2	F1/R3	55°C	F1/R2	58°C	F1/R1
Alpha 2	F1/R1	57°C	-	-	T3/T7	F1/R1	57°C	F1/R2	60°C	T3/T7
Alpha 3	F1/R3	58°C	-	-	F1/R2	F1/R3	57°C	F1/R1	60°C	T3/T7
Alpha 4	F1/R3	55°C	F1/R2	58°C	T3/T7	F1/R3	55°C	F1/R2	58°C	F1/R1
Alpha 5	F1/R3	58°C	-	-	F1/R2	F1/R3	55°C	F1/R2	58°C	F1/R1
Alpha 6	F1/R2 F1/R1	60°C 60°C	-	-	F1/R2 F1/R2	F2/R1	57°C	-	-	T3/T7
Alpha 7	F1/R3	60°C	F1/R3	60°C	F1/R2	F1/R3	57°C	-	-	T3/T7
Alpha 8	F1/R3	55°C	F1/R3	55°C	F1/R3	F1/R3	55°C	F1/R2	58°C	F1/R1
Alpha 9	F1/R1	57°C	-	-	T3/T7	F1/R1	57°C	F1/R2	60°C	T3/T7
Beta 1	F1/R1	57°C	-	-	T3/T7	F1/R1	57°C	F1/R2	60°C	T3/T7
Beta 2	F1/R1	57°C	F1/R2	60°C	T3/T7	F2/R2	57°C	-	-	T3/T7

6.3 Results

6.3.1 Toxicity Bioassays

Bee mortality was recorded 24 hours after topical application. The results of topical application with two neonicotinoids are presented in figures 6.1 and 6.2. No

mortality was observed with *A. m. caucasica* worker bees when treated with any dose of either thiacloprid or acetamiprid. However a mortality dose response curve was observed for *A. m. buckfast* worker bees to both compounds (Figure 6.1 and Figure 6.2) generating LC_{50} values of 1.86 ± 0.61 µg and 7.6 ± 4 µg respectively.

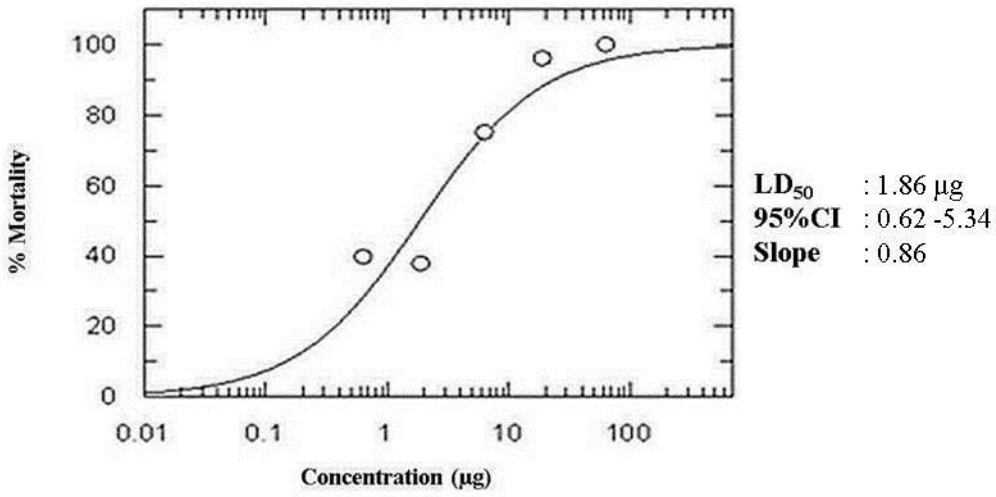


Figure 6.1 Dose-response curve from topical bioassay; thiacloprid against *A. m. buckfast*

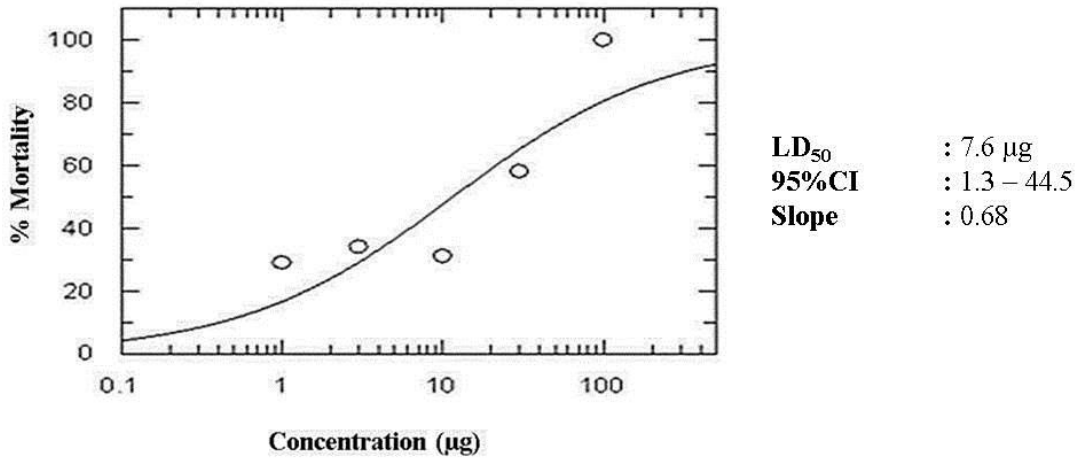


Figure 6.2 Dose-response curve from topical bioassay; acetamiprid against *A. m. buckfast*

Toxicity bioassays were repeated but forager bees and nurse bees of *A. m. ligustica* were assayed separately. Thiacloprid was more toxic to forager bees of *A. m. ligustica* (LD_{50}: 0.5429 +/- 0.3545 µg) compared to the nurse bees of same subspecies ($LD_{50:}$ 58.9748 +/- 39.0658 µg) (Figure 6.3). As indicated by the rejection of the null hypothesis, (a single parameter for both data sets) there is a statistically significant difference between forager and nurse bees of *A. m. ligustica* ($P< 0.0001$) in response to thiacloprid (Appendix 9.6).

Additionally, whilst PBO synergised thiacloprid (95%) equally for both *A. m. ligustica* worker and nurse bees, EN 16/5-1 synergised thiacloprid more for nurse bees (92%) compared to foragers (26%) (Figure 6.4 and Figure 6.5). Dose response curves as shown in figure 6.3 displays the % mortality of tested forager and nurse bees against thiacloprid concentrations. At some concentrations the mortality of nurse bee replicates overlapped the forager bee mortality response to the same concentrations. This error might be related to collection of bees from the hive. After allowing the foragers to leave the hive, frames were shaken for nurse bee collections thus a few forager bees may have remained in the hive at this point and so were included in the experimental nurse bee group.

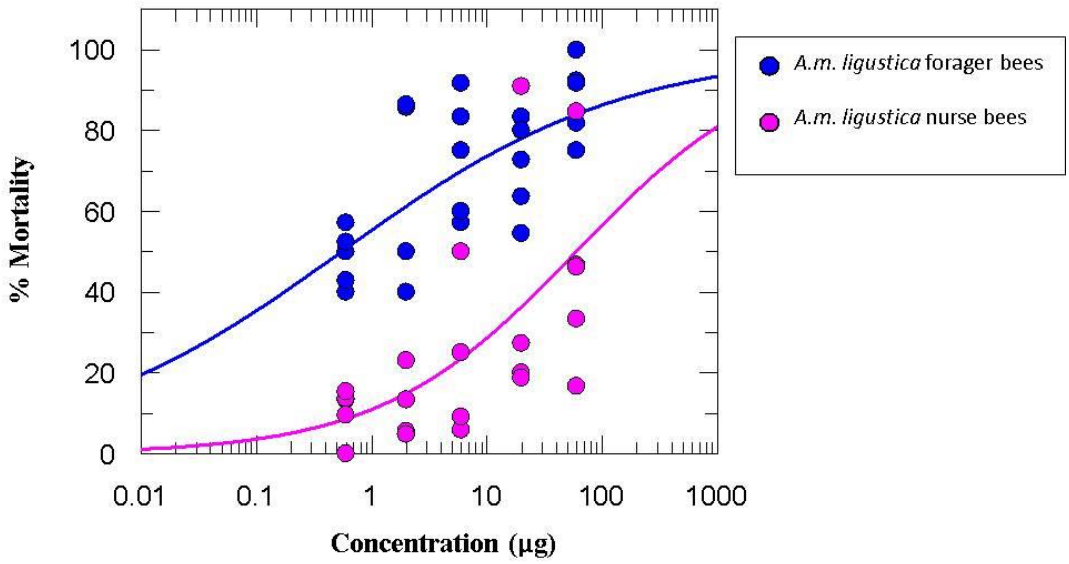


Figure 6.3 Dose-response curves from topical bioassays; thiacloprid against forager and nurse bees from *A. m. ligustica*.

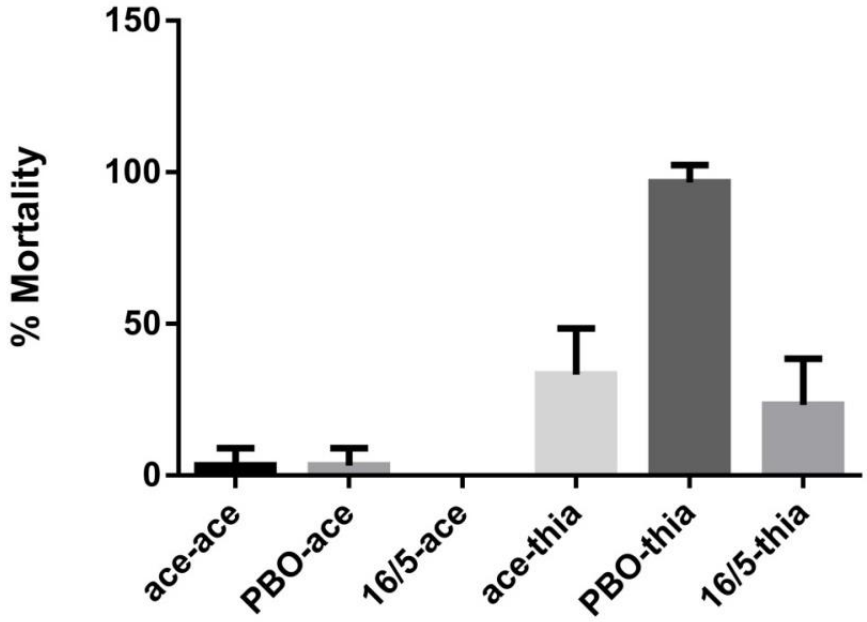


Figure 6.4 Synergistic effect of PBO and EN 16/5-1 mixed with a discriminating dose of thiacloprid on *A. m. ligustica* (forager bees) (Error bars represent standard error of mean).

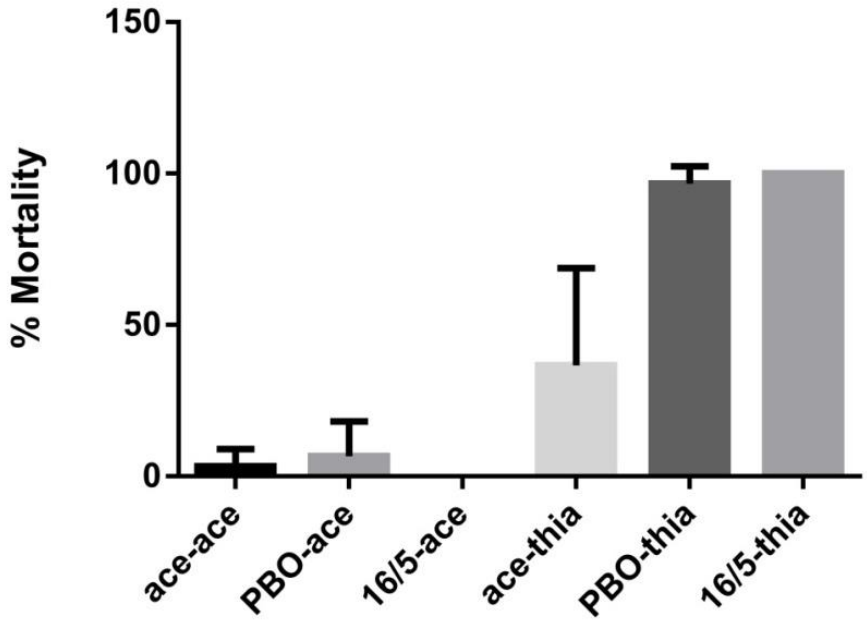


Figure 6.5 Synergistic effect of PBO and EN 16/5-1 mixed with a discriminating dose of thiacloprid on *A. m. ligustica* (nurse bees) (Error bars represent standard error of mean).

6.3.2 Haem peroxidise assay (Quantification of haem group of different bee races)

This assay does not directly measure oxidase activity but instead quantifies the total haem content. A higher haem content is assumed to reflects up-regulation of haem containing P450(s). Comparisons of total haem of 3 different bee races are shown in Figure 6.6. *A. m. caucasica* has the highest haem content (0.191 µg haem/mg protein) compared to *A. m. buckfast* (0.099 µg haem/mg protein) and *A. m. ligustica* (0.056 µg haem/mg protein). The non-paired t-test results also show that the haem content of *A. m. caucasica* is significantly higher than haem content of other two tested races ($P<0.05$) while there is no significant difference of haem content between *A. m. ligustica* and *A. m. buckfast* ($P>0.05$) (Appendix 9.7).

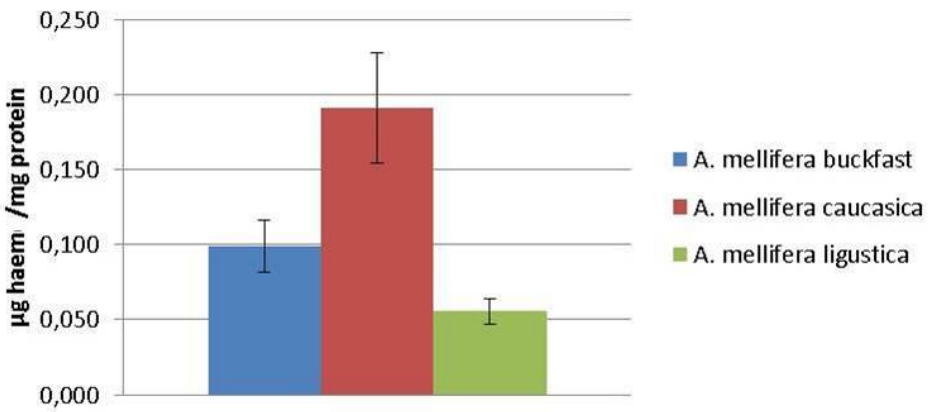


Figure 6.6 Haem content for 3 different honey bee races (Error bars represent standard error of mean).

6.3.3 Comparison of Two Honey Bee Races by Microarray Analysis

In the microarray experiment gene expression in *A. m. buckfast* and *A. m. caucasica* were compared. Total RNA was extracted from pooled samples of 5 bees using TRIzol Reagent (Ambion). Four independent extractions were performed for each race. When RNA integrity of these samples was assessed by electrophoresis there was evidence of a significant degree of RNA degradation, which presumably occurred during transport of bees from Turkey to the UK, despite the samples being transported in RNA*later*™ (Figure 6.7).

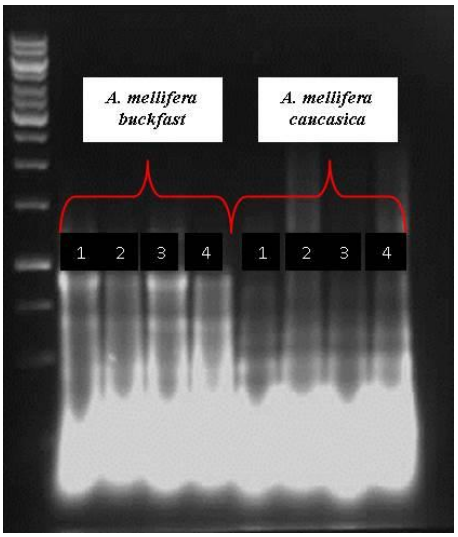


Figure 6.7 RNA gel image of *A. mellifera buckfast* and *A. mellifera caucasica*

This was of some concern because the RNA extracted from *caucasica* bees appeared to be more degraded than that extracted from *buckfast* bees. As it was too late in the season to obtain fresh samples, microarray analysis was continued after purifying these samples further by running them through an RNA purification column (Qiagen) and carefully normalising the amount of starting RNA, after measuring RNA concentration by spectrophotometry. Microarray analysis was carried out as described previously in section 2.7.8, Chapter 2 and the results are shown in Appendix 9.4.

6.3.4 qRT-PCR validation

Microarray results were validated for selected candidate genes using qRT-PCR. Although RNA concentration was normalised for the two races prior to cDNA synthesis using a nanodrop spectrophotometer, the results of expression analysis of several house-keeping genes indicated that there are significant differences in the cDNA quantity/quality obtained with lower C_ts (threshold cycle) observed for *A. mellifera caucasica* for all genes (Figure 6.8 and Figure 6.9).

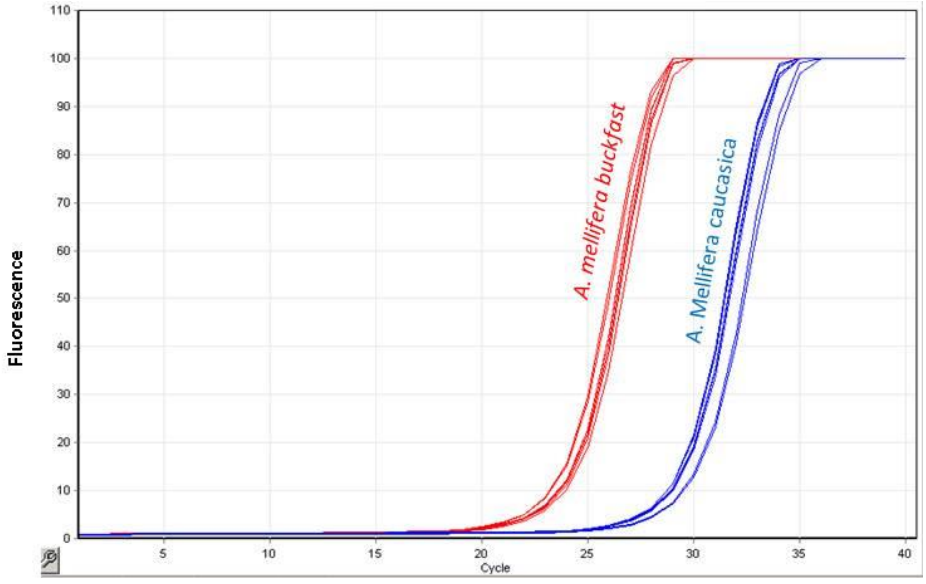


Figure 6.8 Cts of reference genes (ef1)

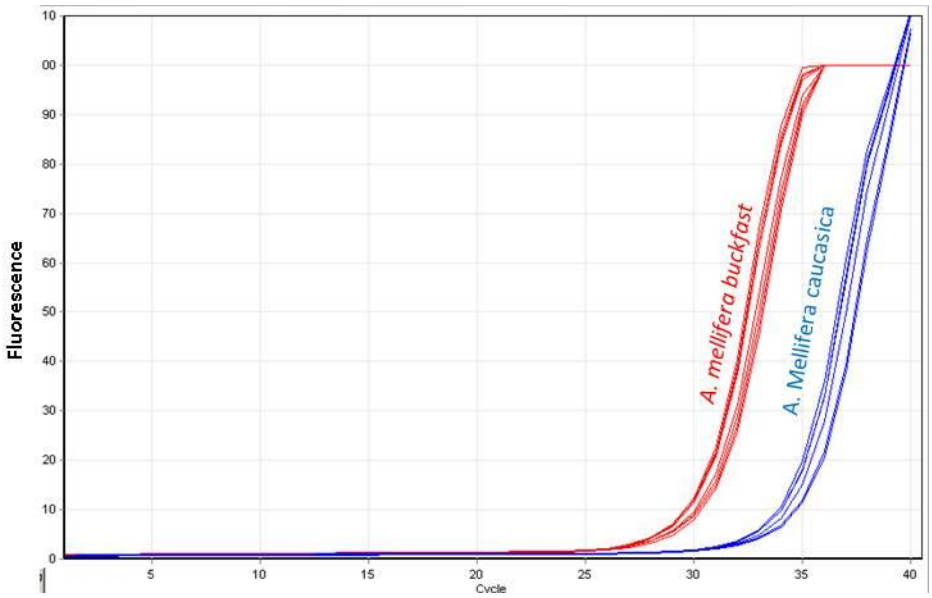


Figure 6.9 CTs of reference genes (tbp)

This is likely to be a result of the differential RNA degradation which may have affected the outcome of the microarray experiments. Nevertheless, in the microarray study a nAChR alpha 6 subunit was up-regulated ~8-fold in *A. m. caucasica* compared to *A. m. buckfast* and, after normalisation for differences in RNA quantity using reference genes in qRT-PCR, these results were confirmed, using two different primers sets (Figure 6.10).

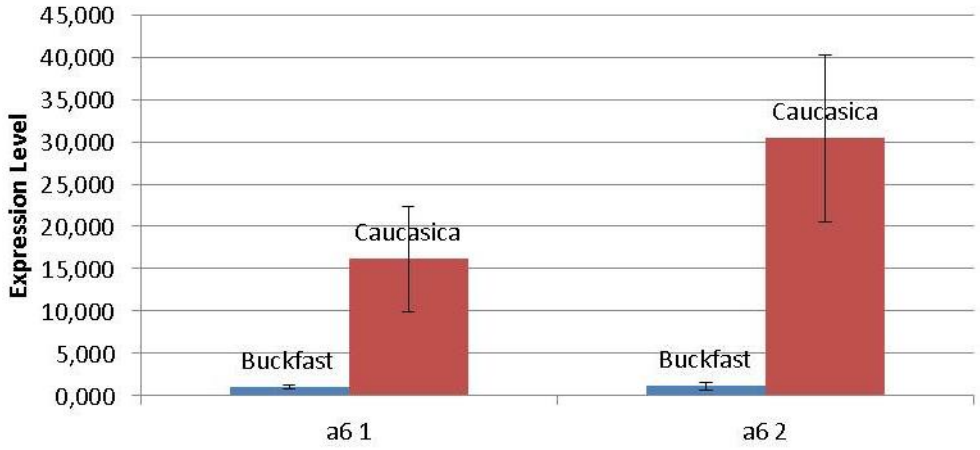


Figure 6.10 Expression levels determined with qRT-PCR for nAChR alpha 6 subunit (Error bars represent 95% confidence limits).

6.3.5 Sequence Analysis of Nicotinic Acetylcholine Subunits in Two Honey Bee Races

Using the published *A. mellifera* genome data, 11 AChR subunits from two races (*A. m. buckfast* and *A. m. caucaisca*) were screened by sequence analysis (Appendix 9.5). Sequences were aligned using Geneious with the *A. mellifera* genome sequence as the template. To date this analysis has identified 5 splice variants in Alpha 4 subunit in *buckfast*, 2 splice variants in alpha 5 in *caucasica* and 2 splice variants in alpha 7 in *buckfast*. No non-synonymous nucleotide changes were identified in nAChR subunit genes between both races.

6.4 Discussion

A. m. buckfast and *A. m. caucasica* subspecies showed different toxicity responses to two cyano-substituted neonicotinoids (thiacloprid and acetamiprid). *A. m. caucasica* exhibited greater tolerance to both assayed neonicotinoids compared to *A. m. buckfast*. Several factors might be involved in the sensitivity difference observed such as physiological differences (different cuticle penetration properties or varying body sizes, or differences in development timings) or biochemical differences (metabolic processes) between the two bee races. Previous work has suggested P450 metabolism may enhance the tolerance of honey bees against cyano-substituted neonicotinoids as the toxicity of such compounds to bees can be significantly increased using detoxification enzyme inhibitors (Iwasa *et al*., 2004). In addition biochemical examination of bee esterase did not show binding to thiacloprid (see Chapter 3). Higher haem content in *A. m. caucasica* may be an indicator of a greater metabolic activity which provides protection against cyano-substituted neonicotinoids, and this may be a race-specific characteristic of *A. m. caucasica*

bees. The transcriptome profiling revealed that a subunit of the nAChR (the nAChR target-site) was overexpressed in *A. m. caucasica* compared to *A. m. buckfast*, while a large number of P450s and esterases were down-regulated. Although rapid P450 detoxification of cyano neonicotinoids appears to be the primary mechanism for relative tolerance of bees, target-site insensitivity might act as an additional mechanism of tolerance.

Target-site resistance to neonicotinoids has been described in other insect species. For example Bass *et al*. (2011) identified multiple mechanisms of resistance in a field population of *Myzus persicae* (peach-potato aphid) to imidacloprid. These included metabolic resistance due to P450-mediated detoxification but also target site resistance as a result of mutation of a key residue in the loop D region of a nAChR β1 subunit.

In another study, a point mutation in two nAChR alpha subunits (Nlα1 and Nlα3) in a brown planthopper laboratory-selected strain of *Nilaparvata lugens* were reported to confer resistant to imidacloprid (Liu *et al*., 2005). However, to date, field populations with resistance to this compound appear to exclusively result from P450 detoxification (Wen *et al*., 2009; Puinean *et al*., 2010).

In reference to these findings, the nAChR of *A. m. caucasica* and *A. m. buckfast* was examined to identify if any mutations could be found to implicate target-site resistance. In the honey bee genome 9 nAChR alpha subunits and 2 beta subunits have been described. However, in my sequencing studies, no non-synonymous mutations were observed between the two races. However, several splice variants were detected in screened nAChR subunits of both sub species *A. m. caucasica* and

A. m. buckfast. Alternative splicing of nAChR subunits has been implicated in resistance to insecticidal nAChR agonists previously. For example, a spinosad resistant strain of diamondback moth *Plutella xylostella* has a mutation resulting in mis-splicing of the nAChR Pxα6 which results in a truncated protein (Baxter *et al*., 2010). However I did not observe alternative splicing resulting in frame-shifts or truncated nAChR subunits in the honey bee race sequences.

Although the nAChR subunit alpha 6 was overexpressed in *A. m. caucasica* compared to *A. m. buckfast,* it is important to point out that the degraded RNA observed in these experiments might have affected the quality of results. Thus, as a future work, the microarray results should therefore be interpreted with caution and ideally repeated using fresh material. If overexpression of the nAChR subunit alpha 6 can be confirmed it would be interesting to carry out functional analyses to explore if this has any effect on resistance to neonicotinoids. One way to do this would be to use the GAL4/UAS system to create transgenic *Drosophila* that express honey bee nAChR subunit alpha 6 at different levels and then examine the sensitivity of the transgenic lines to neonicotinoids. In such an experiment it would be useful to knock-out the endogenous *Drosophila* nAChR subunit alpha 6 (which would not be lethal to Drosophila), this could be done by RNAi or alternatively using the new CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 (CRISPR-associated protein-9 nuclease) technology (Bassett and Liu, 2014).

Insensitivity differences to thiacloprid were also observed between forager and nurse bees of *A. m. ligustica* with nurse bees showing higher tolerant to thiacloprid. This significant insensitivity on nurse bees has been detected in other studies where young bees were found to be more tolerant to pesticides (Wahl and Ulm, 1983; Rortais *et*

al., 2005; Wu *et al*., 2011). This might be explained simply as age-related weakness or could be related to sub-lethal exposure to insecticides/xenobiotics that older (forager) bees may be exposed to during foraging activity. Synergism studies carried out in the presence and absence of PBO and EN 16/5-1 suggested that esterases may provide extra protection to nurse bees against thiacloprid. Further studies are needed to confirm the involvement of esterase to metabolism in nurse bees. This could include an investigation into the interaction of nurse bee esterase and neonicotinoid by "interference assay".

Understanding the mechanism of enzyme detoxification/target-site sensitivity may contribute to the development of a novel technique for pest control and any findings on race-based/labour division based sensitivity will be the first example confirmed using both molecular and biochemical techniques. It would also become an important attribute to be considered by bee-keepers when choosing the race of bees best suited for their particular purpose and that would also have important ramifications for the pesticide registration procedure.

7. GENERAL DISCUSSION

The work of this thesis has provided significant additional evidence for the involvement of phase 1 detoxification enzymes (esterases and P450s) in honey bee 'defence' against the insecticides tau-fluvalinate and thiacloprid. Synergist bioassay results using PBO and EN 16/5-1 suggested that these detoxification mechanism(s) may play an important role in protecting honey bees from tau-fluvalinate and thiacloprid toxicity.–P450s were found to contribute the most protection to both compounds and this was further supported by the "interference assay" with neither insecticide interacting with semi-purified bee esterases. Similar binding studies with another pyrethroid (alpha-cypermethrin) and neonicotinoid (imidacloprid) revealed these compounds also failed to bind to honey bee esterases.

This is in contrast to herbivorous insects that have been assayed using the same interference assay. The resistance-associated esterase, E4, was used as a positive control in the thesis, and in other herbivores (*Bemisia* tabaci, *Plutella xylostella*, *Helicoverpa armigera*) semi-purified esterase(s) has always been found to interact with a variety of xenobiotics (G. Moores, pers comm).

Taken together the synergist and interference assays strongly suggest that P450s are the primary mechanism of detoxification of these insecticides in honey bees.

Specific P450s involved in honey bee detoxification of tau-fluvalinate have been identified previously with *in vitro* characterisation of eight honey bee P450s of the CYP3 clan revealing that three members of the CYP9Q family have the capacity to metabolise tau-fluvalinate and also the organophosphate coumaphos (Mao *et al*.,

2011). However, to date, the P450s involved in the detoxification of cyano-substituted neonicotinoids like thiacloprid have not been identified.

The honey bee genome contains 46 P450 genes and to identify which of these is/are involved in insecticide detoxification requires a high-throughput approach. In this thesis, therefore, an induction strategy followed by whole transcriptome profiling was adopted to identify genes up-regulated in response to exposure to a sub-lethal, topical, dose of thiacloprid.

This is the first demonstration with honey bees of a sub-lethal dose of an insecticide providing a protective effect to subsequent exposure of the same insecticide. In a similarly structured study by Johnson *et al*. (2012), no effect on honey bee toxicity was detected for tau-fluvalinate (pyrethroid) in individuals fed with phenobarbital, xanthotoxin, salicylic acid and indole-3-carbinol; in contrast, quercetin fed individuals showed a reduced sensitivity. However, this study did not include the effect of a tau-fluvalinate feeding on subsequent sensitivity to the same chemical compound. Beyond honey bees, tolerance of *Aedes aegypti* against a sub lethal dose of the pyrethroid permethrin, the organophosphate temephos, the herbicide atrazine and other xenobiotics (fluoranthene and copper) has been explored using a similar approach to this thesis. Larval tolerance to permethrin was moderately enhanced following exposure to xenobiotics while larval tolerance to temephos improved moderately after exposure to atrazine, copper and permethrin, however, none of the insecticides provided a protective effect on larval tolerance to subsequent exposure to the same insecticides (Poupardin *et al*., 2008).

The induction bioassay was planned to monitor the phenotypic effects on honeybees exposed by feeding to sub-lethal doses for 24 hours. This experimental design and

the results obtained revealed that 24 hours of exposure reduced bee sensitivity to an immediate subsequent exposure to a much higher dose of thiacloprid, however this was not a permanent phenomenon and by the 48 hours time point bees fed thiacloprid were no more tolerant than controls. It would be interesting to explore, in future, if longer/continual initial exposure times resulted in a longer 'protective effect'. This type of longer, low-level exposure more closely resembles the type of exposure scenario bees might encounter in the field, and it would be interesting to carry out similar experiments at the field scale. The findings of such a study could theoretically also inform a new approach in apiculture of designing complementary-diets where honey bees can be fed with sub-lethal doses of certain compounds to establish colony defence to that compound. However, such a strategy would require careful examination of possible sub-lethal effects on bees from the low level exposure doses used and any carry through into bee honey. Although theoretically possible in reality such an approach is unlikely to garner widespread support. Interestingly a protective effect from other stress factors such as diseases, parasites or involvement of other pesticides or phytochemicals could not be demonstrated in the laboratory, so replicating similar studies in field conditions are necessary.

The results of the induction bioassay may be interpreted as sub-lethal exposure of thiacloprid activating the transcription of one or more genes encoding detoxification/defence and these subsequently return to constitutive levels, or lower than constitutive levels, at the later time points.

Although identification of the specific genes of honey bees that regulate the metabolism of detoxification enzymes is an important research topic, only a very limited number of studies related to detoxification gene induction have been

published. However, several studies, including this report, show that detoxification capacity can be induced by specific agents. Yu *et al*. (1984) exposed honey bees orally to sub-lethal doses of 5 insecticides (permethrin, metoxychlor, carbaryl, malathion, diflubenzuron) to assess the effects on expression of detoxification enzymes. Surprisingly dietary exposure did not show any effect of detoxification enzyme activity except in the case of permethrin, which was found to significantly induce GST activity; Kezic *et al.* (1992) investigated P450 induction in bees after the 9^{th} day of dietary exposure to benzo-(α)-pyrene, and found that benzo-(α)-pyrene monooxidase activity was increased 5-25 fold in honey bees. In addition, Mao *et al*. (2011) found that CYP9Q3 and CYP9Q2 were induced after exposure to tau-fluvalinate and bifenthrin respectively. Johnson *et al*., (2012) found that when honey bees were exposed to honey extracts, it resulted in the up regulation of the CYP6AS sub family of clade 3. Derecka *et al*. (2013) demonstrated that exposure to a sub lethal dose of pesticides induced the expression of metabolic enzymes of honey bees with CYP6AS3, CYP6AS4, CYP6AS14, CYP6AS15, CYP6AR1, CYP9R1, and CYP9S1 upregulated in larvae following 15 day feeding with imidacloprid. Coumaphos and fluvalinate upregulated CYP6AS3, CYP6AS4 and CYP9S1 in adult honey bees (Schmehl *et al*., 2014). These findings principally implicated members of the CYP3 clade of P450s.. In this thesis study, CYP6AS5 and CYP6BE1 which also members of CYP3 clade were identified as up-regulated in honey bees fed thiacloprid and have not been found to be up-regulated by any xenobiotic previously (Berenbaum and Johnson, 2015). Furthermore, this thesis study shows that, CYP305D1 of the CYP2 clade and was up-regulated in treated bees. This gene was also shown to be upregulated previously in honey bees after exposure to coumaphos (Scmehl *et al*., 2014).

Additionally CCE8, a carboxylestrase was found to be up-regulated by thiacloprid in this study. Honey bee esterases have been shown to be induced previously by several different groups of insecticides including organophosphates, neonicotinoids, pyrethroids, phenylpyrazoles, and spinosyns (Carvalho *et al*., 2013; Berenbaum and Johnson, 2015). Similarly, esterase E4-like was found to be upregulated by coumaphos (Scmehl *et al*., 2014). As, the results of esterase 'interference assays' conducted in this thesis did not showing any interaction between tested insecticides and honey bee estrases it is likely that the upregulation of this gene represents a generic stress response which in this case provides no protective effect.

Beyond honey bees there are examples of induction studies in other insects which aimed to understand the metabolic basis of insecticide resistance and/or identify the enzymes which are capable of detoxifying xenobiotics. Thiamethoxam induces detoxification enzymes including P450s in a resistant *Bemicia tabaci* strain (Yang *et al*., 2013). Willoughby *et al*. (2006) revealed that in *Drosophila melanogaster* minimal induction of detoxification enzymes were observed after a short exposure to high lethal concentrations of several insecticides (spinosad, diazinon, nitenpyram, lufenuron and dicyclanil), in contrast the natural plant compound caffeine and the barbituate phenobarbital induced a number of CYP and GST genes related to insecticide resistance. Thus, insecticides may not always be the most active inducer of detoxification enzymes which are capable of metabolising them (Poupardin *et al*., 2008).

Similarly, *p*-coumaric acid (constituent of honey) were found to be the most active inducer of detoxification enzymes in honey bees and upregulated fourteen xenobiotic-metabolising P450s (Mao *et al*., 2013; Berenbaum and Johnson, 2015). In

contrast in this thesis thiacloprid upregulated only four candidate P450s (CYP315A1, CYP6BE1, CYP305D1, CYP6AS5) which may participate in xenobiotic metabolism.

For the first time, the ompA+2 technique has been used to successfully express honey bee P450s in *E. coli*, enabling further studies to assess the prescence/absence of pesticide metabolism by particular honey bee P450s. Although functional P450 was obtained for all CYP genes expressed, incubation of thiacloprid and imidacloprid with recombinant P450s and cytochrome b5 failed to produce evidence for the metabolism of these compounds as assessed by parent compound depletion. Similarly, in previous studies coumaphos and fluvalinate were found to up-regulate CYP6AS3, CYP6AS4 and CYP9S1 in honey bees (Scmehl *et al*., 2014); however, once expressed heterologously these P450 enzymes did not metabolize either of these two insecticides (Mao *et al*., 2009).

This negative result may be due to several possible factors: Firstly, the induction strategy used has failed to identify the detoxification enzyme which enhances thiacloprid tolerance. This could be because the detoxification enzyme which is responsible for metabolising neonicotinoids may not be induced but rather is constitutively expressed at levels sufficient to provide protection to honey bees against this compound. Alternatively, because the activity of recombinant enzymes could not be validated using two model substrates, it is not clear whether the lack of insecticide metabolism was due to lack of an active enzyme or through the enzyme being unable to metabolise the insecticide. However for all recombinant P450s expressed a clear P450 peak was observed in CO-difference spectrum assays suggesting correctly-folded P450 was obtained. This provides evidence that the latter scenario is perhaps more likely.

Mao *et al*. (2011) described the importance of such findings for selectively induced P450 genes by a specific induction agent which would help to develop monitoring strategies for honey bee exposure to specific pesticides while foraging and to differentiate between in-hive exposure to non-target acaricides applied to control *Varroa* and to exposure to non-target pesticides used on agricultural crops.

In addition, identification of the specific detoxification genes that metabolise insecticides could be used to design new less toxic pesticides/synergists to minimise severe damage to non-target organisms. These new strategies would aim to design chemicals which have the ability to inhibit pest detoxification activities without damaging the honey bee defence mechanism or possibly even stimulate its detoxification capacity.

In humans a great deal of research focuses on the metabolism of xenobiotics, especially therapeutic drugs, by P450s. As an example human CYP3A4 is the most studied drug-metabolised model P450 and has a broad substrate detoxification capacity. Similarly, finding generalist bee detoxification enzymes and identifying a specific model substrate can provide an opportunity to develop high-throughput *in vitro* screening tools which detect insecticides that are substrates/inhibitors and therefore may be metabolised. Furthermore identification of P450s involved in insecticide metabolism would allow the P450/insecticide interaction to be studies using homology modelling. Such studies may guide the synthesis of the new chemical via rational design and allow critical catalytic sites in P450s unique for pests and honey bees to be identified.

In summary identification of the specific honey bee genes that regulate the metabolism of the detoxification enzymes or the alternative target-sites will enable agrochemical R&D to develop safer and target-specific products.

Bee pollinators carry out a vital ecosystem service and their wellbeing is essential to ensure future food security. Because they are insects, bees may be as sensitive to insecticides as the target-pests. However, there are a number of examples where this is not the case, and indeed certain members of the pyrethroid (tau-fluvalinate) and organophosphate (coumaphos) class of insecticides show low toxicity to bees and are used as in-hive treatments to control *Varroa destructor,* a parasitic mite of honey bees. Another example is cyano-substituted neonicotinoids (thiacloprid and acetamiprid) that have been shown to be orders of magnitude less acutely toxic to honey bees than nitro-substituted compounds (imidacloprid, clothianidin, thiamethoxam, dinotefuran, nitenpyram) (Iwasa *et al*., 2004).

Differences between the sub-classes of the neonicotinoids could direct the future focus on insecticide development. Since a nitro group is known to be more toxic to honey bees, by focussing on the development of safer alternatives incorporating a cyano group, which targets insect pest nAChRs rather than the honey bee nAChRs, it may be possible to create a more suitable environment for the registration of neonicotinoids regarding honey bee health.

7.1 Future Work

As a result of the findings of this PhD study, several outstanding questions require further investigation:

- Further examination of the 'protective effect' of honey bee exposure to low levels of thiacloprid: This work has shown that bees exposed to thiacloprid for 24 hours are less sensitive to subsequent immediate exposure. The duration of this 'protective effect' could be explored using longer exposure times of using different concentrations of insecticides. Furthermore the design of similar field-level experiments is required to show if a similar trend is seen at this scale.

- Examination of the proteolytic activity of honey bee: Examining CYP3A4 oxidase activity in combination with honey bee microsomes i.e. to monitor fluorometrically how the activity of CYP3A4 is affected by the presence of honey bee microsomes using a model substrate such as 7-EC. Such studies will confirm or otherwise the presence of protease activity that inhibits P450 activity within honey bee microsomes.

- Co-expression of honey bee P450s with honey be CPR: In this PhD study honey bee P450s were co-expressed with mosquito CPR, which might affect the biotransformation activity of P450. Therefore co-expression of honey bee P450s with honey bee CPR might produce enhanced activity against insecticides.

- More sensitive analysis of insecticide metabolism by candidate P450s.

  In this study metabolism was assessed by measuring parent compound depletion. A more sensitive detection methodology could be used to monitor the appearance of specific P450-mediated insecticidal metabolites using LC-MS/MS. This would require the use of metabolite standards which were not available during the course of this PhD.

- CCE8 expression in alternative expression systems: CCE8 expression was not achieved using the *E. coli* system; eukaryote expression systems have been successfully applied to esterases including *Helicoverpa armigera* esterase expression in baculovirus (Teese *et al*., 2013) and *Tribolium casteneum* esterase expression in methylotrophic yeast *Pichia pastoris* (Delroisse *et al*., (2005). CCE8 expression in baculovirus or yeast expression systems may be worth considering in future.

- Model substrate characterisation: Further investigation of alternative model substrates of recombinant honey bee P450s is required which would facilitate future insecticide screening.

- Interference Assay: Employing the interference assay for screening the binding of nurse and forager bee esterases on neonicotinoids; if binding is found, it will be valuable to identify candidate esterases as important detoxification enzymes.

- Examination of the nAChR sub-units: Examination of mis-spliced variants of nAChRs from different honey bee races (with different susceptibility to insecticide) may lead to identification of an alternative defence mechanism to nAChR agonist insecticides.

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9. APPENDICES

9.1 Solutions, Buffers and Media

9.1.1 Antibiotics, media, electrophoresis buffers

Antibiotics

50 mg/ml Ampicillin in water

25 mg/ml Chloramphenicol in ethanol

Luria Bertani (LB) medium

10 g of Bacto-tryptone

5g of yeast extract

10 g of NaCl

Deonized water (to 1 liter)

pH adjusted to 7 and autoclaved

LB agar

LB medium

20 g of Bacto agar

Terrific Broth (TB)

47.6 g terrific broth powder

8 mL glycerol

Deonized water (to 1 liter)

Autoclaved

50x TAE (Tris Acetate EDTA) buffer

242 g Tris base

100 ml 0.5 M EDTA pH 8.0

Deonized water (to 1 liter)

When diluted, the 1X solution contains 40 mM Tris, 20 mM acetic acid, 1 mM EDTA.

10x TBE (Tris Borate EDTA) buffer

Concentrated solution of 10X TBE in deionized water was obtained from National Diagnostic. When diluted, the 1X solution contains 0.089M Tris base, 0.089M boric acid (pH 8.3) and 2mM Na_2EDTA.

9.1.2 Solutions for preparation of bacterial membranes

2X TSE buffer

100 mM Trizma base, pH 7.6

500 mM sucrose

0.5 mM EDTA

Filter sterilized and store at 4°C

Lysozyme

20 mg/mL in water, freshly prepared

Spheroplast resuspension buffer

100 mM potassium phosphate, pH 7.6

6 mM magnesium acetate

20% (v/v) glycerol

0.1 mM dithiothreitol (DTT)

Filter-sterilized. Solution was prepared without DTT and store at room temperature, and DDT was added from a separate 1 M stock (stored in aliquots at −20°C) just before use.

Protease inhibitors

0.2 M (100mM) phenylmethylsulfonyl fluoride (PMSF), in ethanol (X100 stock); 10 mg/mL of aprotinin, in 10 mM HEPES, pH 8.0 (X10,000 stock); 10 mg/mL leupeptin, in water (X10,000 stock). Stock solutions of protease inhibitors are stored at −20°C.

9.1.3 Solutions for preparation of cytochrome b5

Phosphate buffered saline (PBS)

137 mM NaCl, 10 mM phosphate at pH 7.4

Buffer A

75 mM Tris at pH 8 containing 0.1 mM EDTA, 10 µg/ml aprotinin, and 1 mM PMSF. For 50 ml, add 3.75 ml of 1 M Tris for pH 8, 0.5 ml of 10 mM EDTA at pH 8, 50 µl of 10 mg/ml aprotinin stock, and 250 µl of 200 mM PMSF in ethanol (add 28.7 µl of ethanol per mg of PMSF) to 45.5 ml of deionised water.

Buffer B2

20 mM Tris at pH 8, 2 mM 2-mercaptoethanol (2ME), 20% glycerol (v/v), and 0.1% (w/v) CHAPS. For 50 ml, add 1 ml of 1 M Tris for pH 8, 7 µl of 2ME (in fume-hood), 0.05 g of CHAPS and 10 ml of glycerol to 39 ml of deionised water.

Buffer B3

As with B2, but with 0.1 M imidazole. Prepare a 0.5 M (34 g/l) imidazole stock solution at pH 8 (add HCl while monitoring pH before making up to a final volume) and filter through a syringe. Use 10 ml of this stock and 29 ml of deionised water for 50 mL.

Buffer C (b5 storage buffer)

10 mM Tris at pH 7.5, 20% (v/v) glycerol, 0.5 mM EDTA, 0.1 mM DTT, and 0.05% (w/v) CHAPS. For 50 ml, add 0.5 ml of 1 M Tris for pH 7.5, 10 ml of glycerol, 2.5 ml of 10 mM EDTA at pH 8, 0.001 g of DTT, 0.025 g of CHAPS, and make up to 50 ml with deionised water.

9.1.4 Solutions for spectral determination

P450 spectrum buffer (2X stock)

200 mM Tris-HCl, pH 7.4

20 mM CHAPS

40% (v/v) glycerol

2 mM EDTA

Filter-sterilized and stored at 4°C.

9.2 Microarray Analysis for Induction Experiment 1

9.2.1 Genes identified by microarray as differentially transcribed between 1h treated (neonicotinoid/acetone) samples

ProbeName	p-value	Regulation	Fold change	Description
CUST_7297_PI425798793	0.010733	up	4.786921	sodium calcium exchanger 3
CUST_7865_PI425798793	0.004494	up	4.6502337	serine threonine-protein kinase sbk1
CUST_5381_PI425798793	0.018708	up	4.457718	guanine nucleotide-binding protein subunit beta 1
CUST_4166_PI425798793	0.01412	up	3.7678204	ras-related and estrogen-regulated growth inhibitor
CUST_5654_PI425798793	0.007943	up	3.6812813	protein fam46a
CUST_9366_PI425798793	0.024454	up	3.667787	receptor-type tyrosine-protein phosphatase r
CUST_1699_PI425798793	0.013827	up	3.5489256	guanine nucleotide-binding protein g subunit alpha
CUST_9901_PI425798793	0.031895	up	3.5088837	isoform c
CUST_7488_PI425798793	0.003326	up	3.5001063	voltage-dependent l-type calcium channel subunit beta-2
CUST_6768_PI425798793	0.006894	up	3.3167112	moxd1 homolog 1-like
CUST_4231_PI425798793	0.003783	up	3.316596	protein lin-10
CUST_8546_PI425798793	0.02436	up	3.243571	postreplication repair protein hrad18p
CUST_8228_PI425798793	0.004417	up	3.1261861	kv channel-interacting protein 4
CUST_1444_PI425798793	0.030679	up	3.062578	heparan sulfate n-deacetylase n-sulfotransferase
CUST_7400_PI425798793	0.00866	up	2.9893198	isoform a
CUST_6819_PI425798793	0.016199	up	2.9656339	f-box lrr-repeat protein 16
CUST_220_PI425798793	0.027258	up	2.9087846	potassium sodium hyperpolarization-activated cyclic nucleotide-gated channel 4
CUST_6669_PI425798793	0.004677	up	2.9058588	aftiphilin isoform 1
CUST_237_PI425800172	0.005437	up	2.8929057	CYPAA1
CUST_1222_PI425798793	0.046722	up	2.8699787	proline synthetase co-transcribed bacterial-like protein
CUST_4004_PI425798793	0.023215	up	2.8373952	mothers against decapentaplegic-like protein 4
CUST_5470_PI425798793	0.048042	up	2.7222314	disco-interacting protein 2
CUST_6789_PI425798793	0.004279	up	2.7124064	egf- fibronectin type-iii and laminin g-like domain-containing protein
CUST_3806_PI425798793	0.019587	up	2.7120137	neurexin isoform b
CUST_6276_PI425798793	0.017909	up	2.6342287	mitochondrial ubiquitin ligase activator of nfkb 1
CUST_7675_PI425798793	0.011607	up	2.6053603	acetylcholine receptor subunit alpha-like 2
CUST_5553_PI425798793	0.01342	up	2.6013956	alpha gamma epsilon
CUST_3336_PI425798793	0.035661	up	2.583453	isoform c
CUST_5248_PI425798793	0.032529	up	2.5769362	myosin iiia
CUST_8809_PI425798793	0.023689	up	2.5726	dipeptidyl peptidase
CUST_262_PI425798793	0.012211	up	2.553966	cysteine serine-rich nuclear protein 2
CUST_1023_PI425798793	0.036961	up	2.5527952	nmda receptor isoform e
CUST_3880_PI425798793	0.002709	up	2.5419142	tripartite motif-containing protein 71-like
CUST_3243_PI425798793	0.044888	up	2.5316353	amp dependent coa ligase
CUST_8256_PI425798793	0.033604	up	2.5216749	alpha-2-macroglobulin-like 1
CUST_4634_PI425798793	0.04595	up	2.5158482	pyruvate dehydrogenase
CUST_5375_PI425798793	0.021767	up	2.4476688	calcium-activated potassium channel alpha chain
CUST_6822_PI425798793	0.010474	up	2.393839	serotonin receptor
CUST_8342_PI425798793	0.027994	up	2.3602626	angiotensin-converting enzyme
CUST_1112_PI425798793	0.011576	up	2.3437738	protein vav
CUST_3375_PI425798793	0.028684	up	2.334226	cytokine receptor
CUST_6782_PI425798793	0.025637	up	2.3321173	hepatoma-derived growth factor
CUST_2055_PI425798793	0.007865	up	2.3202736	membrane associated ring finger
CUST_5021_PI425798793	0.01574	up	2.2792106	peripheral plasma membrane protein cask-like isoform 1
CUST_2974_PI425798793	0.006557	up	2.2788162	mkl myocardin-like protein 1
CUST_819_PI425798793	0.008188	up	2.2655263	myosin-rhogap myr
CUST_6490_PI425798793	0.006891	up	2.2642448	camp-dependent protein kinase type ii regulatory subunit
CUST_7939_PI425798793	0.04134	up	2.2550404	glutamate decarboxylase
CUST_2742_PI425798793	0.01922	up	2.2498593	protein btg1
CUST_2653_PI425798793	0.014069	up	2.2475896	protein fam49b
CUST_1604_PI425798793	0.024058	up	2.2360082	transmembrane protein 38a
CUST_9425_PI425798793	0.046063	up	2.1780593	cd63 antigen
CUST_3948_PI425798793	0.023512	up	2.1644561	26s proteasome non-atpase regulatory subunit 11
CUST_10018_PI425798793	0.035467	up	2.1601832	tpa_inf: venus kinase receptor
CUST_9485_PI425798793	0.031598	up	2.1538413	isoform a
CUST_7330_PI425798793	0.007028	up	2.1500232	groucho-like protein
CUST_8858_PI425798793	0.038219	up	2.136555	ap-2 complex subunit mu-1
CUST_10100_PI425798793	9.79E-04	up	2.1307929	probable nuclear hormone receptor hr38
CUST_6994_PI425798793	0.033845	up	2.0769582	longitudinals lacking isoforms a b d l
CUST_10072_PI425798793	0.033493	up	2.0755143	protein jagged-1
CUST_1777_PI425798793	0.048921	up	2.0702293	rhomboid family member 1
CUST_5829_PI425798793	0.003435	up	2.0536528	phosphofurin acidic cluster sorting protein 2
CUST_2580_PI425798793	0.009873	up	2.043548	maguk p55 subfamily member 7
CUST_5499_PI425798793	0.044448	up	2.0409653	galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase i
CUST_196_PI425798793	0.013107	up	2.037705	voltage-gated potassium channel
CUST_64_PI425800172	0.040366	up	2.0266533	CCE3_GB19866
CUST_5693_PI425798793	0.031864	up	2.0234327	tetratricopeptide repeat protein 14

CUST_8241_PI425798793	0.02765	up	2.013083	protein scai-like
CUST_4031_PI425798793	0.021992	up	2.009252	pipsqueak
CUST_9340_PI425798793	0.028587	down	-2.0031161	odorant-binding protein
CUST_8272_PI425798793	0.045757	down	-2.0291111	proton-coupled amino acid transporter 4
CUST_2517_PI425798793	0.019649	down	-2.0361173	upf0184 protein c9orf16-like
CUST_4370_PI425798793	0.018101	down	-2.1150079	tes14
CUST_88_PI425798793	0.035789	down	-2.1150546	casein kinase ii subunit alpha
CUST_4403_PI425798793	0.00503	down	-2.1879268	mucoepidermoid carcinoma
CUST_5771_PI425798793	3.41E-04	down	-2.229174	malate dehydrogenase
CUST_7467_PI425798793	0.024024	down	-2.2656353	doublesex- and mab-3-related transcription factor a2
CUST_5072_PI425798793	0.00985	down	-2.266004	unkempt protein
CUST_6359_PI425798793	0.015326	down	-2.3462105	ef-hand domain-containing protein cg10641-like
CUST_7122_PI425798793	0.003202	down	-2.3663533	probable nucleolar gtp-binding protein 1
CUST_5222_PI425798793	8.83E-06	down	-2.3806221	disks large 1 tumor suppressor protein
CUST_7344_PI425798793	0.001547	down	-2.4076295	morn repeat-containing protein 3
CUST_435_PI425798793	0.021117	down	-2.4617956	homeobox protein b-h1
CUST_3126_PI425798793	0.006364	down	-2.5796573	glucose dehydrogenase
CUST_5818_PI425798793	0.029934	down	-2.8680818	cytochrome c oxidase subunit va
CUST_1924_PI425798793	0.009095	down	-2.9166398	defensin
CUST_4160_PI425798793	0.003587	down	-2.939596	major royal jelly protein 3
CUST_4206_PI425798793	0.023382	down	-3.0589104	scavenger mrna-decapping enzyme
CUST_7235_PI425798793	0.023386	down	-3.0733514	major royal jelly protein 3
CUST_9585_PI425798793	0.003939	down	-3.2095392	transcriptional adapter 3-like
CUST_5591_PI425798793	0.01431	down	-4.058212	major royal jelly protein 3
CUST_7917_PI425798793	0.017072	down	-4.066629	major royal jelly protein 3
CUST_1821_PI425798793	0.024243	down	-5.3423085	outer membrane protein
CUST_4118_PI425798793	0.022173	down	-8.511289	superoxide dismutase

9.2.2 Genes identified by microarray as differentially transcribed between 3h treated (neonicotinoid/acetone) samples

ProbeName	p-value	Regulation	Fold change	Description
CUST_247_PI425798793	0.010895832	up	5.547489	trypsin inhibitor like cysteine rich domain containing protein
CUST_3548_PI425798793	0.001678284	up	3.9009383	cysteine-rich venom
CUST_9788_PI425798793	0.021804081	up	3.3960483	loc100170577 protein
CUST_173_PI425798793	0.048496258	up	3.2618349	major royal jelly protein 3
CUST_8592_PI425798793	0.033318084	up	2.9624407	1-phosphatidylinositol- -bisphosphate phosphodiesterase epsilon-1
CUST_7747_PI425798793	0.039179113	up	2.90226	viral a-type inclusion
CUST_8815_PI425798793	0.011987592	up	2.639624	immune deficiency
CUST_7694_PI425798793	0.026038697	up	2.637517	vacuolar protein sorting-associated protein 8-like protein
CUST_3484_PI425798793	0.013860128	up	2.5683146	glucose dehydrogenase
CUST_1924_PI425798793	0.037255697	up	2.5456228	defensin
CUST_2297_PI425798793	0.021540679	up	2.4789007	major royal jelly protein 3
CUST_8119_PI425798793	0.04585064	up	2.3938794	equilibrative nucleoside transporter 4
CUST_7081_PI425798793	0.042608418	up	2.3484716	PREDICTED: hypothetical protein LOC100576118 [Apis mellifera]
CUST_3747_PI425798793	0.018547371	up	2.3220384	glucose dehydrogenase
CUST_1454_PI425798793	0.03865754	up	2.286891	ubiquitin-conjugating enzyme e2-230k
CUST_1700_PI425798793	0.03022541	up	2.191358	long wave opsin
CUST_1527_PI425798793	0.020368654	up	2.14928	deah (asp-glu-ala-his) box polypeptide 35
CUST_5745_PI425798793	0.03061435	up	2.138745	ws dgat mgat
CUST_7332_PI425798793	4.97E-04	up	2.130464	trna modification gtpase mitochondrial
CUST_2579_PI425798793	0.005724432	up	2.0782223	39s ribosomal protein mitochondrial
CUST_982_PI425798793	0.001416966	up	2.0757914	uncharacterized protein c14orf118-like protein
CUST_5938_PI425798793	0.011112488	up	2.0300066	PREDICTED: hypothetical protein LOC724746 [Apis mellifera]
CUST_96_PI425798793	0.010346935	up	2.019559	polycomb protein scm
CUST_2622_PI425798793	0.04753748	down	-2.0010383	lethal giant
CUST_2101_PI425798793	0.004411719	down	-2.0138924	polypeptide n-acetylgalactosaminyltransferase 5
CUST_8913_PI425798793	0.02753207	down	-2.0144675	anion exchange protein slc4a2
CUST_9205_PI425798793	0.035459135	down	-2.0227416	like-glycosyltransferase
CUST_334_PI425798793	0.004300451	down	-2.025464	uncharacterized protein c21orf59 homolog
CUST_7624_PI425798793	0.014684644	down	-2.041426	phosphatidylserine synthase
CUST_2135_PI425798793	0.010302767	down	-2.0672367	glutamate receptor 1
CUST_8124_PI425798793	0.045941904	down	-2.1059608	chitin deacetylase-like isoform d
CUST_8017_PI425798793	0.047077447	down	-2.1072052	dimethylanaline monooxygenase-like
CUST_8712_PI425798793	0.021942269	down	-2.1151617	btb poz domain-containing protein 9
CUST_6552_PI425798793	0.009923634	down	-2.1162202	takeout like protein
CUST_9879_PI425798793	0.009377559	down	-2.118461	u7 snrna-associated sm-like protein lsm11-like
CUST_8074_PI425798793	0.04123245	down	-2.1382153	ceramide kinase
CUST_4899_PI425798793	0.002305262	down	-2.1506019	-like protein subfamily c member 16
CUST_2136_PI425798793	0.039292745	down	-2.1646595	protein pygopus

CUST_8279_PI425798793	0.047096364	down	-2.1749442	nad dependent epimerase dehydratase
CUST_1630_PI425798793	0.031342927	down	-2.1818328	sodium-dependent phosphate transporter
CUST_7494_PI425798793	0.031938843	down	-2.1852958	cytoplasmic phosphatidylinositol transfer protein 1
CUST_2094_PI425798793	0.016493998	down	-2.1864464	spatzle 5
CUST_8511_PI425798793	0.019186694	down	-2.1890955	rh-like protein
CUST_4923_PI425798793	0.044578027	down	-2.192101	cytochrome b5
CUST_4925_PI425798793	0.04692916	down	-2.2024152	zinc iron transporter
CUST_884_PI425798793	0.02729844	down	-2.2041411	mite allergen der f 3
CUST_6527_PI425798793	0.010686804	down	-2.2080128	chloride intracellular channel exc-4
CUST_1622_PI425798793	0.04505086	down	-2.2141113	pheromone-binding protein 1
CUST_9456_PI425798793	0.015693266	down	-2.2152958	cral trio domain-containing protein
CUST_8266_PI425798793	0.014298315	down	-2.2185054	isoform r
CUST_8268_PI425798793	0.047693733	down	-2.2240942	bifunctional 3 -phosphoadenosine 5 -phosphosulfate synthetase
CUST_5221_PI425798793	0.029660821	down	-2.2515223	coiled-coil domain-containing protein c6orf97-like
CUST_4932_PI425798793	0.045602243	down	-2.290013	ddb1- and cul4-associated factor 15
CUST_4909_PI425798793	0.032066837	down	-2.2924225	lysozyme c-1
CUST_9402_PI425798793	0.00887567	down	-2.3113406	talin-2- partial
CUST_1021_PI425798793	0.022559276	down	-2.3333988	zinc finger protein 652-a
CUST_3975_PI425798793	3.32E-04	down	-2.3535538	zinc finger protein
CUST_1260_PI425798793	0.03882464	down	-2.3732214	isoform b
CUST_5164_PI425798793	0.033564698	down	-2.3987763	-like 1 homeobox protein
CUST_6111_PI425798793	0.024603931	down	-2.4149394	aquaporin
CUST_20_PI425798793	0.039649975	down	-2.4158115	chymotrypsin-1
CUST_3455_PI425798793	0.00500319	down	-2.4383614	cdc42-interacting protein 4-like
CUST_6960_PI425798793	0.012129285	down	-2.473678	e3 ubiquitin-protein ligase march2
CUST_9754_PI425798793	0.03821448	down	-2.5041711	maltase 1
CUST_10040_PI425798793	0.020195676	down	-2.5140288	upf0368 protein cxorf26-like
CUST_3916_PI425798793	0.03496071	down	-2.522396	ctl transporter
CUST_3978_PI425798793	0.026711615	down	-2.5575175	juvenile hormone acid methyltransferase
CUST_9939_PI425798793	0.030466026	down	-2.600827	protein bric-a-brac 2
CUST_59_PI425800172	0.019108819	down	-2.6016052	CCE5_GB15030
CUST_4555_PI425798793	0.03307161	down	-2.6261923	microphthalmia-associated transcription factor
CUST_5308_PI425798793	0.018325917	down	-2.647188	hypothetical protein EAI_15505 [Harpegnathos saltator]
CUST_9850_PI425798793	0.014187377	down	-2.6516306	empty spiracles
CUST_8443_PI425798793	0.026801256	down	-2.6572807	fatty acid 2-hydroxylase
CUST_470_PI425798793	0.001565642	down	-2.670148	high affinity copper uptake protein 1
CUST_5462_PI425798793	0.037256587	down	-2.6912901	isoform c
CUST_4748_PI425798793	0.030083979	down	-2.7089057	potassium-dependent sodium-calcium
CUST_6997_PI425798793	0.008668294	down	-2.7091343	cyclic amp response element-binding protein a-like
CUST_1505_PI425798793	0.037769914	down	-2.7228534	arrestin domain-containing protein 3
CUST_8412_PI425798793	0.005408483	down	-2.7623749	kn motif and ankyrin repeat domain-containing protein 1
CUST_8723_PI425798793	0.002246974	down	-2.8027396	aminopeptidase n
CUST_8321_PI425798793	0.036099084	down	-2.8097558	sodium-dependent multivitamin transporter
CUST_5251_PI425798793	0.028310487	down	-2.825005	tubulin polyglutamylase ttll4-like
CUST_6292_PI425798793	0.008431396	down	-2.8343017	multidrug resistance-associated protein lethal 03659
CUST_3369_PI425798793	0.044521704	down	-2.928311	chitin synthase
CUST_187_PI425800172	0.015580353	down	-2.968103	CYP6AS3
CUST_9842_PI425798793	0.02003154	down	-3.058137	10g08
CUST_6963_PI425798793	0.002298112	down	-3.2358053	chymotrypsin-like protein
CUST_6976_PI425798793	0.029641083	down	-3.2927341	ornithine mitochondrial
CUST_2205_PI425798793	0.030673258	down	-3.4320717	glycyl-trna alpha subunit
CUST_3264_PI425798793	0.029718885	down	-3.4371207	cg7381 cg7381-pa
CUST_8625_PI425798793	0.038416393	down	-3.6984138	protein g12
CUST_2967_PI425798793	0.023993196	down	-4.1990643	calcium and integrin-binding protein 1
CUST_3794_PI425798793	0.040469788	down	-7.254076	synaptotagmin-14

9.2.3 Genes identified by microarray as differentially transcribed between 10h treated (neonicotinoid/acetone) samples

ProbeName	p-value	Regulation	Fold change	Description
CUST_5149_PI425798793	0.0089916	up	2.675777	alpha- -glucosyltransferase alg10-b
CUST_6576_PI425798793	6.38E-04	up	2.30214	hig1 domain family member 2a
CUST_9563_PI425798793	0.0397261	up	2.204715	PREDICTED: hypothetical protein LOC100576410 [Apis mellifera]
CUST_7105_PI425798793	0.0142645	up	2.146234	cell cycle checkpoint protein rad17
CUST_1740_PI425798793	0.0376775	up	2.033679	heat shock protein
CUST_7611_PI425798793	0.0292974	up	2.008059	upf0539 protein cg14977
CUST_6292_PI425798793	0.0173277	down	-2.00198	multidrug resistance-associated protein lethal 03659
CUST_183_PI425800172	0.0078993	down	-2.01238	CYP6AS5
CUST_185_PI425800172	0.0034302	down	-2.01354	CYP6AS4
CUST_4161_PI425798793	0.0175501	down	-2.04301	cation transport regulator-like protein 1
CUST_2863_PI425798793	0.0368153	down	-2.04415	septin isoform g

CUST_184_PI425800172	0.0084713	down	-2.04541	CYP6AS4
CUST_224_PI425800172	0.0336211	down	-2.07389	CYP6AQ1
CUST_1291_PI425798793	0.0420191	down	-2.07426	cellular retinaldehyde-binding protein
CUST_716_PI425798793	0.0118097	down	-2.07542	zinc finger protein 318
CUST_63_PI425800172	0.0317928	down	-2.09257	CCE4_GB10854
CUST_8285_PI425798793	0.0330954	down	-2.09365	phd and ring finger domain-containing protein 1
CUST_2252_PI425798793	0.0494144	down	-2.09859	nucleolar gtp-binding protein 2
CUST_9760_PI425798793	0.0387926	down	-2.1017	fkbp12-rapamycin complex-associated protein
CUST_2532_PI425798793	0.0192375	down	-2.11354	hmg-box protein hmg2l1
CUST_9435_PI425798793	0.0297576	down	-2.11458	neutral ceramidase
CUST_4773_PI425798793	0.0425249	down	-2.12522	innexin inx7
CUST_1122_PI425798793	0.0377178	down	-2.14658	homeotic protein female sterile
CUST_5751_PI425798793	0.0457867	down	-2.15041	chymotrypsin inhibitor
CUST_6654_PI425798793	0.0098413	down	-2.15327	phytanoyl- dioxygenase domain-containing protein 1-like protein
CUST_1970_PI425798793	0.0089234	down	-2.16213	PREDICTED: hypothetical protein LOC100577919 [Apis mellifera]
CUST_3232_PI425798793	0.0185917	down	-2.16239	atp-dependent helicase brm
CUST_8885_PI425798793	0.040924	down	-2.17135	mediator of rna polymerase ii transcription subunit 25
CUST_5521_PI425798793	0.0024439	down	-2.17665	calcium calmodulin-dependent protein kinase type 1
CUST_8343_PI425798793	0.0256019	down	-2.20104	protein prenyltransferase alpha subunit repeat-containing protein 1
CUST_8693_PI425798793	0.0348745	down	-2.22608	5-3 2583
CUST_5205_PI425798793	0.0143332	down	-2.22649	flavone synthase i
CUST_4732_PI425798793	0.038627	down	-2.22756	myosin heavy nonmuscle or smooth muscle
CUST_9169_PI425798793	0.0280329	down	-2.24645	ubiquitin carboxyl-terminal hydrolase 8
CUST_5758_PI425798793	0.0267979	down	-2.29065	mediator of rna polymerase ii transcription subunit 12
CUST_4223_PI425798793	0.027989	down	-2.30517	centrosomal protein of 135 kda
CUST_5151_PI425798793	0.0311573	down	-2.33098	PREDICTED: hypothetical protein LOC727135 [Apis mellifera]
CUST_5110_PI425798793	0.0081381	down	-2.34732	ubiquitin-protein ligase e3b
CUST_423_PI425798793	0.04253	down	-2.36228	large proline-rich protein bat2
CUST_4057_PI425798793	0.0076458	down	-2.3625	PREDICTED: hypothetical protein LOC100576843 [Apis mellifera]
CUST_2147_PI425798793	0.0195042	down	-2.41311	apidaecin
CUST_2232_PI425798793	0.0262928	down	-2.42028	pleckstrin-like proteiny-like domain family b member 2
CUST_867_PI425798793	0.0067102	down	-2.45466	leucine-rich transmembrane protein
CUST_9664_PI425798793	0.0238757	down	-2.49274	bifunctional protein ncoat
CUST_1846_PI425798793	0.012703	down	-2.54578	apidaecin
CUST_7640_PI425798793	0.0402278	down	-2.55829	tyrosine-protein kinase abl
CUST_1751_PI425798793	0.0462197	down	-2.56869	argininosuccinate synthase
CUST_43_PI425800172	0.0145989	down	-2.58322	CCE10_GB15327
CUST_7407_PI425798793	0.002677	down	-2.59319	ccr4-not transcription complex subunit 4
CUST_5393_PI425798793	0.0185967	down	-2.6022	tar-binding protein
CUST_309_PI425798793	0.0041579	down	-2.64577	10-formyltetrahydrofolate dehydrogenase
CUST_8017_PI425798793	0.0325266	down	-2.70762	dimethylanaline monooxygenase-like
CUST_6117_PI425798793	0.0148357	down	-2.72644	peptidyl-prolyl cis-trans isomerase
CUST_8_PI425800189	0.0486213	down	-2.84126	CCE10_GB15327
CUST_7293_PI425798793	0.0056272	down	-2.8445	fk506 binding protein 133kda
CUST_7512_PI425798793	0.0088102	down	-3.01509	apidaecins type 73- partial
CUST_8351_PI425798793	0.0185886	down	-3.08359	15-hydroxyprostaglandin dehydrogenase
CUST_8165_PI425798793	0.0152481	down	-3.08775	vitellogenin
CUST_7792_PI425798793	0.0224281	down	-4.69373	large proline-rich protein bat2

9.3 Microarray Analysis for Induction Experiment 2

9.3.1 Genes identified by microarray as differentially transcribed between 24h treated (neonicotinoid/aceton) samples (0 h).

ProbeName	p-value	Regulation	Fold change	Description
CUST_6604_PI425798793	9.22E-04	up	1.9713323	targeting protein for xklp2
CUST_6995_PI425798793	0.043324564	up	1.7756	adenylate cyclase type 5
CUST_268_PI425798793	0.001737259	up	1.7610627	dna-directed rna polymerase i subunit rpa49
CUST_7467_PI425798793	0.04851442	up	1.6056362	doublesex- and mab-3-related transcription factor a2
CUST_4833_PI425798793	0.033569846	up	1.5604886	dentin sialophosphoprotein
CUST_5208_PI425798793	0.01740102	up	1.5601617	cytochrome p450 315a1

CUST_3876_PI425798793	0.012360056	up	1.5426717	PREDICTED: hypothetical protein LOC724776 [Apis mellifera]
CUST_6131_PI425798793	0.035290223	up	1.5215477	sodium dicarboxylate
CUST_6450_PI425798793	0.03291238	up	1.5189786	forkhead protein forkhead protein domain
CUST_114_PI425800172	0,02338815	up	1,5180756	CYP315A1
CUST_9020_PI425798793	0.021897076	up	1.5013951	likely vesicular transport factor uso1p
CUST_6231_PI425798793	0.02271951	down	-1.5002693	camp-specific 3 -cyclic phosphodiesterase
CUST_1225_PI425798793	0.012310382	down	-1.5253805	morn repeat containing 5
CUST_3735_PI425798793	0.011783974	down	-1.548099	phospholipase membrane-associated-like
CUST_9751_PI425798793	0.024212694	down	-1.5546798	isoform a
CUST_6466_PI425798793	0.024032887	down	-1.5588487	elongation of very long chain fatty acids protein aael008004
CUST_9066_PI425798793	0.024007775	down	-1.5625936	asparagine synthetase
CUST_8142_PI425798793	0.046044823	down	-1.6152343	elongation of very long chain fatty acids protein 6-like
CUST_9438_PI425798793	0.006334751	down	-1.9447331	katanin p60 atpase-containing subunit a-like 2
CUST_9099_PI425798793	0.013743192	down	-2.1038406	two pore potassium channel protein sup-9
CUST_4882_PI425798793	0.017791532	down	-2.755642	tetratricopeptide repeat protein 25

9.3.2 Genes identified by microarray as differentially transcribed between 24h treated (neonicotinoid/aceton) samples (48 h).

ProbeName	p-value	Regulation	Fold change	Description
CUST_1238_PI425798793	0.025606	up	8.141132	platelet glycoprotein v
CUST_7512_PI425798793	0.003339	up	6.147865	apidaecins type 73- partial
CUST_2147_PI425798793	0.004503	up	5.440981	apidaecin
CUST_1846_PI425798793	0.005482	up	5.2217507	apidaecin
CUST_7153_PI425798793	0.036384	up	4.374178	cytochrome b5
CUST_4874_PI425798793	0.013625	up	2.834694	serine protease inhibitor serpin-4
CUST_7526_PI425798793	0.015691	up	2.3446848	serine protease snake
CUST_1665_PI425798793	0.034487	up	2.232396	protein serac1
CUST_10063_PI425798793	0.032066	up	2.2065122	glyceraldehyde-3-phosphate dehydrogenase
CUST_1764_PI425798793	0.01999	up	2.1187086	transferrin
CUST_5811_PI425798793	0.019978	up	2.09544	protein malvolio
CUST_2284_PI425798793	0.022002	up	2.0370576	protein malvolio
CUST_8218_PI425798793	0.043786	up	1.9772508	histamine-gated chloride channel subunit
CUST_7833_PI425798793	0.038769	up	1.9219334	serine protease snake
CUST_4829_PI425798793	0.025807	up	1.8215269	gram negative bacteria binding protein 1
CUST_5763_PI425798793	0.008763	up	1.7943552	isoform e
CUST_6379_PI425798793	0.020011	up	1.7472343	neurobeachin
CUST_9168_PI425798793	0.005947	up	1.7180558	protein
CUST_8231_PI425798793	0.038261	up	1.7103626	PREDICTED: hypothetical protein LOC408280 [Apis mellifera]
CUST_4080_PI425798793	0.020181	up	1.6636832	PREDICTED: hypothetical protein LOC100576863 [Apis mellifera]
CUST_9401_PI425798793	0.022749	up	1.5626922	krueppel-like factor 6
CUST_509_PI425798793	0.029009	down	-1.5087018	tubulin polyglutamylase ttll4-like
CUST_642_PI425798793	0.04983	down	-1.514742	lysosomal alpha-mannosidase (mannosidase alpha class 2b member 1)

CUST_325_PI425798793	0.029218	down	-1.5184697	b9 domain-containing protein 1
CUST_3549_PI425798793	0.043155	down	-1.5395374	abc superfamily atp binding cassette abc protein
CUST_721_PI425798793	0.041707	down	-1.539955	osiris 2 cg1148-pb
CUST_6248_PI425798793	0.040055	down	-1.5538408	PREDICTED: hypothetical protein LOC100576480 [Apis mellifera]
CUST_7259_PI425798793	0.037588	down	-1.5553044	odorant receptor 13a-like
CUST_2769_PI425798793	0.042468	down	-1.5567945	collagen alpha-2 chain-like
CUST_9751_PI425798793	0.04053	down	-1.5681636	isoform a
CUST_4731_PI425798793	0.039713	down	-1.6068443	mitochondrial 2-oxoglutarate malate carrier protein
CUST_3926_PI425798793	0.003196	down	-1.691649	sugar transporter
CUST_6794_PI425798793	0.008408	down	-1.7064772	odorant receptor or1-like
CUST_5108_PI425798793	0.011489	down	-1.7116566	female reproductive tract protease gleanr_2574
CUST_5441_PI425798793	0.02156	down	-1.8329759	mpa2 allergen
CUST_657_PI425798793	0.030361	down	-1.952797	dc-stamp domain-containing protein 1
CUST_9256_PI425798793	0.03966	down	-2.0899084	serine proteinase stubble

9.3.3. Genes identified by microarray as differentially transcribed between 24h treated (neonicotinoid/aceton) samples (96 h).

ProbeName	p-value	Regulation	Fold change	Description
CUST_1463_PI425798793	0.049497	up	6.376693	resolvase
CUST_1087_PI425798793	0.039399	up	5.633321	Chymotrypsin-1 [Camponotus floridanus]
CUST_8706_PI425798793	0.037097	up	3.5076187	mitochondrial import receptor subunit tom40
CUST_7579_PI425798793	0.038965	up	3.4363163	golgin subfamily a member 4
CUST_2798_PI425798793	0.049757	up	2.9664958	inhibitory pou
CUST_8159_PI425798793	0.030399	up	2.4203706	fmrfamide-related neuropeptides-like
CUST_5672_PI425798793	0.04982	up	2.2832217	potassium channel subfamily k member 9
CUST_1020_PI425798793	0.016938	up	1.8158408	carbonic anhydrase
CUST_7536_PI425798793	0.046573	up	1.7571479	hypothetical protein EAI_15441 [Harpegnathos saltator]
CUST_8020_PI425798793	0.002945	up	1.721585	receptor protein tyrosine
CUST_5587_PI425798793	0.029452	up	1.699726	ankyrin repeat
CUST_4483_PI425798793	0.030238	up	1.6795725	microsomal glutathione s-transferase 1
CUST_101_PI425798793	0.004036	up	1.6372229	inwardly rectifying k+
CUST_7524_PI425798793	0.02664	up	1.6332836	opioid-binding protein cell adhesion molecule
CUST_8209_PI425798793	0.037997	up	1.5630051	down syndrome cell adhesion molecule
CUST_4087_PI425798793	0.007829	up	1.5628208	n-acetylneuraminate lyase
CUST_6550_PI425798793	0.045778	up	1.5520744	soluble adenylyl cyclase
CUST_8392_PI425798793	0.012066	up	1.5214038	lrr domain-containing protein
CUST_628_PI425798793	0.012342	down	-1.5195068	t-box transcription factor tbx1
CUST_9803_PI425798793	0.010071	down	-1.5204513	methyl- -binding domain protein 5
CUST_157_PI425800172	0,006223253	down	-1,5258377	CYP9Q1
CUST_158_PI425800172	0,005355946	down	-1,5515327	CYP9Q1
CUST_159_PI425800172	0,004108407	down	-1,56696	CYP9Q1
CUST_5980_PI425798793	0.027902	down	-1.5726794	alkyl hydroperoxide reductase c22 protein
CUST_42_PI425800172	0,018325403	down	-1,7419939	CCE11

9.3.4 Genes identified by microarray as differentially transcribed between 24h treated (neonicotinoid/aceton) samples (144 h).

ProbeName	p-value	Regulation	Fold change	Description
CUST_2179_PI425798793	0.007345277	up	2.8889785	PREDICTED: hypothetical protein LOC411614 [Apis mellifera]
CUST_3548_PI425798793	0.003103808	up	2.4244373	cysteine-rich venom
CUST_6964_PI425798793	0.016088877	up	1.9232169	protein sidekick
CUST_6131_PI425798793	0.041350212	up	1.6362138	sodium dicarboxylate
CUST_1924_PI425798793	0.009486358	up	1.579293	defensin
CUST_5602_PI425798793	0.015709711	up	1.5144125	tp53rk-binding protein
CUST_9477_PI425798793	0.04003594	up	1.5102307	thioredoxin domain-containing protein 3-like protein
CUST_2018_PI425798793	0.02088651	down	-1.5186478	lactosylceramide 4-alpha-galactosyltransferase
CUST_295_PI425798793	0.028327812	down	-1.5826136	sulfakinin receptor
CUST_9020_PI425798793	0.017685506	down	-1.6455889	likely vesicular transport factor uso1p
CUST_7262_PI425798793	0.03082237	down	-1.6624129	crispr-associated helicase cas3
CUST_9097_PI425798793	0.048801497	down	-1.848457	mitochondrial ribosome recycling factor
CUST_1865_PI425798793	0.003078663	down	-2.0232155	dusky- isoform a

9.3.5 Genes identified by microarray as differentially transcribed bees fed sucrose-insecticide that subsequently survived the 0 h topical bioassay versus the non-treated control from the same time point ('survivor' experiment).

ProbeName	p-value	Regulation	Fold change	Description
CUST_2739_PI425798793	0,008332	up	3,340471	sodium-dependent multivitamin transporter
CUST_865_PI425798793	0,040361	up	3,1733584	luciferin 4-monooxygenase
CUST_2668_PI425798793	0,011924	up	2,7343066	fatty acyl- reductase 1
CUST_5851_PI425798793	0,018411	up	2,6648848	pickpocket 16
CUST_5843_PI425798793	0,046257	up	2,3613145	glutamate ionotropic kainate 2
CUST_167_PI425800172	0,019711	up	2,2130027	CYP6BE1
CUST_2921_PI425798793	0,009353	up	2,1903648	CCE8
CUST_168_PI425800172	0,012499	up	2,173628	CYP6BE1
CUST_51_PI425800172	0,008251	up	2,1672835	CCE8
CUST_49_PI425800172	0,007901	up	2,1377225	CCE8
CUST_6277_PI425798793	0,024458	up	2,125866	uncharacterized protein kiaa0825-like protein
CUST_10_PI425800189	0,011164	up	2,1244037	CCE8
CUST_4316_PI425798793	0,034784	up	2,1203434	odorant-binding protein 1
CUST_50_PI425800172	0,009093	up	2,108859	CCE8
CUST_3792_PI425798793	0,002433	up	2,102347	pancreatic triacylglycerol lipase-like
CUST_7831_PI425798793	9,72E-04	up	2,1012146	probable g-protein coupled receptor 158
CUST_6877_PI425798793	0,01685	up	2,0207298	venom serine protease 34
CUST_126_PI425800172	0,00515	up	1,9427272	CYP305D1
CUST_166_PI425800172	0,015335	up	1,896606	CYP6BE1
CUST_2108_PI425798793	0,001027	up	1,888185	sugar transporter

CUST_6536_PI425798793	0,011137	up	1,8763047	CYP6BE1
CUST_125_PI425800172	0,007929	up	1,8594306	CYP305D1
CUST_2882_PI425798793	0,015103	up	1,8542668	CYP305D1
CUST_8843_PI425798793	0,03351	up	1,8523376	protein fam179b
CUST_2724_PI425798793	0,012744	up	1,8512197	glutathione s transferase d1
CUST_124_PI425800172	0,016052	up	1,8471639	CYP305D1
CUST_6682_PI425798793	0,012494	up	1,8187755	multifunctional protein ade2
CUST_8215_PI425798793	0,032531	up	1,7948278	odorant receptor or1-like
CUST_9940_PI425798793	0,011462	up	1,7727959	synaptic vesicle glycoprotein 2b
CUST_7552_PI425798793	0,028927	up	1,7591382	supernumerary limbs
CUST_607_PI425798793	0,005469	up	1,7560664	neuromedin-b receptor
CUST_3123_PI425798793	0,019608	up	1,7548373	sentrin sumo-specific protease senp7
CUST_8078_PI425798793	0,047474	up	1,7481846	keratin-associated protein 4-12-like
CUST_7410_PI425798793	0,007825	up	1,7075233	purine nucleoside phosphorylase
CUST_3340_PI425798793	0,013496	up	1,6914514	coiled-coil domain containing 19
CUST_182_PI425800172	0,006756	up	1,6913733	CYP6AS5
CUST_822_PI425798793	0,008549	up	1,6500429	glycinamide ribonucleotide synthetase-aminoimidazole ribonucleotide synthetase-glycinamide ribonucleotide transformylase
CUST_1830_PI425798793	0,011204	up	1,647102	bifunctional purine biosynthesis protein purh
CUST_135_PI425800172	0,027643	up	1,6465889	CYP301A1
CUST_183_PI425800172	0,003475	up	1,6459757	CYP6AS5
CUST_965_PI425798793	0,001697	up	1,6411326	histone-lysine n-methyltransferase setmar
CUST_53_PI425798793	0,04627	up	1,6407721	radial spoke head protein 3 homolog
CUST_5176_PI425798793	0,03292	up	1,630514	fatty acyl- reductase 1-like
CUST_6508_PI425798793	0,030229	up	1,6272887	4-nitrophenylphosphatase
CUST_4299_PI425798793	0,008606	up	1,6192172	nadph oxidase 5
CUST_181_PI425800172	0,008646	up	1,6171824	CYP6AS5
CUST_1806_PI425798793	0,023932	up	1,6089267	cral trio domain-containing protein
CUST_7582_PI425798793	0,03307	up	1,5909684	ubiquitin carboxyl-terminal hydrolase 1
CUST_1397_PI425798793	0,012735	up	1,5845821	isoform a
CUST_6539_PI425798793	0,013308	up	1,5622213	CYP6AS5
CUST_8355_PI425798793	0,001226	up	1,5514377	zinc metalloprotease
CUST_2041_PI425798793	0,008503	up	1,5508599	acyl- delta desaturase
CUST_3765_PI425798793	0,017659	up	1,543425	rotatin
CUST_4923_PI425798793	0,008841	up	1,5402261	cytochrome b5
CUST_3237_PI425798793	0,011887	up	1,5247549	serine hydroxymethyltransferase
CUST_3987_PI425798793	0,024422	up	1,5185658	homogentisate -dioxygenase
CUST_4308_PI425798793	0,019139	up	1,5138578	kinesin-like protein costal2
CUST_171_PI425798793	0,019072	down	-1,5002978	g-protein coupled receptor
CUST_7323_PI425798793	1,71E-04	down	-1,5036476	domon domain-containing protein cg14681
CUST_7299_PI425798793	0,03333	down	-1,5165423	sodium potassium-transporting atpase subunit beta-2
CUST_9651_PI425798793	0,022884	down	-1,5209029	jmjc domain-containing protein 4-like
CUST_659_PI425798793	0,014305	down	-1,5289698	atpase family aaa domain-containing protein 3
CUST_6533_PI425798793	0,019921	down	-1,5309825	scm-like with four mbt domains 1
CUST_6709_PI425798793	0,010713	down	-1,547853	apidermin 1
CUST_6062_PI425798793	0,037298	down	-1,5529456	ecdysone-induced protein isoform c
CUST_4987_PI425798793	0,016796	down	-1,5536581	PREDICTED: hypothetical protein LOC725148 [Apis mellifera]

CUST_7751_PI425798793	0,011414	down	-1,5540562	fatty acid
CUST_4896_PI425798793	0,031905	down	-1,5635372	ras gtp exchange
CUST_984_PI425798793	0,006639	down	-1,5695513	pih1 domain-containing protein 1
CUST_6285_PI425798793	0,017421	down	-1,571939	hypothetical protein EAI_08187 [Harpegnathos saltator]
CUST_3419_PI425798793	0,003331	down	-1,6051137	atp synthase subunit s-like protein
CUST_783_PI425798793	0,036487	down	-1,6214337	cuticle protein 6
CUST_7066_PI425798793	0,042473	down	-1,6274552	PREDICTED: hypothetical protein LOC409776 [Apis mellifera]
CUST_3823_PI425798793	0,018577	down	-1,6374342	protein takeout
CUST_512_PI425798793	0,039985	down	-1,665058	PREDICTED: hypothetical protein LOC100576198 [Apis mellifera]
CUST_9952_PI425798793	0,03704	down	-1,6775784	dusky- isoform a
CUST_8159_PI425798793	0,044579	down	-1,6825999	fmrfamide-related neuropeptides-like
CUST_3928_PI425798793	0,007446	down	-1,6833814	PREDICTED: hypothetical protein LOC100578025 [Apis mellifera]
CUST_5575_PI425798793	0,012786	down	-1,6879627	dual specificity tyrosine-phosphorylation-regulated kinase
CUST_3406_PI425798793	0,008475	down	-1,7076722	insulin-like growth factor-binding protein complex acid labile chain
CUST_634_PI425798793	0,041529	down	-1,7190806	collagen alpha-2
CUST_4306_PI425798793	0,037394	down	-1,7243892	isoform h
CUST_2613_PI425798793	0,009794	down	-1,7420032	PREDICTED: hypothetical protein LOC100578730 isoform 1 [Apis mellifera]
CUST_9643_PI425798793	0,023003	down	-1,7731405	protein notum-like protein
CUST_7571_PI425798793	0,007616	down	-1,798004	zinc finger with ufm1-specific peptidase domain
CUST_22_PI425800172	0,030528	down	-1,8105357	AChE-2
CUST_8652_PI425798793	0,048051	down	-1,9201459	short-chain dehydrogenase reductase
CUST_4263_PI425798793	0,00919	down	-1,9956423	wd repeat-containing protein c10orf79
CUST_9029_PI425798793	0,00615	down	-2,01419	elongation of very long chain fatty acids protein 6-like
CUST_7486_PI425798793	0,024014	down	-2,1047797	dynein intermediate chain axonemal
CUST_15_PI425798793	0,042165	down	-2,3336186	cyclin b
CUST_5769_PI425798793	0,015138	down	-2,4185452	glucose dehydrogenase
CUST_1591_PI425798793	0,035515	down	-2,421504	sidestep protein
CUST_3811_PI425798793	0,022085	down	-2,5501926	pdz and lim domain protein 3
CUST_3657_PI425798793	0,002785	down	-2,6696415	tpa: cuticle protein

9.4 Microarray Analysis for Comparison of Two *A. mellifera* races

9.4.1 Genes identified by microarray as differentially transcribed between *A. mellifera caucasica* and *A. mellifera buckfast*. (p-value: 0.05)

ProbeName	Fold change	Regulation	Description
CUST_4282_PI425798793	33,532467	up	ikk interacting protein isoform 1 isoform 1
CUST_8592_PI425798793	29,753315	up	1-phosphatidylinositol- -bisphosphate phosphodiesterase epsilon-1
CUST_1850_PI425798793	21,545534	up	protein still isoforms c sif type 2
CUST_8580_PI425798793	18,554037	up	transcription factor sp5
CUST_3042_PI425798793	16,670176	up	sulfotransferase family cytosolic 1b member 1
CUST_2047_PI425798793	15,455329	up	ankyrin repeat domain-containing protein 17
CUST_3317_PI425798793	14,983871	up	-like protein subfamily c member 3
CUST_8446_PI425798793	14,7673	up	rap1 gtpase-activating protein 1

CUST_4114_PI425798793	13,618349	up	uncharacterized protein kiaa1843
CUST_7311_PI425798793	12,70549	up	sodium- and chloride-dependent glycine transporter 2
CUST_307_PI425798793	11,941381	up	thiazole biosynthesis protein
CUST_2414_PI425798793	11,27214	up	preprotachykinin
CUST_10138_PI425798793	10,486459	up	hypothetical protein G5I_02214 [Acromyrmex echinatior]
CUST_3281_PI425798793	10,300925	up	forkhead box protein p2
CUST_1143_PI425798793	9,906644	up	carcinoembryonic antigen-related cell adhesion molecule 1
CUST_6156_PI425798793	9,6251135	up	glutamate
CUST_9757_PI425798793	9,340608	up	c-myc promoter-binding protein
CUST_9488_PI425798793	9,124976	up	PREDICTED: hypothetical protein LOC100578514 [Apis mellifera]
CUST_4311_PI425798793	9,119858	up	u1 small nuclear ribonucleoprotein 70 kda
CUST_3669_PI425798793	8,863236	up	cg31997 cg31997-pa
CUST_413_PI425798793	8,648826	up	major royal jelly protein 9
CUST_7778_PI425798793	8,605673	up	uncharacterized protein kiaa1843
CUST_8404_PI425798793	8,019917	up	inner centromere protein
CUST_5620_PI425798793	7,988599	up	diacylglycerol kinase beta
CUST_235_PI425798793	7,918174	up	protein lethal essential for life-like
CUST_6760_PI425798793	7,8404245	up	nicotinic acetylcholine receptor alpha6 subunit
CUST_6029_PI425798793	7,697769	up	isoform b
CUST_4075_PI425798793	6,965165	up	sterol regulatory element-binding protein 1
CUST_1613_PI425798793	6,7807713	up	discoidin domain-containing receptor 2-like
CUST_4992_PI425798793	6,780687	up	neuroglobin- partial
CUST_243_PI425798793	6,7141423	up	chromatin-remodeling complex atpase chain iswi
CUST_7541_PI425798793	6,6486363	up	protein gpr107
CUST_10057_PI425798793	6,6460724	up	fused lobes
CUST_3998_PI425798793	6,5742083	up	dipeptidase 1
CUST_3671_PI425798793	6,4853196	up	potassium voltage-gated channel subfamily h member 8
CUST_5739_PI425798793	6,207248	up	prp38 pre-mrna processing factor 38 domain containing b
CUST_3360_PI425798793	6,201318	up	isoform r
CUST_9492_PI425798793	6,157097	up	synaptosomal-associated protein 25-like isoform 1
CUST_2905_PI425798793	6,1552854	up	transmembrane protein 151b-like
CUST_8016_PI425798793	6,144512	up	nucleoside diphosphate-linked moiety x motif mitochondrial
CUST_8697_PI425798793	6,13546	up	polycomb protein
CUST_5578_PI425798793	6,02984	up	g-protein coupled receptor 143
CUST_600_PI425798793	5,868349	up	elks rab6-interacting cast family member 1
CUST_536_PI425798793	5,861872	up	wd repeat-containing protein 63
CUST_977_PI425798793	5,7479515	up	protein turtle
CUST_7410_PI425798793	5,7445073	up	purine nucleoside phosphorylase
CUST_7388_PI425798793	5,6991167	up	solute carrier family 35 member c2-like
CUST_4074_PI425798793	5,6233377	up	electron transfer flavoprotein-ubiquinone mitochondrial
CUST_7947_PI425798793	5,5911093	up	e3 ubiquitin-protein ligase hectd1
CUST_6221_PI425798793	5,5101905	up	stringent starvation protein b
CUST_3184_PI425798793	5,44364	up	actin-related protein 2 3 complex subunit 3
CUST_2029_PI425798793	5,359347	up	trifunctional enzyme beta subunit (tp-beta)
CUST_1821_PI425798793	5,3457236	up	outer membrane protein
CUST_9669_PI425798793	5,241773	up	von willebrand factor type egf and pentraxin domain-containing protein 1

CUST_4384_PI425798793	5,187204	up	transcriptional regulating factor 1
CUST_874_PI425798793	5,1773868	up	discoidin domain-containing receptor 2
CUST_435_PI425798793	5,1327186	up	homeobox protein b-h1
CUST_8751_PI425798793	4,8947678	up	g t mismatch-specific thymine dna glycosylase
CUST_2120_PI425798793	4,881628	up	PREDICTED: hypothetical protein LOC100578368 [Apis mellifera]
CUST_1320_PI425798793	4,8502126	up	uv excision repair protein rad23 homolog a
CUST_5344_PI425798793	4,8364058	up	cg13055 cg13055-pa
CUST_4266_PI425798793	4,7884583	up	u6 snrna-associated sm-like protein lsm4
CUST_5885_PI425798793	4,718782	up	zinc finger protein 227
CUST_3274_PI425798793	4,656548	up	sulfate transporter
CUST_6631_PI425798793	4,5974984	up	brefeldin a-inhibited guanine nucleotide-exchange protein 3
CUST_6488_PI425798793	4,522455	up	serine threonine-protein phosphatase pp1-beta catalytic subunit
CUST_1453_PI425798793	4,4226294	up	neuroendocrine convertase 2
CUST_9041_PI425798793	4,395365	up	cue domain-containing protein 2
CUST_7826_PI425798793	4,394957	up	cyclic-nucleotide-gated cation channel
CUST_450_PI425798793	4,325883	up	cytochrome o ubiquinol oxidase subunit iii
CUST_5688_PI425798793	4,280633	up	rho-related btb domain-containing protein 1
CUST_2671_PI425798793	4,15218	up	peroxiredoxin 1
CUST_10122_PI425798793	4,099335	up	PREDICTED: hypothetical protein LOC410449 [Apis mellifera]
CUST_9758_PI425798793	4,070908	up	n- isoform h
CUST_989_PI425798793	4,0214477	up	regulator of g-protein signaling 20
CUST_7601_PI425798793	4,02036	up	PREDICTED: hypothetical protein LOC409327 [Apis mellifera]
CUST_4701_PI425798793	4,0030766	up	uncharacterized protein cg1161
CUST_5459_PI425798793	3,9734368	up	transmembrane and tpr repeat-containing protein 2
CUST_8085_PI425798793	3,8750951	up	amnionless protein
CUST_7352_PI425798793	3,836276	up	cyclin-dependent kinase inhibitor protein
CUST_2662_PI425798793	3,7926352	up	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit dad1
CUST_9332_PI425798793	3,687651	up	soluble guanylyl cyclase alpha 1 subunit
CUST_6576_PI425798793	3,4908772	up	hig1 domain family member 2a
CUST_8692_PI425798793	3,4017885	up	g protein-coupled
CUST_5411_PI425798793	3,283473	up	voltage- gated calcium channel alpha subunit (voltage-dependent t-type calcium channel
CUST_3318_PI425798793	3,2207315	up	growth hormone-inducible transmembrane protein
CUST_1951_PI425798793	3,1573675	up	leucine rich repeat containing 29-like
CUST_6296_PI425798793	3,089434	up	vertebrate growth-associated protein gap-43 homolog
CUST_8838_PI425798793	3,0518377	up	diacylglycerol kinase theta
CUST_3220_PI425798793	3,0502532	up	esr1 protein
CUST_7139_PI425798793	3,0278466	up	protein disulfide-isomerase
CUST_500_PI425798793	3,0209117	up	polypeptide n-acetylgalactosaminyltransferase 5
CUST_8269_PI425798793	3,0189474	up	hypoxia-inducible factor 1 alpha
CUST_9300_PI425798793	2,9966571	up	udp-n-acetylglucosamine--peptide n-acetylglucosaminyltransferase 110 kda subunit
CUST_7137_PI425798793	2,9335222	up	glucose dehydrogenase
CUST_6207_PI425798793	2,8376086	up	polyubiquitin-a-like isoform 1
CUST_9314_PI425798793	2,5659058	up	general receptor for phosphoinositides 1-associated scaffold protein
CUST_1500_PI425798793	2,5474083	up	muconate transport protein
CUST_1183_PI425798793	2,5237482	up	hyperpolarization activated cyclic nucleotide-gated potassium channel
CUST_8621_PI425798793	2,4164288	up	chemosensory protein

CUST_6756_PI425798793	2,3662262	up	15-hydroxyprostaglandin dehydrogenase
CUST_6172_PI425798793	2,2477434	up	creb-regulated transcription coactivator 1
CUST_3885_PI425798793	2,1999733	up	encore protein
CUST_23_PI425798793	2,176429	up	odorant receptor 22c-like
CUST_1101_PI425798793	2,167046	up	organic anion transporter
CUST_5946_PI425798793	2,1374755	up	dual specificity tyrosine-phosphorylation-regulated kinase
CUST_503_PI425798793	2,13312	up	protein tob1
CUST_594_PI425798793	-2,0002928	down	plasma membrane calcium isoform i
CUST_8911_PI425798793	-2,0067947	down	cop9 signalosome complex subunit 5
CUST_32_PI425800172	-2,0282557	down	CCE14
CUST_9291_PI425798793	-2,0301576	down	btb poz domain-containing protein 10
CUST_1340_PI425798793	-2,0308006	down	mitochondrial carrier protein
CUST_2331_PI425798793	-2,039188	down	single-strand selective monofunctional uracil dna glycosylase
CUST_7408_PI425798793	-2,0426185	down	pleckstrin-like proteiny domain-containing family j member 1
CUST_2856_PI425798793	-2,0473793	down	chitin deacetylase 4
CUST_6206_PI425798793	-2,0533595	down	transmembrane protein 222
CUST_3064_PI425798793	-2,072209	down	coiled-coil domain containing 74b
CUST_202_PI425800172	-2,0742695	down	CYP6AS14
CUST_3642_PI425798793	-2,0748029	down	transcription initiation factor tfiid subunit 12
CUST_8852_PI425798793	-2,0813322	down	coiled-coil domain-containing protein 135
CUST_4991_PI425798793	-2,1036706	down	protein fam76a
CUST_6372_PI425798793	-2,108802	down	isoform a
CUST_5446_PI425798793	-2,1110446	down	histone h2a
CUST_2960_PI425798793	-2,11121	down	uncharacterized protein
CUST_4017_PI425798793	-2,1141725	down	alcohol dehydrogenase class-3
CUST_4786_PI425798793	-2,119312	down	gtp cyclohydrolase
CUST_6420_PI425798793	-2,1230037	down	golgi snap receptor complex member 1
CUST_4702_PI425798793	-2,1247602	down	snare-associated protein snapin
CUST_777_PI425798793	-2,141306	down	vacuolar protein sorting-associated protein 37a
CUST_7098_PI425798793	-2,1423845	down	vacuolar protein sorting 37b
CUST_1323_PI425798793	-2,1454496	down	dipeptidyl peptidase iii
CUST_4622_PI425798793	-2,1486638	down	slc39a9-prov protein
CUST_3597_PI425798793	-2,15616	down	porphobilinogen deaminase
CUST_5779_PI425798793	-2,1574056	down	small calcium-binding mitochondrial
CUST_5938_PI425798793	-2,16159	down	PREDICTED: hypothetical protein LOC724746 [Apis mellifera]
CUST_7781_PI425798793	-2,1693602	down	vacuolar atp synthase subunit f
CUST_9055_PI425798793	-2,177115	down	son protein
CUST_5099_PI425798793	-2,1793878	down	outer dense fiber protein 3
CUST_8791_PI425798793	-2,1843536	down	nicotinamide riboside kinase 1
CUST_2688_PI425798793	-2,1864529	down	connector of kinase to ap- isoform a
CUST_4652_PI425798793	-2,1879635	down	isoform a
CUST_9059_PI425798793	-2,1980004	down	f-box only protein 22
CUST_9525_PI425798793	-2,1999013	down	isocitrate dehydrogenase
CUST_1960_PI425798793	-2,2013817	down	membrane protein tms1d
CUST_1461_PI425798793	-2,2017605	down	was protein family-like protein 1
CUST_4906_PI425798793	-2,2018046	down	ataxin 3 variant ref

CUST_5123_PI425798793	-2,2021368	down	excitatory amino acid transporter 3
CUST_4378_PI425798793	-2,203001	down	integrator complex subunit 12
CUST_8387_PI425798793	-2,2034097	down	PREDICTED: hypothetical protein LOC409518 [Apis mellifera]
CUST_4589_PI425798793	-2,205263	down	isoform a
CUST_1095_PI425798793	-2,2071698	down	phosphoglycolate phosphatase
CUST_593_PI425798793	-2,228004	down	seryl-trna synthetase
CUST_6647_PI425798793	-2,2292416	down	c-myc promoter-binding protein
CUST_3505_PI425798793	-2,2325056	down	troponin c type iib
CUST_4069_PI425798793	-2,2359648	down	ras-related protein rab-43
CUST_5777_PI425798793	-2,2362363	down	epidermal growth factor receptor substrate 15-like 1
CUST_1314_PI425798793	-2,2380111	down	splicing arginine serine-rich 7
CUST_8708_PI425798793	-2,252444	down	mrg-binding protein
CUST_121_PI425798793	-2,252583	down	odorant binding protein 12
CUST_1953_PI425798793	-2,2554388	down	ubiquitin protein ligase
CUST_3367_PI425798793	-2,2563207	down	e3 ubiquitin-protein ligase bre1
CUST_7000_PI425798793	-2,2595556	down	hypothetical protein EAG_12683 [Camponotus floridanus]
CUST_1990_PI425798793	-2,2612214	down	short-chain dehydrogenase
CUST_8230_PI425798793	-2,2663262	down	-like 2
CUST_4366_PI425798793	-2,2672079	down	isoform b
CUST_8850_PI425798793	-2,271632	down	endonuclease exonuclease phosphatase family protein
CUST_2941_PI425798793	-2,2743437	down	wd-repeat protein
CUST_8186_PI425798793	-2,2748654	down	smu-1 suppressor of mec-8 and unc-52 homolog (elegans)
CUST_6424_PI425798793	-2,2752235	down	acidic fibroblast growth factor intracellular-binding protein
CUST_3337_PI425798793	-2,3110962	down	set and mynd domain-containing protein 4
CUST_8487_PI425798793	-2,3116212	down	phosphatidylinositol-glycan biosynthesis class f
CUST_8890_PI425798793	-2,313811	down	upf0430 protein cg31712
CUST_8070_PI425798793	-2,3162777	down	ptb domain-containing engulfment adapter protein 1
CUST_1622_PI425798793	-2,3171513	down	pheromone-binding protein 1
CUST_5392_PI425798793	-2,3219035	down	nuclear pore complex protein nup93
CUST_8596_PI425798793	-2,3309402	down	transmembrane protein 115
CUST_112_PI425798793	-2,3349116	down	hypothetical protein EAI_07295 [Harpegnathos saltator]
CUST_4110_PI425798793	-2,3383167	down	ubiquitin carboxyl-terminal hydrolase bap1
CUST_7314_PI425798793	-2,3414853	down	hypothetical conserved protein
CUST_9297_PI425798793	-2,3428485	down	viral a-type inclusion protein
CUST_2468_PI425798793	-2,3532457	down	isoform a
CUST_8352_PI425798793	-2,364249	down	isoform e
CUST_5287_PI425798793	-2,3651435	down	39s ribosomal protein mitochondrial
CUST_8318_PI425798793	-2,3656113	down	probable cytochrome p450 6a14
CUST_3555_PI425798793	-2,3693192	down	serine threonine-protein phosphatase 2a 65 kda regulatory subunit a alpha isoform
CUST_4494_PI425798793	-2,372037	down	pyridoxal-dependent decarboxylase domain-containing protein 1
CUST_9926_PI425798793	-2,3728883	down	alkylated dna repair protein alkb-like protein 4
CUST_8924_PI425798793	-2,381683	down	cysteine mitochondrial
CUST_7171_PI425798793	-2,3825207	down	isoform a
CUST_5428_PI425798793	-2,3882613	down	glutamine-oxaloacetic transaminase
CUST_1244_PI425798793	-2,390972	down	elongation factor ts
CUST_5665_PI425798793	-2,407198	down	coiled-coil domain-containing protein 93

CUST_5455_PI425798793	-2,4105349	down	scf apoptosis response protein
CUST_6150_PI425798793	-2,411699	down	cell adhesion
CUST_5357_PI425798793	-2,414717	down	ica_apime ame: full=icarapin-like ame: full=venom carbohydrate-rich protein flags: precursor
CUST_7573_PI425798793	-2,418628	down	isoform h
CUST_2273_PI425798793	-2,4217453	down	transcription initiation factor tfiid subunit 6
CUST_7303_PI425798793	-2,4273002	down	uroporphyrinogen decarboxylase
CUST_6765_PI425798793	-2,436456	down	atp-binding cassette sub-family b member mitochondrial
CUST_3860_PI425798793	-2,446153	down	deoxyhypusine hydroxylase
CUST_2405_PI425798793	-2,4490683	down	ephrin-b2a-like isoform partial
CUST_517_PI425798793	-2,4589012	down	evolutionarily conserved signaling intermediate in toll mitochondrial
CUST_6228_PI425798793	-2,4671104	down	cd82 antigen
CUST_8376_PI425798793	-2,4678168	down	nadh dehydrogenase
CUST_7005_PI425798793	-2,4688876	down	26s proteasome non-atpase regulatory subunit 13
CUST_3100_PI425798793	-2,4785337	down	wd-repeat protein
CUST_9404_PI425798793	-2,479613	down	sorting and assembly machinery component 50 homolog (cerevisiae)
CUST_4746_PI425798793	-2,4860592	down	eukaryotic translation elongation factor 1 epsilon 1
CUST_7090_PI425798793	-2,4895637	down	ankyrin repeat domain-containing protein 39
CUST_5160_PI425798793	-2,4907167	down	cytochrome c oxidase assembly protein cox15
CUST_147_PI425800172	-2,4907708	down	CY9S1
CUST_3486_PI425798793	-2,490837	down	adp-ribosylation factor-like 2
CUST_4062_PI425798793	-2,493331	down	anaphase-promoting complex subunit 7
CUST_4425_PI425798793	-2,493807	down	coatomer subunit epsilon
CUST_6222_PI425798793	-2,5021787	down	rab11 family-interacting protein 4
CUST_831_PI425798793	-2,5147784	down	methionine-r-sulfoxide reductase
CUST_5607_PI425798793	-2,52321	down	isoform b
CUST_1773_PI425798793	-2,5238817	down	sugar phosphate exchanger 2
CUST_1540_PI425798793	-2,5253065	down	protein mto1-like mitochondrial
CUST_465_PI425798793	-2,530881	down	28s ribosomal protein mitochondrial
CUST_1405_PI425798793	-2,5340374	down	sin3 histone deacetylase corepressor complex component sds3
CUST_4572_PI425798793	-2,5369625	down	complementary sex determiner
CUST_2088_PI425798793	-2,5396156	down	galactokinase 2
CUST_3264_PI425798793	-2,5515409	down	cg7381 cg7381-pa
CUST_3375_PI425798793	-2,5789747	down	cytokine receptor
CUST_2170_PI425798793	-2,59198	down	ribokinase
CUST_2731_PI425798793	-2,5943387	down	zinc finger protein
CUST_3785_PI425798793	-2,597476	down	carbonyl reductase
CUST_2994_PI425798793	-2,602803	down	sodium-dependent phosphate transporter
CUST_1234_PI425798793	-2,6068232	down	homocysteine s-methyltransferase
CUST_2223_PI425798793	-2,6115077	down	cdk5 and abl1 enzyme substrate 1
CUST_21_PI425798793	-2,6156566	down	vacuolar atp synthase subunit d
CUST_1899_PI425798793	-2,6335382	down	PREDICTED: hypothetical protein LOC100576614 [Apis mellifera]
CUST_5519_PI425798793	-2,63655	down	26s protease regulatory subunit s10b
CUST_8104_PI425798793	-2,640344	down	cob yrinic acid -diamide mitochondrial
CUST_698_PI425798793	-2,6506414	down	phosphoinositide 3-kinase regulatory subunit 4
CUST_7198_PI425798793	-2,6565804	down	ribonuclease h2 subunit b
CUST_4801_PI425798793	-2,6583087	down	flavin reductase

CUST_1235_PI425798793	-2,6597092	down	sugar transporter
CUST_3585_PI425798793	-2,6649473	down	mitochondrial ribosomal protein s31
CUST_5013_PI425798793	-2,6670036	down	furin 2
CUST_542_PI425798793	-2,6744814	down	peroxisome proliferator-activated receptor binding protein
CUST_8137_PI425798793	-2,6746526	down	gram-negative bacteria-binding protein 1-2
CUST_629_PI425798793	-2,6792784	down	leucine-rich repeats and immunoglobulin-like domains protein 3
CUST_46_PI425798793	-2,6803458	down	probable actin-related protein 2 3 complex subunit 2
CUST_8734_PI425798793	-2,695933	down	wd-repeat protein
CUST_1636_PI425798793	-2,6999187	down	signal peptidase complex subunit 3
CUST_7030_PI425798793	-2,700543	down	protein fam45b
CUST_8939_PI425798793	-2,7026014	down	ribosomal protein l7ae
CUST_3689_PI425798793	-2,710902	down	mitochondrial carrier protein
CUST_5073_PI425798793	-2,7140431	down	periodic tryptophan protein 1 homolog
CUST_5500_PI425798793	-2,7192225	down	pumilio
CUST_6460_PI425798793	-2,721631	down	x-linked retinitis pigmentosa gtpase regulator-like protein
CUST_5427_PI425798793	-2,7270088	down	splicing factor u2af large subunit
CUST_7199_PI425798793	-2,7377818	down	a disintegrin and metalloproteinase with thrombospondin motifs 7
CUST_7404_PI425798793	-2,7467954	down	PREDICTED: hypothetical protein LOC724644 [Apis mellifera]
CUST_5476_PI425798793	-2,7562556	down	tbc1 domain family member 14
CUST_68_PI425800172	-2,7568207	down	CCE2
CUST_2256_PI425798793	-2,773383	down	mitochondrial solute carrier
CUST_865_PI425798793	-2,773756	down	luciferin 4-monooxygenase
CUST_8248_PI425798793	-2,7766333	down	tailless
CUST_8040_PI425798793	-2,7768734	down	odorant binding protein 14
CUST_9146_PI425798793	-2,7778625	down	delta-aminolevulinic acid dehydratase
CUST_1469_PI425798793	-2,7813323	down	cramped protein
CUST_9381_PI425798793	-2,7928176	down	zinc finger miz domain-containing protein 1
CUST_9222_PI425798793	-2,7930245	down	n-acetylgalactosamine kinase-like
CUST_362_PI425798793	-2,8046722	down	ubiquitin carboxyl-terminal hydrolase isozyme l5
CUST_8231_PI425798793	-2,8058286	down	PREDICTED: hypothetical protein LOC408280 [Apis mellifera]
CUST_2901_PI425798793	-2,8110533	down	thrombospondin type-1 domain-containing protein 4
CUST_4135_PI425798793	-2,8147402	down	sterol regulatory element-binding protein 1
CUST_7015_PI425798793	-2,8210363	down	forkhead box protein n3
CUST_2759_PI425798793	-2,8213944	down	gpi transamidase component pig-s
CUST_3916_PI425798793	-2,8246686	down	ctl transporter
CUST_191_PI425800172	-2,8253715	down	CYP6AS2
CUST_937_PI425798793	-2,8273451	down	headcase protein
CUST_7011_PI425798793	-2,8276074	down	PREDICTED: hypothetical protein LOC100577512 [Apis mellifera]
CUST_2501_PI425798793	-2,8288953	down	ring-box protein 2
CUST_943_PI425798793	-2,831371	down	vit_apime ame: full=vitellogenin flags: precursor
CUST_6767_PI425798793	-2,8357763	down	thymidylate synthase
CUST_739_PI425798793	-2,841961	down	serine threonine-protein kinase lats1
CUST_5921_PI425798793	-2,8519075	down	cg11044 cg11044-pa
CUST_7547_PI425798793	-2,8536956	down	extra macrochaetae
CUST_8253_PI425798793	-2,8795962	down	rag1-activating protein 1-like protein
CUST_7839_PI425798793	-2,8841157	down	probable multidrug resistance-associated protein lethal 03659

CUST_7829_PI425798793	-2,8906438	down	tropomyosin 1 isoform b
CUST_7654_PI425798793	-2,8962255	down	transmembrane protein 85
CUST_4941_PI425798793	-2,9010496	down	nadh dehydrogenase
CUST_2278_PI425798793	-2,916248	down	cytochrome b5 domain-containing protein 2
CUST_848_PI425798793	-2,9189384	down	glyceraldehyde-3-phosphate dehydrogenase
CUST_4373_PI425798793	-2,9195192	down	protein transport protein sec23
CUST_876_PI425798793	-2,9269981	down	dullard-like protein
CUST_6882_PI425798793	-2,9285004	down	coiled-coil-helix-coiled-coil-helix domain-containing protein mitochondrial
CUST_9043_PI425798793	-2,9319358	down	39s ribosomal protein mitochondrial
CUST_7261_PI425798793	-2,9350488	down	3-5 exoribonuclease csl4 homolog
CUST_343_PI425798793	-2,9558246	down	protein kibra-like isoform 2
CUST_5052_PI425798793	-2,9606347	down	mitochondrial-processing peptidase subunit alpha
CUST_6759_PI425798793	-2,9641137	down	coiled-coil domain-containing protein 85c
CUST_315_PI425798793	-2,9704168	down	rna-binding protein 8a
CUST_209_PI425798793	-2,989234	down	f-box only protein 32
CUST_4333_PI425798793	-3,0035737	down	sorting nexin-29
CUST_6387_PI425798793	-3,009809	down	acetyl-coenzyme a transporter 1
CUST_2981_PI425798793	-3,016091	down	renin receptor
CUST_1072_PI425798793	-3,0262678	down	troponin i
CUST_1329_PI425798793	-3,027074	down	suppression of tumorigenicity 1
CUST_936_PI425798793	-3,0292263	down	prophenoloxidase
CUST_2761_PI425798793	-3,0367448	down	golgi-specific brefeldin a-resistance factor
CUST_9322_PI425798793	-3,0386283	down	hypothetical conserved protein
CUST_2079_PI425798793	-3,0555975	down	serologically defined colon cancer antigen 3-like protein
CUST_462_PI425798793	-3,066242	down	cabut
CUST_7723_PI425798793	-3,067868	down	transmembrane protein 147
CUST_9_PI425798793	-3,0734177	down	glial cell differentiation regulator-like
CUST_1055_PI425798793	-3,07397	down	mevalonate kinase
CUST_8207_PI425798793	-3,0800817	down	otu domain-containing protein 7b
CUST_5336_PI425798793	-3,080689	down	larval cuticle protein a3a
CUST_5496_PI425798793	-3,103762	down	protein-l-isoaspartate (d-aspartate) o-methyltransferase
CUST_6744_PI425798793	-3,1085293	down	actin-related protein 2 3 complex subunit 4
CUST_3631_PI425798793	-3,1147978	down	glutamate synthase
CUST_2797_PI425798793	-3,11918	down	general transcription factor 3c polypeptide 2
CUST_8901_PI425798793	-3,1198733	down	monocarboxylate transporter 9
CUST_8337_PI425798793	-3,1227646	down	lipase 1 precursor
CUST_9822_PI425798793	-3,1232853	down	subfamily member 17
CUST_5132_PI425798793	-3,1246142	down	defective proboscis extension
CUST_392_PI425798793	-3,1302466	down	membrane metallo-endopeptidase-like 1
CUST_6102_PI425798793	-3,131828	down	solute carrier family member 37
CUST_3587_PI425798793	-3,1334343	down	zinc finger cchc domain-containing protein 24
CUST_9098_PI425798793	-3,1372705	down	oligopeptidase a
CUST_7997_PI425798793	-3,1437457	down	exonuclease 3 -5 domain-like-containing protein 1
CUST_3774_PI425798793	-3,1572402	down	nadh dehydrogenase 1 alpha 1-like
CUST_7946_PI425798793	-3,159479	down	activating signal cointegrator 1 complex subunit 1
CUST_3843_PI425798793	-3,1604903	down	isoforms a c f g

CUST_7616_PI425798793	-3,1651552	down	aspartyl-trna synthetase
CUST_9106_PI425798793	-3,171164	down	nuclear lim interactor-interacting factor (nli-interacting factor) (nli-if)
CUST_7984_PI425798793	-3,1736844	down	eukaryotic translation initiation factor 3 subunit 4
CUST_814_PI425798793	-3,173952	down	glutaredoxin-related protein 5
CUST_3892_PI425798793	-3,1760514	down	actin-related protein 3
CUST_5272_PI425798793	-3,1800528	down	wd repeat-containing protein 92
CUST_1489_PI425798793	-3,1881735	down	lysine-specific histone demethylase 1a
CUST_4126_PI425798793	-3,18861	down	chloride channel protein 3
CUST_3224_PI425798793	-3,1889746	down	polymerase delta-interacting protein 2
CUST_4972_PI425798793	-3,201896	down	glutathione synthetase
CUST_771_PI425798793	-3,2032933	down	elongation factor 1 delta
CUST_1635_PI425798793	-3,2155123	down	bmp-binding endothelial regulator protein
CUST_5656_PI425798793	-3,2227514	down	upf0369 protein c6orf57-like protein
CUST_4347_PI425798793	-3,2230837	down	leucine-rich repeats and immunoglobulin-like domains protein 3
CUST_5394_PI425798793	-3,229625	down	alpha-aminoadipic semialdehyde mitochondrial
CUST_6716_PI425798793	-3,231887	down	nadh-ubiquinone oxidoreductase subunit
CUST_888_PI425798793	-3,23398	down	cox assembly mitochondrial protein homolog
CUST_7568_PI425798793	-3,2395566	down	extensin-like protein dif10
CUST_5874_PI425798793	-3,2433007	down	v-type proton atpase 116 kda subunit a isoform 1-like
CUST_5178_PI425798793	-3,2462687	down	inositol triphosphate 3-kinase
CUST_5234_PI425798793	-3,2464943	down	eukaryotic translation initiation factor 4b
CUST_7861_PI425798793	-3,2488852	down	dna repair protein xp-c rad4
CUST_181_PI425800172	-3,2489274	down	CYP6AS5
CUST_5115_PI425798793	-3,2576537	down	cg1909-like protein
CUST_8664_PI425798793	-3,2591803	down	ino80 complex subunit b-like
CUST_8643_PI425798793	-3,2738435	down	glutamyl aminopeptidase
CUST_5791_PI425798793	-3,2814019	down	d-beta-hydroxybutyrate mitochondrial
CUST_3546_PI425798793	-3,2820792	down	f-box only protein 21-like
CUST_9105_PI425798793	-3,2838836	down	mitochondrial carrier protein
CUST_6833_PI425798793	-3,284709	down	dna ligase 3
CUST_4043_PI425798793	-3,287955	down	histone h3
CUST_2517_PI425798793	-3,2934208	down	upf0184 protein c9orf16-like
CUST_10041_PI425798793	-3,3206356	down	ubiquinone biosynthesis protein coq4 mitochondrial-like
CUST_5889_PI425798793	-3,3247607	down	probable cytochrome p450 6a14
CUST_3726_PI425798793	-3,3339553	down	cub and sushi domain-containing protein 3
CUST_2956_PI425798793	-3,3362596	down	serine threonine-protein phosphatase 2a regulatory subunit b subunit alpha
CUST_5038_PI425798793	-3,339512	down	upf0545 protein c22orf39-like protein
CUST_9969_PI425798793	-3,3470879	down	dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase
CUST_229_PI425800172	-3,3491082	down	CYP4AZ1
CUST_1816_PI425798793	-3,3497822	down	26s proteasome non-atpase regulatory subunit 7
CUST_7309_PI425798793	-3,3530452	down	cytochrome b-c1 complex subunit mitochondrial
CUST_9566_PI425798793	-3,3530946	down	g protein pathway suppressor 2
CUST_526_PI425798793	-3,3552887	down	translocase of outer membrane 34
CUST_3805_PI425798793	-3,3627372	down	retinoblastoma-binding protein 5
CUST_574_PI425798793	-3,3663743	down	b(+)-type amino acid transporter 1
CUST_5537_PI425798793	-3,3693895	down	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 4

CUST_223_PI425798793	-3,387382	down	plasma membrane calcium-transporting atpase 3
CUST_6840_PI425798793	-3,3878715	down	pyrroline-5-carboxylate reductase 2
CUST_3703_PI425798793	-3,3889897	down	charged multivesicular body protein 6
CUST_2304_PI425798793	-3,3940356	down	stromal cell-derived factor 2
CUST_6497_PI425798793	-3,4000719	down	catenin alpha
CUST_3995_PI425798793	-3,4013474	down	tau-tubulin kinase 1
CUST_6249_PI425798793	-3,4070508	down	isoform a
CUST_7464_PI425798793	-3,4116437	down	39s ribosomal protein mitochondrial
CUST_1168_PI425798793	-3,4181406	down	glutamate cysteine ligase
CUST_2964_PI425798793	-3,4203231	down	proteasome subunit beta type-2
CUST_705_PI425798793	-3,4242442	down	isoform a
CUST_6129_PI425798793	-3,4286187	down	uncharacterized protein c12orf41-like protein
CUST_7796_PI425798793	-3,4539418	down	cytochrome p450
CUST_420_PI425798793	-3,4869652	down	methionine aminopeptidase
CUST_7678_PI425798793	-3,4926295	down	tay bridge
CUST_5577_PI425798793	-3,5168111	down	mesoderm induction early response protein 1
CUST_8495_PI425798793	-3,5193577	down	PREDICTED: hypothetical protein LOC726382 [Apis mellifera]
CUST_5470_PI425798793	-3,5195148	down	disco-interacting protein 2
CUST_2128_PI425798793	-3,5335188	down	charged multivesicular body protein 5
CUST_10011_PI425798793	-3,5343785	down	protein sidekick-1
CUST_183_PI425800172	-3,5375016	down	CYP6AS5
CUST_2168_PI425798793	-3,5579135	down	hypothetical protein SINV_04665 [Solenopsis invicta]
CUST_3468_PI425798793	-3,561992	down	isoform a
CUST_1666_PI425798793	-3,5627053	down	neuronal calcium sensor 2
CUST_8671_PI425798793	-3,5645273	down	coiled-coil-helix-coiled-coil-helix domain-containing
CUST_4187_PI425798793	-3,5713756	down	high affinity camp-specific and ibmx-insensitive 3 -cyclic phosphodiesterase 8a
CUST_6565_PI425798793	-3,5784533	down	zinc finger swim domain-containing protein kiaa0913
CUST_6166_PI425798793	-3,5803578	down	amino acid transporter
CUST_4919_PI425798793	-3,5948846	down	vacuolar atp synthase subunit ac39
CUST_3701_PI425798793	-3,5980232	down	splicing factor 45
CUST_6300_PI425798793	-3,5980527	down	phosphoenolpyruvate carboxykinase
CUST_6677_PI425798793	-3,603277	down	PREDICTED: hypothetical protein LOC552190 [Apis mellifera]
CUST_2276_PI425798793	-3,6036298	down	tumor suppressor candidate 3
CUST_6629_PI425798793	-3,6059716	down	uncharacterized protein c20orf111-like protein
CUST_2971_PI425798793	-3,6214013	down	placental protein 11
CUST_257_PI425798793	-3,6288204	down	maguk p55 subfamily member 6
CUST_6668_PI425798793	-3,62943	down	dna-directed rna polymerase i subunit rpa2
CUST_6454_PI425798793	-3,6362345	down	acylglycerol mitochondrial
CUST_5771_PI425798793	-3,6418648	down	malate dehydrogenase
CUST_1206_PI425798793	-3,6447697	down	plasma glutamate carboxypeptidase
CUST_4514_PI425798793	-3,650609	down	leucine-rich repeat and calponin-like proteiny domain-containing protein 3
CUST_4982_PI425798793	-3,654903	down	uncharacterized protein c8orf55-like protein
CUST_1028_PI425798793	-3,6736348	down	mitochondrial inner membrane
CUST_8799_PI425798793	-3,6739168	down	wd repeat-containing protein 7
CUST_7491_PI425798793	-3,7064168	down	beta-arrestin 1
CUST_1456_PI425798793	-3,717051	down	sterol regulatory element-binding protein cleavage-activating protein

CUST_7585_PI425798793	-3,7187505	down	chromobox protein 5
CUST_8395_PI425798793	-3,7265236	down	adenosine kinase
CUST_9647_PI425798793	-3,7282708	down	monocarboxylate transporter
CUST_6360_PI425798793	-3,736838	down	sap30-binding protein
CUST_8139_PI425798793	-3,7379034	down	thyroid receptor-interacting protein 11
CUST_2813_PI425798793	-3,7445323	down	ubiquinone biosynthesis protein mitochondrial
CUST_250_PI425798793	-3,7449703	down	eukaryotic initiation factor 4a-iii
CUST_6348_PI425798793	-3,7466173	down	n -adenine-specific dna methyltransferase 1
CUST_793_PI425798793	-3,7534566	down	lkb1 interacting protein
CUST_8234_PI425798793	-3,7732108	down	retinal degeneration slow protein
CUST_3087_PI425798793	-3,773462	down	2-oxoglutarate dehydrogenase
CUST_1255_PI425798793	-3,7950935	down	glucose dehydrogenase
CUST_5041_PI425798793	-3,7958555	down	inosine-uridine preferring nucleoside hydrolase
CUST_248_PI425798793	-3,7990205	down	3-hydroxyacyl- dehydrogenase type-2
CUST_7225_PI425798793	-3,8025608	down	mitochondrial import inner membrane translocase subunit tim10
CUST_3425_PI425798793	-3,8041632	down	trafficking kinesin binding 2
CUST_4431_PI425798793	-3,8211799	down	enolase
CUST_4467_PI425798793	-3,8222454	down	protein dj-1
CUST_7080_PI425798793	-3,823601	down	zinc carboxypeptidase a 1
CUST_6344_PI425798793	-3,8302717	down	succinyl-coa synthetase beta chain
CUST_8482_PI425798793	-3,832733	down	translocon-associated delta subunit
CUST_1725_PI425798793	-3,8385417	down	upf0518 protein fam160b1
CUST_9408_PI425798793	-3,8509893	down	oxidative stress induced growth inhibitor 1
CUST_9992_PI425798793	-3,8524175	down	hexokinase
CUST_179_PI425800172	-3,86283	down	CYP6AS7
CUST_186_PI425800172	-3,8657403	down	CYP6AS4
CUST_5044_PI425798793	-3,8659124	down	calponin transgelin
CUST_8858_PI425798793	-3,865956	down	ap-2 complex subunit mu-1
CUST_3786_PI425798793	-3,8755326	down	beta lactamase domain
CUST_2984_PI425798793	-3,8800936	down	proteasome subunit beta 7
CUST_6052_PI425798793	-3,9208612	down	cation-transporting atpase
CUST_5419_PI425798793	-3,9244075	down	e3 ubiquitin-protein ligase huwe1
CUST_4777_PI425798793	-3,9278672	down	h2a histone member v
CUST_9307_PI425798793	-3,951184	down	PREDICTED: hypothetical protein LOC727647 [Apis mellifera]
CUST_8198_PI425798793	-3,9568276	down	phosphatidylinositol 3-kinase regulatory subunit alpha
CUST_2898_PI425798793	-3,98912	down	mediator of rna polymerase ii transcription subunit 15
CUST_6183_PI425798793	-3,99052	down	cordon-bleu 1
CUST_3326_PI425798793	-3,993762	down	mitochondrial carrier protein
CUST_8585_PI425798793	-4,01788	down	reversion-inducing cysteine-rich protein with kazal motifs
CUST_6720_PI425798793	-4,0228815	down	PREDICTED: hypothetical protein LOC409502 [Apis mellifera]
CUST_287_PI425798793	-4,0300055	down	cofilin actin-depolymerizing factor-like protein
CUST_1544_PI425798793	-4,0404243	down	sorbin and sh3 domain-containing protein 1
CUST_1805_PI425798793	-4,0438147	down	wd repeat-containing protein 37
CUST_9866_PI425798793	-4,0533257	down	solute carrier family 23 member 1
CUST_7412_PI425798793	-4,0595098	down	isoform b
CUST_36_PI425800172	-4,063884	down	CCE13_GB18660

CUST_8368_PI425798793	-4,0742607	down	b chain crystallographic studies of nucleosome core particles containing histone sin mutants
CUST_2788_PI425798793	-4,0880914	down	integrin-linked protein kinase
CUST_6275_PI425798793	-4,1179843	down	zinc finger protein 650
CUST_6654_PI425798793	-4,1327744	down	phytanoyl- dioxygenase domain-containing protein 1-like protein
CUST_4319_PI425798793	-4,1483316	down	t-complex protein 1 subunit epsilon
CUST_2645_PI425798793	-4,176508	down	PREDICTED: hypothetical protein LOC725232 [Apis mellifera]
CUST_4719_PI425798793	-4,1854877	down	heparan-alpha-glucosaminide n-acetyltransferase
CUST_192_PI425800172	-4,191722	down	CYP6AS2
CUST_9797_PI425798793	-4,197727	down	nadh-ubiquinone oxidoreductase 75 kda mitochondrial
CUST_3498_PI425798793	-4,2005258	down	importin beta-3
CUST_7277_PI425798793	-4,202723	down	endoa
CUST_8243_PI425798793	-4,2276316	down	adp-ribosylation factor gtpase-activating protein 3
CUST_3186_PI425798793	-4,2284045	down	muscular protein 20
CUST_188_PI425800172	-4,229183	down	CYP6AS3
CUST_53_PI425800172	-4,2346225	down	CCE7_GB13602
CUST_6276_PI425798793	-4,261806	down	mitochondrial ubiquitin ligase activator of nfkb 1
CUST_5834_PI425798793	-4,267766	down	uncharacterized protein kiaa0090-like
CUST_7871_PI425798793	-4,268149	down	voltage-dependent anion-selective channel
CUST_1428_PI425798793	-4,268762	down	mitochondrial manganese superoxide dismutase
CUST_182_PI425800172	-4,2930236	down	CYP6AS5
CUST_3293_PI425798793	-4,295341	down	PREDICTED: hypothetical protein LOC410044 [Apis mellifera]
CUST_4925_PI425798793	-4,318452	down	zinc iron transporter
CUST_7802_PI425798793	-4,342163	down	cg11699-ra protein
CUST_2749_PI425798793	-4,3632693	down	tropomyosin 1
CUST_9645_PI425798793	-4,3769193	down	elongator complex protein 3
CUST_9343_PI425798793	-4,3801737	down	isocitrate dehydrogenase
CUST_6966_PI425798793	-4,3827024	down	glutamate transporter
CUST_78_PI425800172	-4,4036846	down	GSTmic1
CUST_52_PI425798793	-4,4119277	down	calponin transgelin
CUST_2774_PI425798793	-4,4149766	down	low-density lipoprotein receptor-related protein
CUST_1158_PI425798793	-4,4312673	down	nadh-ubiquinone oxidoreductase ashi subunit
CUST_4651_PI425798793	-4,4592285	down	l 01289 long form
CUST_5218_PI425798793	-4,4594736	down	interferon-related developmental regulator 1
CUST_6577_PI425798793	-4,467044	down	microsomal glutathione s-transferase
CUST_475_PI425798793	-4,4687796	down	endothelin-converting enzyme
CUST_1593_PI425798793	-4,53095	down	arylsulfatase b
CUST_738_PI425798793	-4,534731	down	triosephosphate isomerase
CUST_3817_PI425798793	-4,535735	down	isoform a
CUST_1137_PI425798793	-4,5445085	down	probable cytochrome p450 6a14
CUST_5562_PI425798793	-4,5692344	down	myosin heavy muscle isoform 1
CUST_9833_PI425798793	-4,588997	down	protein phosphatase 2c
CUST_619_PI425798793	-4,6100273	down	double-stranded rna-binding protein staufen-like protein 2
CUST_8656_PI425798793	-4,616942	down	chorion b-zip transcription factor
CUST_727_PI425798793	-4,6489325	down	ubx domain-containing protein 8
CUST_8335_PI425798793	-4,6650662	down	glucose dehydrogenase
CUST_8382_PI425798793	-4,667315	down	mannose-1-phosphate guanyltransferase

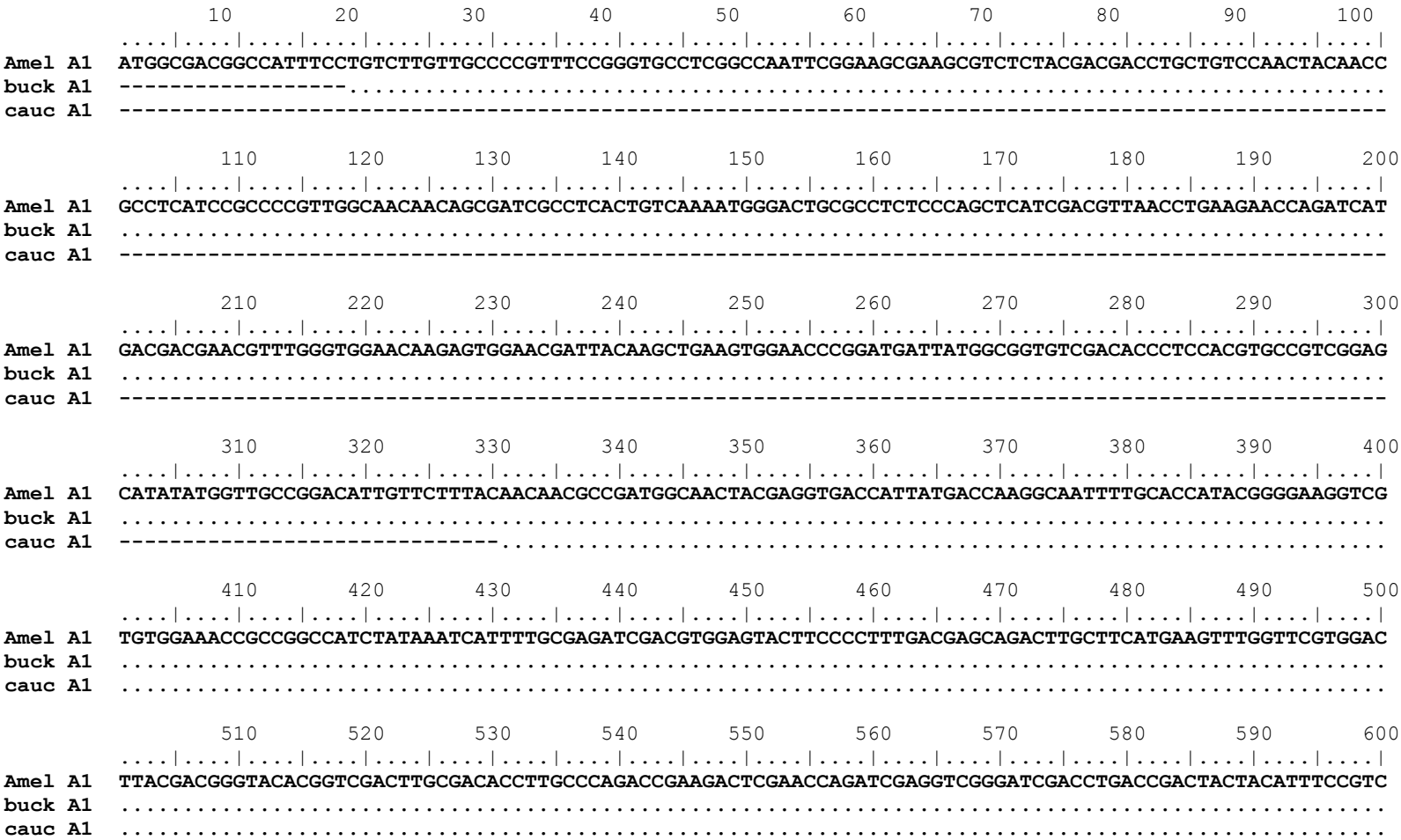
CUST_8443_PI425798793	-4,675484	down	fatty acid 2-hydroxylase
CUST_143_PI425800172	-4,686591	down	CYP15A1
CUST_9033_PI425798793	-4,6964984	down	acyl-coenzyme a:6-aminopenicillanic-acid-acyltransferase 40 kda form
CUST_5654_PI425798793	-4,7044716	down	protein fam46a
CUST_9790_PI425798793	-4,7079144	down	ribonuclease oy
CUST_198_PI425798793	-4,7348495	down	ubiquitin-conjugating enzyme rad6
CUST_5553_PI425798793	-4,745271	down	alpha gamma epsilon
CUST_11_PI425800189	-4,7454104	down	CCE7_GB13602
CUST_9073_PI425798793	-4,750506	down	6-phosphofructo-2-kinase fructose- -bisphosphatase short form
CUST_5773_PI425798793	-4,7549376	down	b-cell receptor-associated protein 31
CUST_4279_PI425798793	-4,773872	down	protein g12
CUST_7687_PI425798793	-4,8261685	down	synaptotagmin 1
CUST_4280_PI425798793	-4,847702	down	muscular protein 20
CUST_1929_PI425798793	-4,8549194	down	guanine nucleotide-binding protein beta 2 (g protein beta2)
CUST_8733_PI425798793	-4,867089	down	sterol o-acyltransferase 1
CUST_4458_PI425798793	-4,8679132	down	dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase
CUST_10106_PI425798793	-4,8783545	down	protein tipe
CUST_4020_PI425798793	-4,8919168	down	zinc finger ccch domain-containing protein 11a
CUST_9848_PI425798793	-4,899198	down	nadh dehydrogenase
CUST_215_PI425800172	-4,9113274	down	CYP6AS10
CUST_1080_PI425798793	-4,9328976	down	ester hydrolase c11orf54-like protein
CUST_7732_PI425798793	-5,0151296	down	lymphoid-restricted membrane isoform cra_a
CUST_7868_PI425798793	-5,0152	down	prefoldin subunit 2
CUST_5236_PI425798793	-5,0233274	down	serine threonine-protein kinase doa
CUST_217_PI425798793	-5,035859	down	insulin-like growth factor-binding protein complex acid labile chain
CUST_3128_PI425798793	-5,086801	down	ensangp00000017418-like protein
CUST_5658_PI425798793	-5,1149855	down	nadh dehydrogenase
CUST_7258_PI425798793	-5,1160336	down	bmp and activin membrane-bound inhibitor homolog
CUST_207_PI425800172	-5,1898584	down	CYP6AS13
CUST_2803_PI425798793	-5,284858	down	dynein light chain cytoplasmic
CUST_4722_PI425798793	-5,2958837	down	transmembrane protein 8
CUST_6014_PI425798793	-5,302268	down	proteasome subunit alpha type-2
CUST_6021_PI425798793	-5,3089137	down	serine threonine-protein kinase mark2
CUST_7007_PI425798793	-5,3196874	down	isoform a
CUST_39_PI425800172	-5,35144	down	CCE12
CUST_6127_PI425798793	-5,3610034	down	dehydrogenase reductase sdr family member 7
CUST_1981_PI425798793	-5,3940864	down	glycogen phosphorylase
CUST_125_PI425798793	-5,402744	down	lysosomal aspartic protease
CUST_5011_PI425798793	-5,421019	down	mitochondrial ribosomal protein l50
CUST_691_PI425798793	-5,4529004	down	chondroitin proteoglycan-2
CUST_6267_PI425798793	-5,47572	down	isoform b
CUST_3558_PI425798793	-5,4957423	down	5 nucleotidase
CUST_7233_PI425798793	-5,5211678	down	solute carrier family 22 member 3
CUST_6730_PI425798793	-5,569195	down	glucosyl glucuronosyl transferases
CUST_3907_PI425798793	-5,6251836	down	tubulin-specific chaperone e
CUST_1710_PI425798793	-5,665791	down	golgin subfamily b member 1-like

CUST_616_PI425798793	-5,7230973	down	perq amino acid-rich with gyf domain-containing protein 2
CUST_1548_PI425798793	-5,723992	down	lupus la
CUST_1958_PI425798793	-5,733195	down	juvenile hormone epoxide hydrolase 1
CUST_2064_PI425798793	-5,7529483	down	t-complex protein 1 subunit delta
CUST_4243_PI425798793	-5,7568555	down	dipeptidyl peptidase
CUST_106_PI425800172	-5,771479	down	CYP336A1
CUST_8763_PI425798793	-5,799202	down	nadh dehydrogenase
CUST_4834_PI425798793	-5,8594956	down	u6 snrna-associated sm-like protein lsm5
CUST_3142_PI425798793	-5,8692026	down	nadh-ubiquinone oxidoreductase 39 kda subunit
CUST_1014_PI425798793	-5,876833	down	protein isoform b
CUST_5850_PI425798793	-5,8805656	down	hypothetical protein EAG_14228 [Camponotus floridanus]
CUST_4727_PI425798793	-5,9220114	down	cytochrome p450 6k1
CUST_9276_PI425798793	-5,96185	down	protein grainyhead
CUST_8525_PI425798793	-5,974938	down	nadh dehydrogenase
CUST_9366_PI425798793	-5,9780955	down	receptor-type tyrosine-protein phosphatase r
CUST_3242_PI425798793	-6,0126324	down	glucosyl glucuronosyl transferases
CUST_5823_PI425798793	-6,017715	down	aldo-keto reductase
CUST_4316_PI425798793	-6,0275326	down	odorant-binding protein 1
CUST_10049_PI425798793	-6,0447216	down	calcium-independent phospholipase a2-gamma
CUST_3015_PI425798793	-6,1027837	down	venom acid phosphatase
CUST_4166_PI425798793	-6,144366	down	ras-related and estrogen-regulated growth inhibitor
CUST_154_PI425800172	-6,158186	down	CYP9Q2
CUST_223_PI425800172	-6,188366	down	CYP6AQ1
CUST_2220_PI425798793	-6,2242804	down	mitochondrial ribosomal protein l50
CUST_8211_PI425798793	-6,289498	down	isoform a
CUST_1018_PI425798793	-6,438594	down	peptidyl-prolyl cis-trans rhodopsin-specific isozyme
CUST_3675_PI425798793	-6,440037	down	ras-related protein rab-5b-like
CUST_5509_PI425798793	-6,4742994	down	conserved insect protein
CUST_206_PI425800172	-6,518891	down	CYP6AS13
CUST_9719_PI425798793	-6,5917616	down	organic cation transporter
CUST_4510_PI425798793	-6,6127834	down	adenylate cyclase
CUST_6655_PI425798793	-6,6306534	down	nadh dehydrogenase
CUST_4739_PI425798793	-6,638666	down	karyopherin alpha 6
CUST_4433_PI425798793	-6,640064	down	water dikinase
CUST_774_PI425798793	-6,6840096	down	nucleolar protein c7b
CUST_3372_PI425798793	-6,7949424	down	protein kinase dc2
CUST_6254_PI425798793	-6,815934	down	cytochrome p450 9e2
CUST_184_PI425800172	-6,979884	down	CYP6AS4
CUST_7497_PI425798793	-6,9915004	down	phosphorylase b kinase gamma catalytic skeletal muscle isoform
CUST_5290_PI425798793	-7,0253105	down	peptidoglycan-recognition protein s2
CUST_2665_PI425798793	-7,027776	down	ecdysteroid udp-glucosyltransferase
CUST_6938_PI425798793	-7,085281	down	mitochondrial cytochrome c oxidase subunit 6b
CUST_4257_PI425798793	-7,1145344	down	smoothelin-like protein 2
CUST_9151_PI425798793	-7,1943254	down	probable cytochrome p450 6a14
CUST_5708_PI425798793	-7,2413197	down	mature t-cell proliferation 1 neighbor
CUST_7754_PI425798793	-7,2773786	down	receptor expression-enhancing protein 5

CUST_6503_PI425798793	-7,2797565	down	PREDICTED: hypothetical protein LOC100578955 [Apis mellifera]
CUST_6559_PI425798793	-7,3665266	down	eupolytin
CUST_8334_PI425798793	-7,4118977	down	trehalase
CUST_5130_PI425798793	-7,634765	down	basic helix-loop-helix zip transcription factor
CUST_2243_PI425798793	-7,903392	down	p53 regulated pa26 nuclear protein
CUST_197_PI425798793	-8,140963	down	membrane-associated transporter protein
CUST_6519_PI425798793	-8,15039	down	protein isoform a
CUST_6911_PI425798793	-8,20804	down	ubiquitin-protein ligase e3a
CUST_4822_PI425798793	-8,299138	down	myb protein
CUST_8356_PI425798793	-8,328981	down	isoform a
CUST_747_PI425798793	-8,365029	down	troponin c
CUST_8492_PI425798793	-8,388883	down	long-chain-fatty-acid coa ligase
CUST_5492_PI425798793	-8,42016	down	chymotrypsin-2
CUST_5564_PI425798793	-8,46128	down	female-specific doublesex isoform f2
CUST_6128_PI425798793	-8,512053	down	aminopeptidase n
CUST_538_PI425798793	-8,650761	down	isoform e
CUST_369_PI425798793	-8,731529	down	octopamine receptor beta-2r
CUST_6410_PI425798793	-8,856543	down	polymerase ii (dna directed) polypeptide h
CUST_7253_PI425798793	-8,860517	down	lysozyme isoform 1
CUST_5852_PI425798793	-8,862886	down	inwardly rectifying k+ channel protein
CUST_7844_PI425798793	-9,025035	down	PREDICTED: hypothetical protein LOC724993 [Apis mellifera]
CUST_5375_PI425798793	-9,472136	down	calcium-activated potassium channel alpha chain
CUST_912_PI425798793	-9,538921	down	synaptotagmin-like protein 5
CUST_6610_PI425798793	-9,665585	down	troponin c
CUST_224_PI425800172	-9,756338	down	CYP6AQ1
CUST_880_PI425798793	-9,903043	down	sodium potassium-dependent atpase beta-2 subunit
CUST_35_PI425800172	-9,943121	down	CCE13_GB18660
CUST_4895_PI425798793	-10,121311	down	nuclear hormone receptor ftz-f1 beta
CUST_1699_PI425798793	-10,143931	down	guanine nucleotide-binding protein g subunit alpha
CUST_7631_PI425798793	-10,33982	down	muscle-specific protein isoform i
CUST_5838_PI425798793	-10,382632	down	PREDICTED: peritrophin-1-like [Apis mellifera]
CUST_15_PI425800189	-10,409016	down	CCE3_GB19866
CUST_9091_PI425798793	-10,631098	down	alpha-amylase
CUST_4209_PI425798793	-11,071764	down	uncharacterized protein
CUST_6861_PI425798793	-11,286233	down	28s ribosomal protein mitochondrial
CUST_7219_PI425798793	-11,328418	down	gtp-binding protein sar1b
CUST_1603_PI425798793	-12,278866	down	glycerol-3-phosphate dehydrogenase
CUST_1584_PI425798793	-12,379415	down	ring finger protein nhl-1
CUST_366_PI425798793	-13,319147	down	held out wings
CUST_9057_PI425798793	-13,693938	down	carboxypeptidase a
CUST_212_PI425800172	-13,829649	down	CYP6AS11
CUST_7095_PI425798793	-14,274029	down	ice_drome ame: full=caspase ame: full=drice contains: ame: full=caspase subunit p21 contains: ame: full=caspase subunit p12 flags: precursor
CUST_71_PI425800172	-14,546093	down	CCE1_GB16342
CUST_65_PI425800172	-15,053115	down	CCE3_GB19866
CUST_211_PI425800172	-16,128645	down	CYP6AS11
CUST_2536_PI425798793	-16,675543	down	carboxypeptidase a-like

CUST_4568_PI425798793	-17,238838	down	thymus-specific serine protease
CUST_9733_PI425798793	-18,05587	down	PREDICTED: hypothetical protein LOC408608 [Apis mellifera]
CUST_9901_PI425798793	-19,71263	down	isoform c
CUST_70_PI425800172	-20,303305	down	CCE1_GB16342
CUST_5304_PI425798793	-20,47923	down	maltase 1
CUST_5441_PI425798793	-21,472687	down	mpa2 allergen
CUST_8632_PI425798793	-24,529428	down	teratocyte released chitinase
CUST_5469_PI425798793	-43,227585	down	protein g12

9.5 Sequence alignment of *A. m. mellifera, A. m. buckfast* and *A. m. caucasica* nAChR subunits



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                610       620       630       640       650       660       670       680       690       700
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A1   GAGTGGGACATCATAAAAGTGCCTGCCGTGAGGAACGAGGCGTTCTACATATGCTGCGAGGAGCCCTACCCCGATATCGTGTTCAATATCACCCTACGCC
buck A1   ....................................................................................................
cauc A1   ....................................................................................................

                710       720       730       740       750       760       770       780       790       800
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A1   GCAAGACCCTCTTCTACACGGTGAACCTGATCATACCGTGCGTGGGCATATCTTTTCTCTCGGTGCTGGTGTTCTACCTGCCGAGCGACAGCGGGGAGAA
buck A1   ...................................................................................T........
cauc A1   ....................................------------------------------------------------------------

                810       820       830       840       850       860       870       880       890       900
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A1   GGTCTCGCTCTCGATCTCGATCCTGCTGTCGTTGACGGTGTTCTTCCTACTGCTCGCCGAGATAATCCCGCCCACGTCGCTAACGGTGCCGTTGCTCGGC
buck A1   ......------------------------------------------------------------------------------------------
cauc A1   --------------------------------------------------------------------------------------------------
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                 10        20        30        40        50        60        70        80        90       100
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A3    ATGATGAAGAGCCTGGTGGGGATCATGTGGATAGTGTTGGTGCTCATATCAGGATGCTCGGGAAATCCGGACGCGAAGCGGCTGTACGACGACCTCCTGT
buck A3    ----------------------------------------.....................................................
                 110       120       130       140       150       160       170       180       190       200
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A3    CGAATTACAACAAGCTGGTTCGTCCAGTAGTCAACGTCACAGACGCCCTCACCGTTAAAATCAAGCTCAAACTCTCTCAGTTGATCGACGTAAATCTGAA
buck A3    ........................G.....................................................................

                 210       220       230       240       250       260       270       280       290       300
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A3    AAATCAAATCATGACAACGAACCTCTGGGTAGAACAGTCATGGTACGATTACAAGTTAAAATGGGATCCAAAGGAATATGGTGGGGTGGAAATGCTACAC
buck A3    ....................................................................................................

                 310       320       330       340       350       360       370       380       390       400
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A3    GTGCCATCCGATCATATATGGAGGCCCGATATAGTTTTGTACAACAACGCCGACGGTAATTTCGAGGTGACGCTGGCAACGAAGGCGACGCTGAATTACA
buck A3    ....................................................................................................
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                410       420       430       440       450       460       470       480       490       500
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A3    CAGGGAGGGTCGAATGGAAGCCACCGGCCATATACAAATCCTCTTGCGAGATCGACGTAGAATACTTTCCGTTCGACGAGCAAACGTGCGTCATGAAGTT
buck A3    ..................................................................................................

                510       520       530       540       550       560       570       580       590       600
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A3    CGGCTCGTGGACTTATGACGGCTTCCAGGTTGATCTTCGACACATCGACGAAATTCGTGGCAAAAATGTCGTCGACATCGGCGTTGATCTGTCCGAGTTT
buck A3    ..................................................................................................


                610       620       630       640       650       660       670       680       690       700
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A3    TATACTTCCGTCGAGTGGGATATCCTAGAAGTCCCAGCCGTGCGAAATGAAAAATTTTACACGTGCTGCGACGAGCCCTACCTCGACATCACGTTCAACA
buck A3    ..................................................................................................

                710       720       730       740       750       760       770       780       790       800
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A3    TCACCATGAGACGGAAGACTCTATTTTACACCGTCAATCTTATAATACCGTGTATGGGTATCTCGTTTCTCACGGTGTTGGTCTTCTATCTGCCAAGCGA
buck A3    ..................................................................................................
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```
                 10        20        30        40        50        60        70        80        90       100
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A4    GGGCTCGAGGGGGACTAGTGCGTCGGTCATAGTGGAGGAGACAGGGGTCCACGATGCCCCCCATAATAGGGGAAACGTTGCGTGTATGGTTCCTCTCGGC
buck A4.1  ----------------------------------------------------.....................................................
buck A4.2  ----------------------------------------------------.....................................................
buck A4.3  ----------------------------------------------------.....................................................
buck A4.7  ----------------------------------------------------.....................................................
buck A4.8  ----------------------------------------------------.....................................................
cauc A4    ---------------------------------------------------------------------.....................................
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```
                110       120       130       140       150       160       170       180       190       200
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A4    GTTGGTGGTTCACGGTGCTGTCGCTGGCAATCCGGACGCGAAACGCCTTTACGACGACTTGCTTTCTAATTATAACAAACTAGTGCGCCCTGTTGTGAAT
buck A4.1  ..................................................................C.............................
buck A4.2  ..................................................................C.............................
buck A4.3  ..................................................................C.............................
buck A4.7  ..............................................C.....................C.............................
buck A4.8  ..................................................................C.............................
cauc A4    ..................................................................C.............................

                210       220       230       240       250       260       270       280       290       300
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A4    ACCTCGGACGTGCTACGCGTGTGCATCAAGTTGAAACTCTCCCAGCTCATCGACGTGAATTTGAAGAATCAAATCATGACGACGAATCTATGGGTGGAAC
buck A4.1  ..........................................................................G.........
buck A4.2  ..........................................................................G.........
buck A4.3  ..........................................................................G.........
buck A4.7  ..............................................C...........................G.........
buck A4.8  ..........................................................................G.........
cauc A4    ..........................................................................G.........

                310       320       330       340       350       360       370       380       390       400
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A4    AGTCATGGTACGATTACAAGTTACGATGGGAGCCGAAGGAGTACGGAGGAGTTAAAATGTTACACGTGCCATCCGATCACATATGGCGGCCCGATATAGT
buck A4.1  ..----------------------------------------------------------------------------------------------
buck A4.2  ....................C.....................................................G.....................
buck A4.3  ....................C.....................................................G.....................
buck A4.7  ....................C.....................................................G.....................
buck A4.8  ....................C.....................................................G.....................
cauc A4    ....................C.....................................................G.....................

                410       420       430       440       450       460       470       480       490       500
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A4    CCTCTACAACAACGC------------------------------GGACGGCAACTTCGAGGTGACCTTGGCCACGAAGGCCACCATCTACCATCAAGG
buck A4.1  ----------------------------------------------------------------------------------------------
buck A4.2  ............----------------------------------------------------------------------------------
buck A4.3  ..............------------------------------.....................................................C.........
buck A4.7  ..............CCAGAACCACTAATGTGCTCGCTCGCAGCGC.....................................................T.......
buck A4.8  ............----------------------------------------------------------------------------------
cauc A4    ............----------------------------------------------------------------------------------
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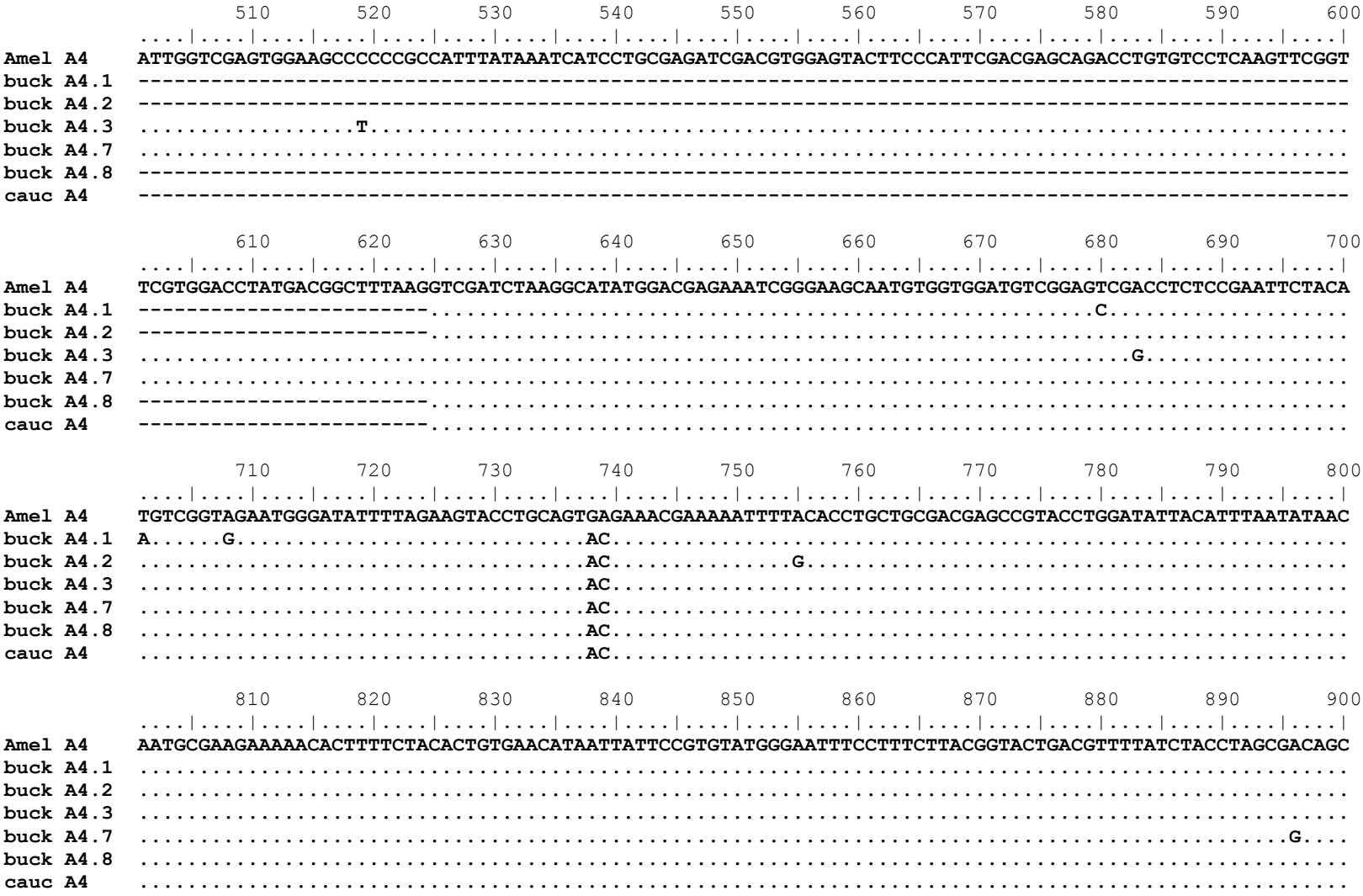
510 520 530 540 550 560 570 580 590 600
Amel A4 ATTGGTCGAGTGGAAGCCCCCCGCCATTTATAAATCATCCTGCGAGATCGACGTGGAGTACTTCCCATTCGACGAGCAGACCTGTGTCCTCAAGTTCGGT
buck A4.1 ----------
buck A4.2 ----------
buck A4.3 ....T.....
buck A4.7 ....
buck A4.8 ----------
cauc A4 ----------
610 620 630 640 650 660 670 680 690 700
Amel A4 TCGTGGACCTATGACGGCTTTAAGGTCGATCTAAGGCATATGGACGAGAAATCGGGAAGCAATGTGGTGGATGTCGGAGTCGACCTCTCCGAATTCTACA
buck A4.1 ----------.....C.....
buck A4.2 ----------.....
buck A4.3 .....G.....
buck A4.7 .....
buck A4.8 ----------.....
cauc A4 ----------.....
710 720 730 740 750 760 770 780 790 800
Amel A4 TGTCGGTAGAATGGGATATTTTAGAAGTACCTGCAGTGAGAAACGAAAAATTTTACACCTGCTGCGACGAGCCGTACCTGGATATTACATTTAATATAAC
buck A4.1 A.....G.....AC.....
buck A4.2 .....AC.....G.....
buck A4.3 .....AC.....
buck A4.7 .....AC.....
buck A4.8 .....AC.....
cauc A4 .....AC.....
810 820 830 840 850 860 870 880 890 900
Amel A4 AATGCGAAGAAAAACACTTTTCTACACTGTGAACATAATTATTCCGTGTATGGGAATTTCCTTTCTTACGGTACTGACGTTTTATCTACCTAGCGACAGC
buck A4.1 .....
buck A4.2 .....
buck A4.3 .....
buck A4.7 .....G....
buck A4.8 .....
cauc A4 .....

```
                910       920       930       940       950       960       970       980       990       1000
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A4    GGGGAAAAGGTCACGCTCTCCATTTCCATTCTCATCAGTCTTCACGTGTTTTTCCTGCTGGTCGTCGAGATCATTCCACCTACGTCGCTGGTCGTGCCGC
buck A4.1  ..................----------------------------------------------------------------------------
buck A4.2  ..................----------------------------------------------------------------------------
buck A4.3  ..................----------------------------------------------------------------------------
buck A4.7  ..................----------------------------------------------------------------------------
buck A4.8  ..................----------------------------------------------------------------------------
cauc A4    .....----------------------------------------------------------------------------------------
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```
                 10        20        30        40        50        60        70        80        90        100
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A5    GGACAGGCGGTCTCGCGTTTAAGTGGTCCATCAATTCCATGTCGCCTTTGGTCCTGTTCTTTCATTACGGAGTTTTGGCCATCATTTTCGGGAACGGTTT
buck A5    ----------------------------------------.....C.....................................................
cauc A5    ----------------------------------------------------------------------------------------------------
cauc A5.2  ------------------------------------------------------.......G.....................................

                 110       120       130       140       150       160       170       180       190       200
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A5    TGGGGATGAACACGAATATAGATTGACGAAATATCTGCTCGATGGATACGACGCCGGTGTACGTCCAGCCGAGAATTCCTCCCAACCCCTGGCGGTCGTC
buck A5    ....................................................................................................
cauc A5    ----------------------------------------------------------------------------------------------------
cauc A5.2  ....................................................................................................

                 210       220       230       240       250       260       270       280       290       300
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A5    TTTGGCCTTTCTCTTCATCACATAATCGACGTGGACGAGAAGAATCAGATACTGACGACGAATTGCTGGGTGACACAGATTTGGACGGACCATCACCTGA
buck A5    ....................................................................................................
cauc A5    --------------------------------....................................................................
cauc A5.2  ....................................................................................................

                 310       320       330       340       350       360       370       380       390       400
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A5    AATGGAACGCCTCGGAGTTCGCGGGGATCCGAGTGATTCGTGTTCCTTACAATCGCGTCTGGAGGCCGGACACGATCCTCTACAACAACGCCGATCCACA
buck A5    ....................................................................................................
cauc A5    ....................................................................................................
cauc A5.2  ....................................................................................................
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                410       420       430       440       450       460       470       480       490       500
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A5    GTACAGCTCGGCAGTCATAAATACGAACGTGATAGTGAGTCACACGGGGGAGGTGGTGTGGTTGAGCCATGGGATATTTCGCAGCAGCTGCGACATCGAC
buck A5    ..................................................................................................
cauc A5    ..................................................................................................
cauc A5.2  ..........................................................A.......................................

                510       520       530       540       550       560       570       580       590       600
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A5    GTCGAGTTCTTCCCCTTCGACGAGCAACGCTGTGTGTTGAAATGGGCCTCCTGGACGTACGATGGATACCAATTGGAGTTGGAGAAGCAAAGCGAGCAAG
buck A5    ..................................................................................................
cauc A5    ..................................................................................................
cauc A5.2  ..................................................................................................

                610       620       630       640       650       660       670       680       690       700
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A5    GAGACGTGAGCAATTATCAAGCGAACGGTGAATTCGACTTGGTCAATTTCTCCGCGAGAAGGAACGTGGAGTATTACTCCTGCTGCCCGGAACCGTATCC
buck A5    ....................................C.....................................T.....................
cauc A5    ....................................C.....................................T.....................
cauc A5.2  ....................................C.....................................................

                710       720       730       740       750       760       770       780       790       800
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A5    GGACATCACTTACGAGATACGATTGCGACGTCGACCGATGTTTTACGTCTTCAATCTGATACTTCCATGCATACTCATCAACAGTGTCGCCCTGTTGGTA
buck A5    ..................................................................................................
cauc A5    T.....................................C.....................................--------------------
cauc A5.2  T.....................................C.....................................................

                810       820       830       840       850       860       870       880       890       900
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A5    TTCTACGTGCCGTCTGAATCGGGGGAAAAAGTCACCCTCGGGATTTCGGCCCTCCTCTCTATGACGGTCTTCCTAATGACTATTCGCGAGTCACTGCCGC
buck A5    ..........................................................----------------------------------------------
cauc A5    --------------------------------------------------------------------------------------------------
cauc A5.2  ........................----------------------------------------------------------------------------
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```
            10        20        30        40        50        60        70        80        90       100
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A6    ATGCGCGCAAGTAGTGTATTACAAGCAGAGAGTGATGTGTCATCGTGCGTTATTTTTGGTGTATTATTTGTGTTATTTTCCTTTCTGAGGACACGTACGA
buck A6    ----------------------------------------------------------------------------------------------------

            110       120       130       140       150       160       170       180       190       200
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A6    AGCTCCAACCTACTTATTTTCATCATACATACATCATATATGAAAGCCTGTGCGGACGTCACGAGAAACGTTTGTTGAACGAGCTGTTGTCGTCATACAA
buck A6    ----------------------------------------------------------------------------------------------------

            210       220       230       240       250       260       270       280       290       300
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A6    CACTCTGGAGCGGCCCGTCGCCAATGAGAGCGAGCCTCTCGAAGTGAAATTTGGCATCACGCTACAGCAGATCATAGATGTGGATGAGAAGAATCAAATA
buck A6    ----------------------------------------------------------------------------------------------------

            310       320       330       340       350       360       370       380       390       400
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A6    TTAACTACGAACGCGTGGCTCAAGTTGGAATGGGTGGACTACAACCTCCAATGGAACGAGTCCGAGTACGGAGGTGTAAAGGACCTTCGAATTACACCAA
buck A6    ----------------------------------------------------TGT.T.A.A....GA...TGA.GTGTC.T.G.-.CG...TTTTTG

            410       420       430       440       450       460       470       480       490       500
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A6    ACAAGCTCTGGAAGCCAGACATTCTCATGTACAACAGTGCGGATGAGGGTTTCGACGGGACATACCAAACAAACGTGGTAGTCACGCATAACGGCAGTTG
buck A6    GTGTAT.A.TTGT.TT.----..T..C.T.CTG.GGACA..T.C..A.C.CCAAC.TATTT..TTTC.T..T..A.AC--A...TAT..GGAATGG..G.

            510       520       530       540       550       560       570       580       590       600
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A6    CCTGTACGTTCCTCCGGGCATCTTCAAGAGCACTTGCAAGATAGACATCACTTGGTTCCCCTTTGACGACCAACACTGTGACATGAAGTTCGGATCCTGG
buck A6    A..AC.ACC...AAT..A-------.C...TC.GA.T.C.G-..GTG.A.---A.GA..TT-----...ATT....C---.A.CA.GC.CT...AG.CA.

            610       620       630       640       650       660       670       680       690       700
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A6    ACCTACGACGGCA-ACCAGGTCGACCTCGTGCTCAGCTCGGAGACGGGCGGTGACCTGTCCGACTTTATCACGAATGGAGAATGGTACCTGATCGGAATG
buck A6    ..A.T.TCAT.T.C.A.....................................................................................

            710       720       730       740       750       760       770       780       790       800
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A6    CCGGGCAAGAAGAACACGATAACGTACCAATGCTGTCCGGAGCCTTACGTCGACGTCACATTCACCATACAGATCCGTAGAAGGACCCTCTACTATTTTT
buck A6    .....................................................................................................
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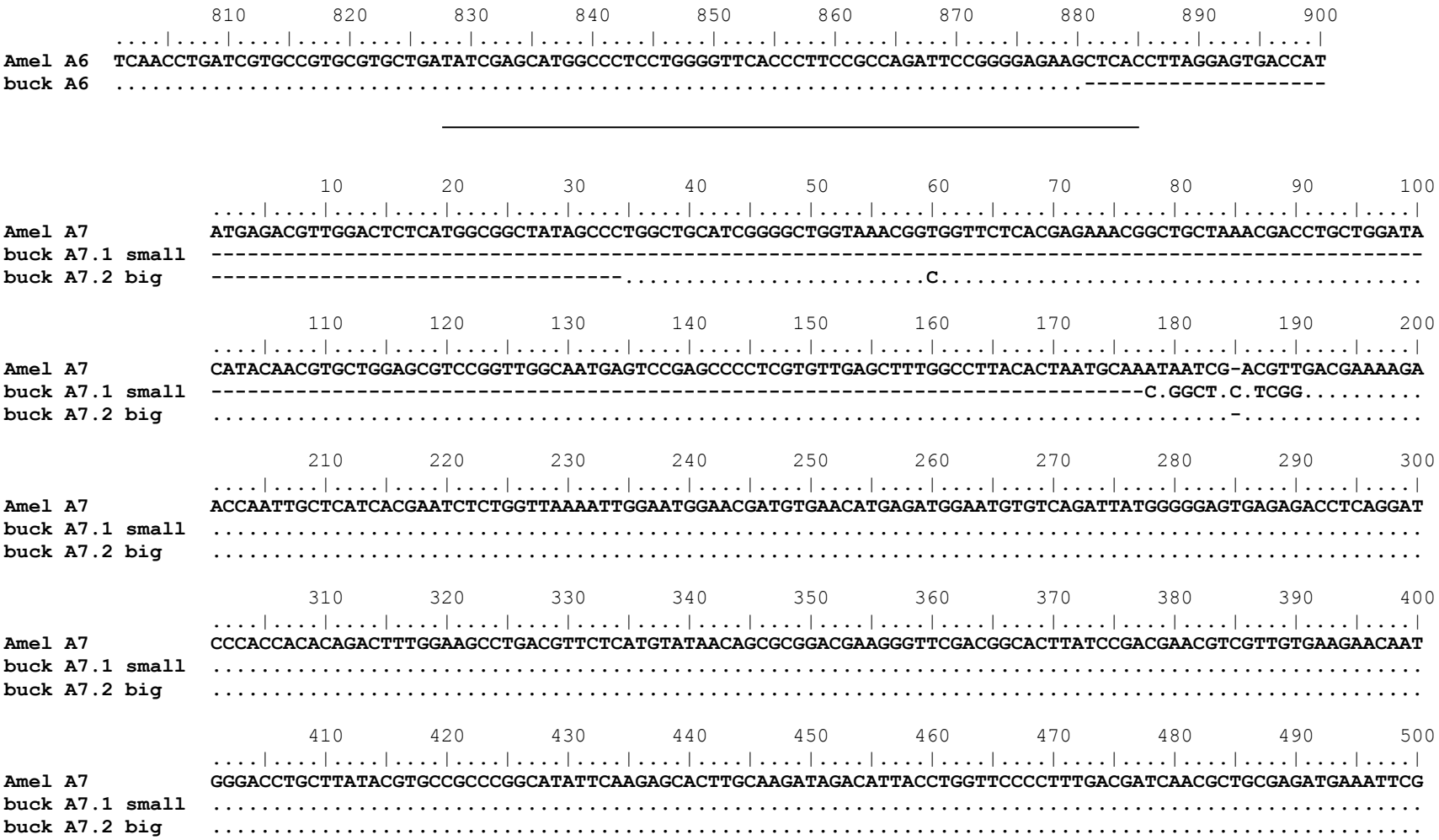
810 820 830 840 850 860 870 880 890 900
Amel A6 TCAACCTGATCGTGCCGTGCGTGCTGATATCGAGCATGGCCCTCCTGGGGTTCACCCTTCCGCCAGATTCCGGGGAGAAGCTCACCTTAGGAGTGACCAT
buck A6 ----------
10 20 30 40 50 60 70 80 90 100
Amel A7 ATGAGACGTTGGACTCTCATGGCGGCTATAGCCCTGGCTGCATCGGGGCTGGTAAACGGTGGTTCTCACGAGAAACGGCTGCTAAACGACCTGCTGGATA
buck A7.1 small ----------
buck A7.2 big ----------.....C.....
110 120 130 140 150 160 170 180 190 200
Amel A7 CATACAACGTGCTGGAGCGTCCGGTTGGCAATGAGTCCGAGCCCCTCGTGTTGAGCTTTGGCCTTACACTAATGCAAATAATCG-ACGTTGACGAAAAGA
buck A7.1 small ----------C.GGCT.C.TCGG.....
buck A7.2 big .....-.....
210 220 230 240 250 260 270 280 290 300
Amel A7 ACCAATTGCTCATCACGAATCTCTGGTTAAAATTGGAATGGAACGATGTGAACATGAGATGGAATGTGTCAGATTATGGGGGAGTGAGAGACCTCAGGAT
buck A7.1 small .....
buck A7.2 big .....
310 320 330 340 350 360 370 380 390 400
Amel A7 CCCACCACACAGACTTTGGAAGCCTGACGTTCTCATGTATAACAGCGCGGACGAAGGGTTCGACGGCACTTATCCGACGAACGTCGTTGTGAAGAACAAT
buck A7.1 small .....
buck A7.2 big .....
410 420 430 440 450 460 470 480 490 500
Amel A7 GGGACCTGCTTATACGTGCCGCCCGGCATATTCAAGAGCACTTGCAAGATAGACATTACCTGGTTCCCCTTTGACGATCAACGCTGCGAGATGAAATTCG
buck A7.1 small .....
buck A7.2 big .....

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                    510       520       530       540       550       560       570       580       590       600
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A7             GCTCCTGGACGTACGACGGCTTTCAGTTGGACCTGCAACTGCAAGACGAAGCGGGAGGTGACATCAGCAGTTTCATCACTAACGGCGAGTGGGATCTGTT
buck A7.1 small     ..............................................................................................................
buck A7.2 big      ..............................................................................................................

                    610       620       630       640       650       660       670       680       690       700
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A7             GGGGGTGCCTGGTAAAAGAAACGAAATTTATTACAATTGCTGCCCAGAACCGTATATAGATATAACGTTCGTGGTGATCATCAGAAGGCGAACTCTTTAC
buck A7.1 small     .........C.....................................................................G.........
buck A7.2 big      .........C.....................................................................G.........

                    710       720       730       740       750       760       770       780       790       800
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A7             TATTTCTTCAACCTGATCGTGCCGTGTGTCCTGATTGCCAGCATGGCCGTTCTGGGATTCACCTTGCCACCCGATTCCGGCGAGAAGCTATCTCTAGGGG
buck A7.1 small     ..............................................................................------
buck A7.2 big      ..............................................................................................................

                    810       820       830       840       850       860       870       880       890       900
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A7             TAACCATCCTCTTGTCCCTCACTGTGTTCCTGAATATGGTGGCCGAGACAATGCCAGCGACTTCGGACGCCGTGCCTCTGCTGGGGACGTACTTCAACTG
buck A7.1 small     ------------------------------------------------------------------------------------------------------
buck A7.2 big       A.......--------------------------------------------------------------------------------------------
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                     10        20        30        40        50        60        70        80        90       100
                     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A8              TTTATATTTATTAACTTACACTTAAAAATTACTATATAATATTGGCAGATAAACGTATTTTTATTGGGAGTGGTCATTGCCATCTCAACCCTATATAAAT
buck A8              ------------------------------------------------------------------------------------------------------
cauc A8              ------------------------------------------------------------------------------------------------------

                    110       120       130       140       150       160       170       180       190       200
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A8             TTGTAAATAATTATGTTTAAAATGCAAATATTGACGCTTGGTGTTCTTTTTAATACTCTTCATATTATATATACAGTGTTGCTGGATTGAAAATTTTCGAAG
buck A8             ------------------------------.....................................................................
cauc A8             ------------------------------------------------------------------------------------------------------
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                210       220       230       240       250       260       270       280       290       300
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A8    CAAATCCTGACACAAAGAGACTTTATGATGACTTATTATCGAATTATAATAGACTTATACGACCTGTTATGAACAATACTGAAACCTTGACAGTTCAACT
buck A8    ....................................................................................................
cauc A8    ----------------------------------------------------------------------------------------------------.GGTGT.

                310       320       330       340       350       360       370       380       390       400
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A8    CGGTTTAAAATTATCACAATTAATTGAAATGAATTTAAAAAATCAAGTGATGACCACCAATGTCTGGGTAGAACAGAGATGGAATGATTATAAACTAAAA
buck A8    ....................................................................................................
cauc A8    .TT.....T.C.-CT...--..T.AT.T.CA.....................................................................
                410       420       430       440       450       460       470       480       490       500
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A8    TGGAATCCAGAAGAATACGGTGGCGTGGAAATGCTATATGTACCTTCCGAAAATATTTGGTTACCAGATATTGTTCTATATAATAATGCTGACGGTAATT
buck A8    ....................................................................................................
cauc A8    ....................................................................................................

                510       520       530       540       550       560       570       580       590       600
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A8    ATGAAGTGACGCTTATGACAAAAGCTACATTAAAATATACTGGTGATGTATCTTGGAAACCACCTGCAATTTATAAATCATCTTGTGAAATTAATGTAGA
buck A8    ....................................................................................................
cauc A8    ....................................................................................................

                610       620       630       640       650       660       670       680       690       700
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A8    ATATTTCCCATTCGACGAACAATCGTGTATTATGAAATTCGGTTCATGGACTTATAATGGCGCTCAGGTAGATTTAAAACATATGAAACAAGAAGCTGGT
buck A8    ....................................................................................................
cauc A8    ....................................................................................................

                710       720       730       740       750       760       770       780       790       800
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A8    AGCAATTTAGTTGCAAAAGGAATAGATTTAAGCGATTTCTATTTATCAGTAGAATGGGATATTTTAGAAGTACCAGCATCGAGAAATGAAGAATATTATC
buck A8    ....................................................................................................
cauc A8    ....................................................................................................

                810       820       830       840       850       860       870       880       890       900
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A8    CATGTTGCACAGAACCCTATTCTGATATCACTTTTAATATTACAATGCGAAGAAAAACATTATTCTATACGGTTAACTTAATAATTCCTTGCGTGGGTAT
buck A8    ....................................................................................................
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cauc A8  ..........................................................................................
                910       920       930       940       950       960       970       980       990       1000
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel A8  TACATTCCTTACAGTACTTGTATTTTATCTACCAAGTGATTCTGGTGAAAAAGTATCATTATGTTCTTCCATTCTCCTTTCATTGACGGTATTCTTTTTA
buck A8  ..........................................................................T----------------------
cauc A8  ..........................................-----------------------------------------------------
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                                10        20        30        40        50        60        70        80        90        100
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel B1                  GGGGTTGGCCACGGGTGGGACACCGCCTGAAACCTGTCCAAAAAATGCATAATATTTGCTCGAGGCTCGGGCGAATTCTGCTCATCTCCGCCGTTTTCTG
cauc B1  (head)          ----------------------------------------------------------------------------------------------------

                                110       120       130       140       150       160       170       180       190       200
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel B1                  CGTCGGTCTCTGCTCCGAGGATGAGGAAAGATTGGTGCGAGACTTGTTCAGAGGTTACAACAAACTCATTAGACCCGTGCAGAACATGACAGAGAAAGTG
cauc B1  (head)          -.....................................................................................................

                                210       220       230       240       250       260       270       280       290       300
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel B1                  CACGTGAATTTTGGCCTCGCTTTCGTGCAATTGATCAACGTGAACGAGAAAAATCAAATTATGAAGTCGAACGTTTGGCTGAGATTCATCTGGACGGATT
cauc B1  (head)          .....................................................................................................

                                310       320       330       340       350       360       370       380       390       400
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel B1                  ATCAGCTGCAATGGGACGAGGCAGACTATGGCGGTATCGGGGTACTTAGATTACCACCCGACAAAGTATGGAAACCTGACATCGTGTTGTTTAACAACGC
cauc B1  (head)          .....................................................................................................

                                410       420       430       440       450       460       470       480       490       500
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel B1                  GGACGGGAACTACGAGGTGCGATACAAGAGCAACGTGCTCATCTATCCGAACGGCGACGTCCTCTGGGTGCCTCCGGCCATCTACCAGAGTTCCTGCACC
cauc B1  (head)          ..........................................................................A..........................

                                510       520       530       540       550       560       570       580       590       600
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel B1                  ATCGACGTCACGTACTTCCCGTTCGACCAGCAAACCTGCATCATGAAATTCGGCTCCTGGACGTTCAACGGCGACCAAGTGTCCCTGGCTTTGTACAACA
cauc B1  (head)          ...........T........C.....................................................................................
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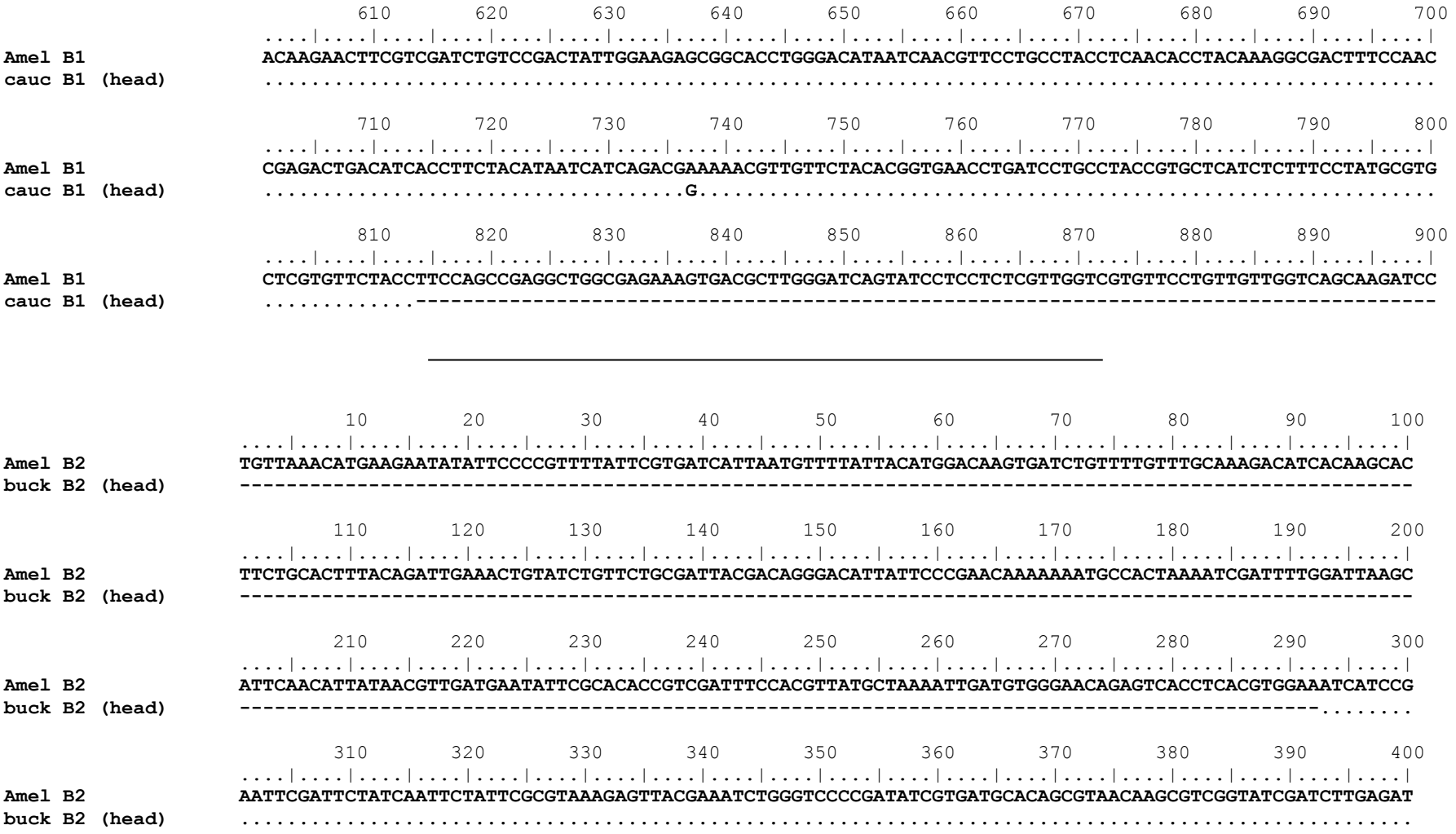
610 620 630 640 650 660 670 680 690 700
Amel B1 ACAAGAACTTCGTCGATCTGTCCGACTATTGGAAGAGCGGCACCTGGGACATAATCAACGTTCCTGCCTACCTCAACACCTACAAAGGCGACTTTCCAAC
cauc B1 (head) .....
710 720 730 740 750 760 770 780 790 800
Amel B1 CGAGACTGACATCACCTTCTACATAATCATCAGACGAAAAACGTTGTTCTACACGGTGAACCTGATCCTGCCTACCGTGCTCATCTCTTTCCTATGCGTG
cauc B1 (head) .....G.....
810 820 830 840 850 860 870 880 890 900
Amel B1 CTCGTGTTCTACCTTCCAGCCGAGGCTGGCGAGAAAGTGACGCTTGGGATCAGTATCCTCCTCTCGTTGGTCGTGTTCCTGTTGTTGGTCAGCAAGATCC
cauc B1 (head) .....----------
10 20 30 40 50 60 70 80 90 100
Amel B2 TGTTAAACATGAAGAATATATTCCCCGTTTTATTCGTGATCATTAATGTTTTATTACATGGACAAGTGATCTGTTTTGTTTGCAAAGACATCACAAGCAC
buck B2 (head) ----------
110 120 130 140 150 160 170 180 190 200
Amel B2 TTCTGCACTTTACAGATTGAAACTGTATCTGTTCTGCGATTACGACAGGGACATTATTCCCGAACAAAAAAATGCCACTAAAATCGATTTTGGATTAAGC
buck B2 (head) ----------
210 220 230 240 250 260 270 280 290 300
Amel B2 ATTCAACATTATAACGTTGATGAATATTCGCACACCGTCGATTTCCACGTTATGCTAAAATTGATGTGGGAACAGAGTCACCTCACGTGGAAATCATCCG
buck B2 (head) ----------.....
310 320 330 340 350 360 370 380 390 400
Amel B2 AATTCGATTCTATCAATTCTATTCGCGTAAAGAGTTACGAAATCTGGGTCCCCGATATCGTGATGCACAGCGTAACAAGCGTCGGTATCGATCTTGAGAT
buck B2 (head) .....

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                         410       420       430       440       450       460       470       480       490       500
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel B2             GCCCTCTGTCGAATGCATAGTGTTCAATTCTGGCACTATACTTTGCGTACCGTTCACTACGTATACACCTGTTTGCGAATATGATCACACTTGGTGGCCT
buck B2 (head)      ..................................................................................................

                         510       520       530       540       550       560       570       580       590       600
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel B2             TACGATATATTGAACTGCACTATACACATTGCCTCGTGGTCCCACGGAAGCAACGAGATTAAATTAAACTCTTTGGATACTGAACAAATTTTAGATGATA
buck B2 (head)      ..................................................................................................

                         610       620       630       640       650       660       670       680       690       700
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel B2             TGTACAATAATAACACGGAATGGGAGATAGTGCATATGTCTCACAGCGAGAGCACGATAGATTCCAAATTTGGTTTAGGTTTTACCACTGATTTGTTATC
buck B2 (head)      ..................................................................................................

                         710       720       730       740       750       760       770       780       790       800
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel B2             TTATAATATTCTGCTGAGAAGACATTACTCCATGAACAGTACGACGTATGTGACTTTGACCATAGTATTAATGACCATGACATTGATGACATTATGGTTA
buck B2 (head)      ..................................................................................................

                         810       820       830       840       850       860       870       880       890       900
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel B2             GAGCCAAGTTCTACGGAGCGTATGATCATAGCAAATCTGAATTTTATTCTGCATCTATTTTGCTTGTTAGATGTGCAATGGAGGATTCCTTTCAATGGAA
buck B2 (head)      ..................................................................................................

                         910       920       930       940       950       960       970       980       990       1000
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Amel B2             TTCAAATGCCAAACTTGATGGTGTTCTATGAGAAATCTCTTGCCCTAGCCGCATTCTCGCTTATGTTGACAAGCATCTTGCGATATTTGCAAGAATTACA
buck B2 (head)      ..................................................-------------------------------------------------
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9.6 Results from the extra sum of squares test; thiacloprid against forager and nurse bees from *A. m. ligustica*.

	Foragers	Nurse	Global (shared)
Comparison of Fits			
Null hypothesis			2 parameters same for all data sets
Alternative hypothesis			2 parameters different for each data set
P value			< 0.0001
Conclusion (alpha = 0.05)			Reject null hypothesis
Preferred model			2 parameters different for each data set
F (DFn, DFd)			32.49 (2,46)
2 parameters different for each data set			
Best-fit values			
Bottom	= 0.0	= 0.0	
Top	= 100.0	= 100.0	
LogIC50	-0.2642	1.770	
HillSlope	0.3543	0.5125	
IC50	0.5442	58.88	
Span	= 100.0	= 100.0	
Std. Error			
LogIC50	0.2621	0.2817	
HillSlope	0.08898	0.1936	
95% Confidence Intervals			
LogIC50	-0.8064 to 0.2780	1.187 to 2.353	
HillSlope	0.1702 to 0.5384	0.1119 to 0.9130	
IC50	0.1562 to 1.897	15.39 to 225.3	
Goodness of Fit			
Degrees of Freedom	23	23	
R square	0.4538	0.3240	
Absolute Sum of Squares	4293	11493	
Sy.x	13.66	22.35	
Constraints			
Bottom	Bottom = 0.0	Bottom = 0.0	
Top	Top = 100.0	Top = 100.0	
2 parameters same for all data sets			
Best-fit values			
Bottom	= 0.0	= 0.0	
Top	= 100.0	= 100.0	
LogIC50	0.9105	0.9105	0.9105
HillSlope	0.3421	0.3421	0.3421
IC50	8.138	8.138	8.138
Span	= 100.0	= 100.0	
Std. Error			
LogIC50	0.2179	0.2179	0.2179
HillSlope	0.1117	0.1117	0.1117
95% Confidence Intervals			
LogIC50	0.4719 to 1.349	0.4719 to 1.349	0.4719 to 1.349
HillSlope	0.1173 to 0.5669	0.1173 to 0.5669	0.1173 to 0.5669
IC50	2.964 to 22.34	2.964 to 22.34	2.964 to 22.34
Goodness of Fit			
Degrees of Freedom			48
R square	-0.9664	-0.3310	0.1885
Absolute Sum of Squares	15455	22629	38084
Sy.x			28.17
Constraints			
Bottom	Bottom = 0.0	Bottom = 0.0	
Top	Top = 100.0	Top = 100.0	
LogIC50	LogIC50 is shared	LogIC50 is shared	
HillSlope	HillSlope is shared	HillSlope is shared	
Number of points			
Analyzed	25	25	

9.7 Unpaired t-test results for haem content of 3 different honey bee races

Table Analyzed	Data 1
Column B	Caucasian
vs.	vs.
Column A	Buckfast
Unpaired t test	
P value	0.0451
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.434 df=7
How big is the difference?	
Mean ± SEM of column A	0.0986 ± 0.01703 N=5
Mean ± SEM of column B	0.1905 ± 0.03693 N=4
Difference between means	0.0919 ± 0.03775
95% confidence interval	0.002629 to 0.1812
R squared	0.4584
F test to compare variances	
F,DFn, Dfd	3.762, 3, 4
P value	0.2331
P value summary	ns
Significantly different? (P < 0.05)	No
Column C	Italian
vs.	vs.
Column A	Buckfast
Unpaired t test	
P value	0.0522
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=2.278 df=8
How big is the difference?	
Mean ± SEM of column A	0.0986 ± 0.01703 N=5
Mean ± SEM of column C	0.0554 ± 0.008334 N=5
Difference between means	-0.0432 ± 0.01896
95% confidence interval	-0.08692 to 0.0005241
R squared	0.3935
F test to compare variances	
F,DFn, Dfd	4.176, 4, 4
P value	0.1951
P value summary	ns
Significantly different? (P < 0.05)	No
Column C	Italian
vs.	vs.
Column B	Caucasian
Unpaired t test	
P value	0.0052
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.999 df=7
How big is the difference?	
Mean ± SEM of column B	0.1905 ± 0.03693 N=4
Mean ± SEM of column C	0.0554 ± 0.008334 N=5
Difference between means	-0.1351 ± 0.03379
95% confidence interval	-0.2150 to -0.05520
R squared	0.6955
F test to compare variances	
F,DFn, Dfd	15.71, 3, 4
P value	0.0223
P value summary	*
Significantly different? (P < 0.05)	Yes