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Insecticidal and synergistic properties of *Piper nigrum* seed extracts investigated using acute toxicity assays and gene expression profiling of *Drosophila melanogaster*

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Insecticidal and synergistic properties of *Piper nigrum* seed extracts investigated using acute toxicity assays and gene expression profiling of *Drosophila melanogaster*

Helen Rose Jensen

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Dedication

To Jérôme-Antoine

and

To my ever cheerful and inspired Ferme Maraîchère Ways Mills team, Tony Scott and Natalie Raiche, in hopes that this may help cut down the workload at Salon Chez Fenouille ☺

Abstract

The overall objective of this study was to investigate the insecticidal and synergistic properties of an ethylacetate extract of *Piper nigrum* L. (Piperaceae) seeds used alone and in conjunction with pyrethrin, a botanical insecticide extracted from the flowers of *Chrysanthemum cinerariaefolium* Benth. & Hook. (Asteraceae). There were three specific objectives pursued in this research. (1) Test whether a *P. nigrum* extract is an effective synergist when used in conjunction with pyrethrin against two test insects, *Musca domestica* L. (Diptera: Muscidae) and *Drosophila melanogaster* (Diptera: Drosophilidae). (2) Determine the effect of an insecticidal dose of a *P. nigrum* extract upon gene induction and suppression in the model insect *Drosophila melanogaster* in order to obtain novel information concerning the insect response to a *P. nigrum* extract when it is used as an insecticide. (3) Determine the effects of pyrethrin, a *P. nigrum* extract and a synergistic combination of pyrethrin and a *P. nigrum* extract upon gene induction and suppression in *Drosophila melanogaster* in order to obtain information concerning the insect response to the use of a *P. nigrum* extract as a pyrethrin synergist compared to the response to either a *P. nigrum* extract or pyrethrin used alone.

The use of a *P. nigrum* extract as a synergist for pyrethrin resulted in high synergist ratios of 13.9 for *M. domestica* and 11.6 for *D. melanogaster*. The treatment of *D. melanogaster* adult females with an insecticidal concentration of a *P. nigrum* extract led to the upregulation of the mRNA encoding six cytochrome P450 genes related to phase I metabolism and two glutathione-S-transferase genes related to phase II metabolism. The treatment of *D. melanogaster* with sublethal concentrations of a *P. nigrum* extract, pyrethrin and pyrethrin plus a *P. nigrum* extract resulted in the differential expression of a group of seven genes common to at least two of the three treatments

including *l(2)efl,* which is associated with the defense response and CG13091 with oxidoreductase activity.

It was concluded that *P. nigrum* extract is a highly promising candidate for a novel botanical synergist for pyrethrin and could potentially be used as a replacement for piperonyl butoxide in certain pyrethrum formulations. The upregulation of mRNA transcripts encoding cytochrome P450 detoxification enzymes by a *P. nigrum* extract may indicate possible target proteins to enhance the toxicity of insecticides and synergists. The seven common genes differentially expressed in two or three of the sublethal treatments with pyrethrin, *P. nigrum* extract or pyrethrin plus *P. nigrum* extract merit further study of their functions relating to detoxification processes and defense responses. Future work should include a detailed, tissue-specific characterization of the genes of interest identified in this study and an investigation of the activity of the *P. nigrum* extract against the CYP enzymes associated with the *Cyp* genes that were differentially expressed in response to a *P. nigrum* extract.

Résumé

L'objectif de cette étude était d'investiguer les propriétés insecticides et synergiques d'un extrait d'acétate d'éthyle des graines de *Piper nigrum* L. (Piperaceae) utilisé seul et en conjonction avec le pyrèthre, un insecticide botanique extrait des fleurs de *Chrysanthemum cinerariaefolium* Benth. & Hook. (Asteraceae). Trois objectifs spécifiques ont été poursuivis. (1) Évaluer le potentiel d'un extrait de *P. nigrum* en tant que synergiste pour le pyrèthre contre deux insectes : *Musca domestica* L. (Diptera : Muscidae) et *Drosophila melanogaster* (Diptera : Drosophilidae). (2) Déterminer l'effet d'une concentration insecticide d'un extrait de *P. nigrum* sur l'expression différentielle des gènes de *D. melanogaster* pour obtenir des informations nouvelles concernant la réaction physiologique de l'insecte à un extrait de *P. nigrum* lorsqu'il est utilisé en tant qu'insecticide. (3) Déterminer les effets du pyrèthre, d'un extrait de *P. nigrum* et d'un mélange de pyrèthre et d'un extrait de *P. nigrum* sur l'expression différentielle des gènes de *D. melanogaster* afin d'évaluer l'effet synergique d'un extrait de *P. nigrum* sur le pyrèthre comparé à l'utilisation d'un extrait de *P. nigrum* ou du pyrèthre employés seuls.

L'utilisation d'un extrait de *P. nigrum* en tant que synergiste pour le pyrèthre a donné comme résultat une hausse de la toxicité du pyrèthre par un facteur de 13,9 pour *M. domestica* et par un facteur de 11,6 pour *D. melanogaster*. Des femelles adultes de *D. melanogaster* traitées avec une concentration insecticide d'un extrait de *P. nigrum* ont démontré une hausse des niveaux d'ARNm codant pour les enzymes de détoxification, en particulier six cytochromes P450 intervenant dans la phase I du métabolisme et deux glutathion-S-transférases intervenant dans la phase II du métabolisme. Les traitements de *D. melanogaster* avec des concentrations sub-léthales d'un extrait de *P. nigrum*, de pyrèthre et d'un mélange de pyrèthre et d'un extrait de *P. nigrum* ont provoqué une expression différentielle d'un groupe de sept gènes communs à au moins deux des trois

traitements. Il y a eu une hausse des niveaux de ARNm codant pour le gène *l(2)efl* qui est associé à la défense et pour le gène CG13091 impliqué dans l'oxydoréduction.

En conclusion, l'extrait de *P. nigrum* possède un haut potentiel en tant que nouveau synergiste botanique pour le pyrèthre et pourrait remplacer le pipéronyle butoxyde dans certaines formulations de pyrèthre. La hausse des niveaux d'ARNm reliés aux enzymes de détoxification cytochrome P450 pourrait indiquer des protéines cibles pour augmenter la toxicité des insecticides et des synergistes. Les sept gènes exprimés à des niveaux différents dans deux ou trois des traitements sub-léthales avec le pyrèthre, l'extrait de *P. nigrum* ou le mélange de pyrèthre et de l'extrait de *P. nigrum* mériteraient des études complémentaires concernant leurs fonctions dans les processus de détoxification et de défense. Les perspectives pour les travaux futurs incluent la caractérisation détaillée des gènes d'intérêt identifiés dans cette étude au niveau des différents tissus de l'insecte et une étude de l'activité de l'extrait de *P. nigrum* contre les enzymes des cytochromes P450 associés aux gènes des cytochromes P450 qui ont présenté des variations de leur niveau d'expression en présence de l'extrait de *P. nigrum*.

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List of Abbreviations

bp	base pair
CDSSF	Core DNA Sequencing and Synthesizing Facility
CDMC	Canadian Drosophila Microarray Center
CMRS	Canadian Molecular Research Services
Cyp	Cytochrome P450 gene
CYP	Cytochrome P450 protein
DEPC	diethyl pyrocarbonate
cDNA	complimentary deoxyribonucleic acid
EST	expressed sequence tag
EtOH	ethanol
GITC	guanidine thiocyanate
GMO	genetically modified organism
HPLC	high performance liquid chromatography
IPM	integrated pest management
Kb	Kilobase (equivalent to 1000 bp)
KPa	Kilopascal
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PSI	pounds per square inch
PSMO	polysubstrate monooxygenase
QPCR	quantitative polymerase chain reaction
RCF	relative centrifugal force
RT-QPCR	reverse transriptase quantitative polymerase chain reaction
SSC	standard sodium citrate

Notes

(1) Please note that the term "*Piper nigrum*" employed with regards to the experiments conducted in this thesis refers to an ethylacetate extract of the seeds of the *P. nigrum* plant.

Chapter 1: General Introduction

1.1 Introduction

It has been suggested that the relationship between humans and domestic plants is an example of co-evolution with the plants evolving towards reduced defense by secondary metabolites in favour of more vigorous growth and reproduction. In exchange, humans assure the defense of the plant by controlling competing plant species and herbivorous insects in a relationship similar to the mutualism between ants and acacia trees (Rindos, 1984). The development of agriculture may have been a means to assure a measure of control over the availability and yield of staple foods during a period of rapid expansion of the human population (Cohen, 1977). Currently, with an ever increasing human population, societies are yet again faced with the need to produce large quantities of food.

Numerous arthropod species are major competitors for human food resources. Over the course of the past few centuries agriculture has become increasingly industrialized and the monoculture systems that are widely used favor the proliferation of pest insects. The globalization of trade and travel has also increased the rate of introduction of exotic insect species with no natural enemies. The major solution to insect problems in the past 70 years has been the use of synthetic insecticides. However, the indiscriminate application of these products has led to insect resistance and contamination of the food chain. More recently, genetically modified organisms (GMOs) have been proposed as a means to combat insect pest problems while reducing the use of insecticides. They have been met with conflicting opinions in the scientific community concerning their safety. The general public has also been reluctant to embrace this technology.

A pest control solution which should be re-considered is the integrated pest management (IPM) approach. In IPM there is an attempt to reduce the use of insecticides through good cropping practices. When insecticide use is deemed necessary, products derived from natural sources are preferred due to their reduced residence time in the environment. Botanical insecticides are particularly promising due to the diversity of insecticidal secondary metabolites synthesized by plants. In this study the insecticidal neotropical plant *Piper nigrum* L. (Piperaceae) is studied with regards to its potential as a novel synergist for the botanical insecticide pyrethrin.

1.2 Literature review

1.2.1 Insect defenses

Herbivorous insects pose a considerable threat to plant survival and create intense selection pressure for the evolution of secondary metabolites with toxic or repellent characteristics. This has led to a wide radiation of plant and insect species with complex attack and counterattack mechanisms for the prevention of herbivory on the one hand, and the conquest of plant defenses on the other (Ehrlich and Raven, 1964). While plants have evolved an extensive pharmacopia of secondary metabolites with a broad spectrum of biological activities, insects have responded with many specialized enzymes for the detoxification of these phytochemicals. Currently, the most widely studied of these enzymes are the cytochrome P450 monooxygenases (CYP) and the glutathione-S-transferases.

The CYP enzymes are a large class of hemoproteins with the capacity to bind and metabolize a wide variety of substrates, including plant toxins and xenobiotics (Lewis, 2001). CYP enzymes have evolved from their original function of detoxifying molecular oxygen approximately 1400 million years ago (Lewis, 2001) to their current multitude of functions involving endogenous and exogenous metabolism. Located in the

smooth endoplasmic reticulum of insect cells, these enzymes are particularly concentrated in the gut and the fat body (Petersen *et al.*, 2001). A common characteristic of all CYP enzymes is a conserved sequence of amino acids (FXXGXXXCXG) which forms a heme-binding decapeptide (Danielson *et al.*, 1997). The CYP enzymes that are implicated in the elimination of toxins act in phase I metabolism by oxidoreduction of lipophilic compounds in order to facilitate their elimination (Lewis, 2001). In *Drosophila melanogaster* there are an estimated 90 CYP enzymes belonging to numerous families including CYP 4,6,9,12,15,18 and 28 (Scott *et al.*, 1998). Currently, all CYP 6 enzymes are exclusive to insects and are often implicated in insecticide metabolism (Tijet *et al.*, 2001). Many cases of evolved insecticide resistance are attributed to selection pressure for the overexpression of specific CYP enzymes (Brandt *et al.*, 2002). In *Drosophila, Cyp6g1* is over-expressed in field collected DDT resistant strains, a trait that also confers resistance to the neonicotinoid imidacloprid, an entirely different class of insecticide (Daborn *et al.*, 2001). This hints at the broad base of substrate binding capacity possessed by some P450 enzymes. Once this type of resistance to one insecticide has evolved, cross-resistance to others is therefore likely to occur.

The glutathione-S-transferases are a family of enzymes found in all eukaryotes. They play a major role in phase II metabolism by catalyzing the covalent linkage of glutathione to a large number of lipophilic substrates including products of endogenous metabolism, xenobiotics and plant toxins (Syvanen *et al.*, 1994) and thereby facilitating elimination. Insect glutathione-S-transferases have classically been implicated in insecticide resistance to organophosphates and organochlorines (Ku *et al.*, 1994, Cochrane *et al.*, 1992). Pyrethroid resistance has been linked to increased expression of specific glutathione S-transferases such as *nlgst1-1* in the rice brown planthopper *Nilaparvata lugens* (Vontas *et al.*, 2002). Induction of glutathione-S-transferase activity

accompanies the treatment of *Tenebrio molitor* with decamethrin (Kostaropoulos *et al.*, 2001). Induction of glutathione-S-transferase activity has also been noted to occur in *Drosophila melanogaster* exposed to *trans*- and *cis*-stilbene oxide as substrates. It is apparent that glutathione-S-transferase is important in detoxification processes both in terms of evolved constitutive overexpression and also in short-term inducible responses. Recent studies have suggested a role of these enzymes in insect defense against oxidative stress. In *Drosophila melanogaster* it has been reported that DmGSTS1-1 has a high glutathione-conjugating activity for products of lipid peroxidation such as 4-hydroxynonenal (Singh *et al.*, 2001). Gene expression data has also found the *DmGSTD1* gene to be upregulated in response to increasing age and oxidative stress as measured by treatment with paraquat, a free-radical generating agent (Zou *et al.*, 2000).

Other enzyme families involved in detoxification processes are the ABC transporters and the esterases. ATP-binding cassette (ABC) transporters (also referred to as P-glycoproteins) have been linked to multidrug resistance in mammalian tumor cells (Schinkel and Jonker, 2003) where they function as pumps for the purpose of excreting xenobiotics from cells (Schinkel *et al.*, 1996). ABC-transporters have also been characterized in insects and function as a blood-brain barrier in the tobacco hornworm, (*Manduca sexta*) to protect the central nervous system from nicotine (Murray *et al.*, 1994). Multiple insecticide resistance in the tobacco budworm (*Heliothis virescens*) has been associated with increased expression of p-glycoprotein (Lanning *et al.*, 1996). Resistance to the fungal compound amanitin in certain wild populations of *Drosophila melanogaster* has also been associated with constitutive overexpression of an ABC-transporter gene (Begun and Whitley, 2000). Human P-glycoprotein is inhibited by piperine, one of the most abundant phytochemical constituents of *P. nigrum*.

In addition to metabolism by CYP enzymes, metabolism of many classes of insecticides such as organophosphates, organochlorines and pyrethroids can occur

through the activity of carboxylesterases (Devonshire and Moores, 1982). Metabolism of the pyrethroids by these enzymes occurs through hydrolysis and cleavage of the ester bond (Chang and Whalon, 1986). Pyrethroid resistance in the Colorado potato beetle (*Leptinotarsa decemlineata*) has been associated with an increase in carboxylesterase activity (Argentine *et al.*, 1995, Anspaugh *et al.*, 1995).

1.2.2 Insecticides of plant origin

Considering the variety of molecular mechanisms of insecticide resistance it is essential to implement insect-control policies which aim to reduce the development of resistance in order to reduce the need for greater application volumes. It is also necessary to address current problems with resistant insects. Botanical products show strong potential as a partial solution to these problems due to their inherently synergistic nature stemming from the presence of multiple structurally related secondary metabolites. This phytochemical diversity and redundancy has been found to inhibit the evolution of resistance to neem extract in *Myzus persicae* although resistance to the neem compound azadirachtin can evolve relatively rapidly (Feng and Isman, 1995). A study conducted using the Colorado potato beetle, *Leptinotarsa decemlineata*, found that a *Piper tuberculatum* extract was effective against a strain with resistance to a range of synthetic insecticides including cypermethrin, a pyrethroid (Scott *et al.*, 2003). Therefore, the use of a botanical product may provide an alternative control method that is not affected by the insecticide-resistant genotype and may also help prevent the future evolution of resistance. This study will address two specific botanical insecticides: the natural pyrethrins from *Chrysanthemum cinerariaefolium* Benth. & Hook. (Asteraceae) and *Piper nigrum* L. (Piperaceae) (figure 1.1).

Natural pyrethrin is a widely used botanical insecticide. The six insecticidal pyrethrin esters (pyrethrin I and II, cinerin I and II and jasmolin I and II) are extracted

(a)

(b)

Figure 1.1: (a) *Chrysanthemum cineraeriafolium* Benth. & Hook. (Asteraceae) and (b) *Piper nigrum* L. (Piperaceae). Photos by J. T. Arnason, reproduced with permission.

from the pyrethrum daisy, *Chrysanthemum cinerariaefolium* (figure 1.2). The insecticidal activity of the pyrethrins is characterized by an extremely rapid knockdown in insects. These compounds have a neurotoxic mode of action due to their high affinity for binding site seven of the voltage-gated sodium channel (Zlotkin, 1999). This results in a delayed closing of the sodium channel and a prolonged tail current which causes repetitive neuronal discharge followed by blocking (Soderlund, 1995).

Black pepper or *Piper nigrum* (Piperaceae) is grown commercially as a spice crop. Plants of the genus *Piper* make frequent appearances in ethnobotanical reports. Usage categories for these plants include, among others, use as narcotics (*P. methysticum*), fish poisons (*P. piscatorum*), insecticides (*P. guineense*) and stomach remedies (*P. aduncum*) (McFerren and Rodriguez, 1998, Parmar *et al.*, 1997). Their wide range of biological activities can be attributed to an extremely complex phytochemistry which includes approximately six hundred known compounds including alkaloids, lignans and neolignans (Parmar *et al.*, 1997).

The insecticidal activity of *Piper* plants has been attributed largely to the presence of piperamides (figure 1.3) (Su and Horvat, 1981). Notable among these are piperine, pellitorine, pipercide and guineensine (Miyakado *et al.*, 1985), pipernonaline (Yang *et al.*, 2002) as well as 4,5-dihydropiperlonguminine, piperlonguminine and 4,5-dihydropiperine (Scott *et al.*, 2002). Other amides from *Piper* species have been identified as antifungals (Vasques da Silva *et al.*, 2002, Navickiene *et al.*, 2000). Piperine and pellitorine have insecticidal and knockdown activity towards the American cockroach (*Periplaneta americana*) which has been attributed to an effect on the central nervous system that is characterized by repetitive neuronal discharge in a manner quantitatively similar to the effect of the pyrethrins (Hatakoshi *et al.*, 1984). The isobutylamide piperovatine has been determined to interact with site 2 of the voltage-

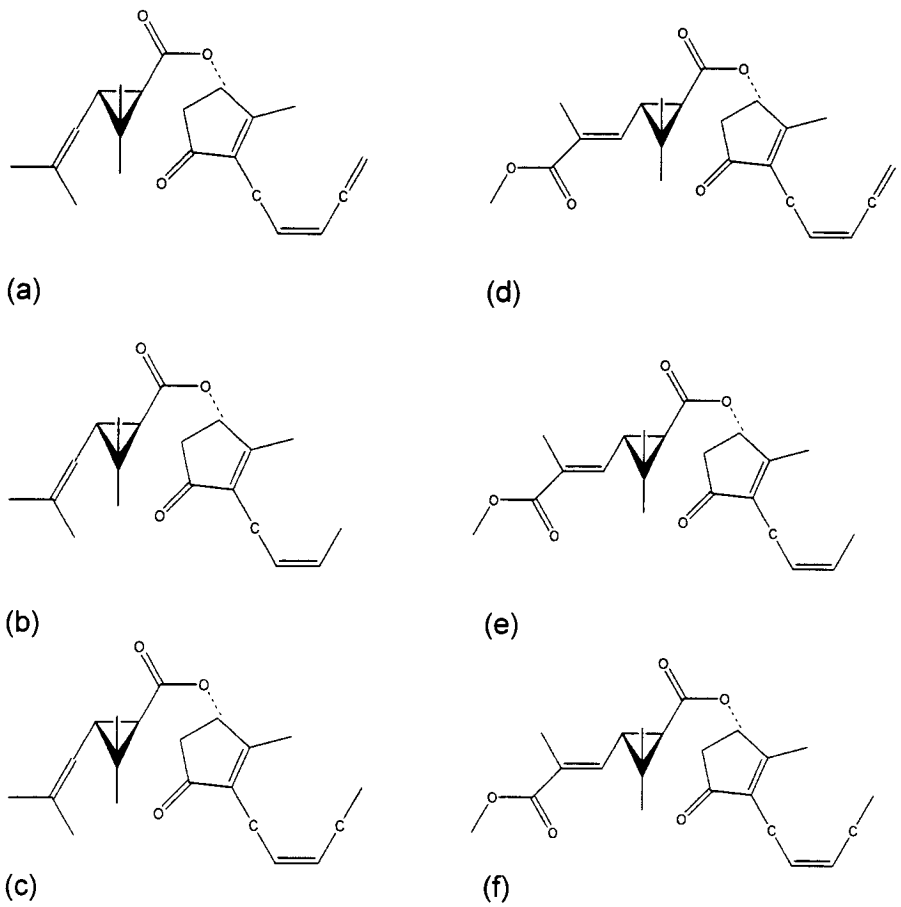

Figure 1.2: Molecular structures of the six natural pyrethrin esters, (a) pyrethrin I, (b) cinerin I, (c) jasmolin I, (d) pyrethrin II, (e) cinerin II and (f) jasmolin II.

(a)

(b)

(c)

(d)

(e)

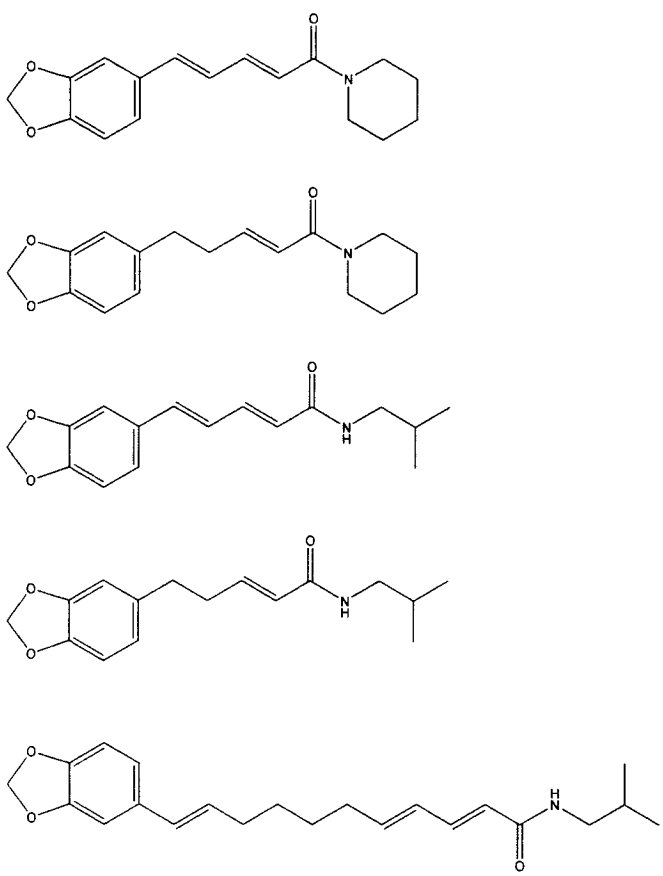

Figure 1.3: Molecular structures of five representative piperamides present in *P. nigrum*, (a) piperine, (b) 4,5-dihydropiperine, (c) piperlonguminine, (d) 4,5-dihydropiperlonguminine and (e) pipercide. Note the presence of the methylenedioxyphenyl functional group on all the molecules and the isobutylamide functional groups on (c), (d) and (e).

gated sodium channel (McFerren *et al.*, 2002). Certain piperamides are bifunctional due to the presence of both an amide moiety and a methylenedioxyphenyl moiety with polysubstrate monooxygenase inhibiting properties (Scott *et al.*, 2003, Bhardwaj *et al.*, 2002). *P. nigrum* is of interest both as an insecticide due to its strong insecticidal activity and also as a synergist due to the polysubstrate monooxygenase activity exhibited by certain piperamides.

1.2.3 Synergy and potentiation

Synergy as discussed in this thesis will be defined in the broad sense according to the work of Gaddum (1985) wherein synergy is said to occur when the combined effect of two or more active agents is greater than the sum of its constituents measured separately. In a more specific definition of the term it can be said that if two active constituents are combined and result in an increased toxicity, this is referred to as potentiation, whereas synergy refers to a situation in which one of the constituents used alone has no toxic effect but results in increased toxicity when administered in conjunction with a toxic product (Hewlett, 1968). In terms of insect control, there are three aspects of synergy which are of interest. These are: (1) the increased toxicity of an insecticide by the addition of a synergist, (2) the overcoming of evolved resistance to an insecticide by the addition of a synergist and, (3) the analogue synergy that frequently exists between the multiple phytochemicals present in a botanical extract.

The formulation of an insecticide with a synergist is a common practice and allows a reduction in application volumes. The natural pyrethrins are generally synergized with a product that inhibits polysubstrate monooxygenase activity such as piperonyl butoxide (PBO) or N-octyl-bicycloheptene dicarboxiimide (MGK-264) (figure 1.4). PSMO inhibition is associated with the presence of a methylenedioxyphenyl (MDP) functional group (Hodgson and Philpot, 1974). The response of cytochrome P450

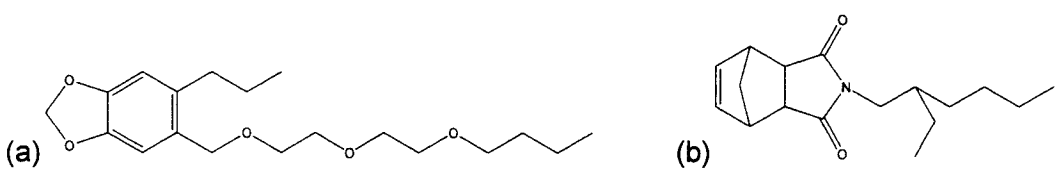

Figure 1.4: Molecular structures of the synthetic synergists (a) piperonyl butoxide (PBO) and (b) N-octyl-bicycloheptene dicarboxiimide (MGK-264).

activity to many MDP synergists such as PBO is biphasic, with an initial suppression followed by induction (Hodgson et al, 1995). Interestingly, piperine has a monophasic effect on cytochrome P450 and results in decreased activity even twenty-four hours after treatment (Dalvi and Dalvi, 1991). Products which inhibit esterase activity (S,S,S-tributyl phosphorotrithioate) and glutathione-S-transferase activity (diethyl malate) also function as insecticide synergists (Ahmad and Hollingworth, 2004). Compounds with a butynyl side chain have also been identified as synergists for permethrin and carbofuran (Pap *et al.*, 2001). The use of a mixture of two different insecticides can also lead to an increase in toxicity referred to as potentiation. This type of mixture generally involves insecticides of different classes such as carbamates and pyrethroids (Corbel *et al.*, 2003). An additional benefit of such synergistic combinations is the effect they may produce in resistant strains of insects.

In cases of evolved insecticide resistance, the addition of a synergist may partially or completely overcome the problem. Generally this approach is successful when resistance is due to the constitutive overexpression of a specific detoxification enzyme which can be inhibited by the synergist although there is a reported case where a resistance which was not linked to increased cytochrome P450 activity was still synergizable by piperonyl butoxide (Kennaugh *et al.*, 1993). The resistance to synthetic pyrethroids among most field populations of the pear psylla (*Cacopsylla pyri L.*) can be greatly reduced using piperonyl butoxide (Buès *et al.*, 2003). In strains of the oriental fruit fly (*Bactrocera dorsalis*) selected for resistance to specific organophosphates, carbamates or pyrethroids it was found that piperonyl butoxide was able to significantly reduce the LD_{50} values for five out of the 10 insecticides tested (Hsu *et al.*, 2004). Studies with PSMO and esterase inhibiting synergists used against pyrethroid-resistance in the German cockroach (*Blatella germanica* L.) have also demonstrated partial success (Cochran, 1994). Combinations of pyrethroid and organophosphate

insecticides show potentiation (Corbel *et al.*, 2002) and are effective against pyrethroid-resistant mosquitoes (*Anopheles gambiae*) (Darriet *et al.*, 2003). It is often possible to overcome insecticide resistance with the use of synergists but not in all cases.

Many plant species are characterized by a certain level of phytochemical redundancy. This has been hypothesized in many cases to be linked to the presence of an analogue synergism between the different secondary metabolites (Berenbaum and Zangerl, 1996). It has been demonstrated in the case of *P. nigrum* that combinations of at least three different insecticidal amides with the MDP functional group are synergistic (Scott *et al.*, 2002). It has also been demonstrated that *Piper* amides from *Piper cenocladum* which do not possess the MDP functional group are also synergistic (Dyer *et al.*, 2003). Synergy has been noted to occur in binary mixtures of feeding deterrents (Akhtar and Isman, 2003) and has been noted to delay the decrease in effectiveness which can occur over time. The natural product dillapiol (present in Indian dill, *Anethum graveolans*) is strongly synergistic when used with pyrethrin in acute toxicity assays (Bhuiyan *et al.*, 2001).

1.2.4 Microarrays past, present and future

A large proportion of the research presented in this thesis focuses on the use of DNA microarray technology for the study of the activity of the botanical insecticide pyrethrin and the synergy between *Piper nigrum* and pyrethrin. The use of DNA microarray methodology as a novel tool for investigating gene expression in a variety of organisms is growing in importance. Microarray technology has become increasingly accessible and sophisticated but the analysis stage of microarray experiments is still problematic. Large data sets are produced and numerous pre-processing steps are necessary to normalize and sometimes re-scale the data prior to analysis (Bowtell and Sambrook, 2003). The complexity of certain experimental designs requires the use of

powerful multivariate statistical procedures in order to obtain answers to the biological questions posed. These problems are currently diminishing due to an extensive literature devoted to these subjects. A growing interest in this field has led to the elaboration of specialized software tools for data storage and analysis. A comprehensive set of guidelines known as MIAME (Minimum Information About a Microarray Experiment) (Brazma *et al.*, 2001) has been established with the objective of providing a common language for microarray researchers. The use of MIAME allows experiments to be clearly defined and facilitates the interpretation of data and comparison of results between different experiments.

The whole genome of *Drosophila melanogaster* has been sequenced, making the organism a good candidate for use as a model insect and a number of commercial oligonucleotide arrays and cDNA arrays are available to *Drosophila* researchers. Previous *Drosophila* microarray experiments have provided novel information concerning physiological processes. A large proportion of research has focused upon cell differentiation and development. Microarrays have been employed to investigate the role of transcriptional repressors in the development of ovarian follicle cells (Cayirlioglu *et al.*, 2003). In a study of tissue specific gene expression during embryogenesis, microarrays were used as a means of corroborating the results of *in situ* hybridization of 2179 genes to fixed *Drosophila* embryos (Tomancak *et al.*, 2002). This is an unusual use of microarrays, which are generally used as a means of exploring a vast subject with the aim of determining genes of interest for further study using RT-PCR, Northern hybridizations and other methodologies characterized by lower volumes of data and superior precision. A more specific study of embryogenesis has examined the genes involved in mesoderm development. Normal mesoderm development is initiated by the *twist* transcription factor and leads to the formation of heart and somatic muscle and many other cell types (Furlong *et al.*, 2001). The authors compared gene

expression in embryos with *twist* loss of function, embryos with ubiquitous *twist* expression and wild type embryos. Hundreds of genes were found to be implicated in mesoderm development (Furlong *et al.*, 2001).

An equally important moment of organized genetic chaos in the life of an insect is metamorphosis, a pivotal stage of the lifecycle which enables the larval insect to become an adult and reproduce. This process involves cellular proliferation, tissue remodeling, cell migration and apoptosis occurring in an orderly and civilized manner under the control of various physiological arbiters including the moulting hormone 20-hydroxyecdysone (White *et al.*, 1999). An ambitious microarray experiment examined patterns of gene expression in *Drosophila* larvae prior to the initial pulse of 20-hydroxyecdysone and throughout the puparium formation and prepupal phases following it. Cluster analysis allowed the allocation of 534 genes to 35 groups with similar expression patterns during metamorphosis (White *et al.*, 1999).

A microarray experiment represents an initial probing of a biological question from which further, hypothesis driven work must follow in order to allow concise conclusions. Additional areas which have been studied include the response of gene expression to starvation (Zinke *et al.*, 2002), the effects of sex, age and genotype upon transcriptional profiles (Jin *et al.*, 2001), the difference between males and females in terms of cytochrome P450 expression (Kasai and Tomita, 2003) and variations in gene transcription in response to circadian rhythms (Etter and Ramaswami, 2002, Lin *et al.*, 2002).

Very few microarray studies have targeted the response of *Drosophila* to an external stressor. The effects of inoculation with Gram negative and Gram positive bacteria (*Escherichia coli* and *Micrococcus luteus*) have been examined (Irving *et al.*, 2001). Maximum expression of antimicrobial peptides was found to occur six hours post-infection regardless of the bacterium in question. In response to bacterial immune

challenge the expression levels of 543 genes (of a total of 13 600 present on the array) changed by a factor of 2 or more (Irving *et al.*, 2001). These results indicate the complexity of the immune response in *Drosophila*. This study confirmed the up-regulation of genes coding for numerous defense and immunity proteins as well as many genes not previously implicated in immune response, producing new leads for study.

The central role of CYP enzymes in insecticide resistance is well documented (Lewis, 2001) but to date only one microarray study of this subject has examined differences in constitutive gene expression between different susceptible and resistant strains of *Drosophila melanogaster* (Le Goff *et al.*, 2003). According to this work, a single gene, *Cyp 6g1*, is upregulated in field selected strains of DDT-resistant *D. melanogaster* despite the fact that several *Cyp* genes such as *Cyp 12d1* and *Cyp 6a8* can confer DDT resistance when selected for in a laboratory situation. This indicates that not all CYP enzymes are created equal and certain forms may confer a broader range of cross-resistance to xenobiotics which in turn leads to strong selection for up-regulation of these specific genes in insects exposed to multiple insecticides and contaminants. An important area of study which has not yet been adequately explored via microarrays is the elucidation of the effect of botanical insecticides upon insect gene induction and suppression. This is an interesting lead to follow due to the complexity of the phytochemicals in question and the co-evolutionary relationship between insects and plants.

1.3 Rationale, hypotheses and objectives

1.3.1 Rationale

There is an increasing demand for insecticides with reduced risk to the natural environment and to the human food chain. The only insecticides that are acceptable to the rapidly expanding organic farming community are those which are formulated solely

with botanical products. At the 50th annual joint meeting of the Entomological Society of Canada, the Entomological Society of America and the Société d'entomologie du Québec (2000), the Québec-based organic farmers specifically stated the need for botanical synergists to replace PBO in pyrethrum formulations. There is also a need to address current cases of insecticide resistance by identifying products that are not affected by cross-resistance. Furthermore, currently used products could be rendered more effective through the development of novel synergists which allow a reduced application rate. The plant kingdom is an important source of natural insecticidal chemicals which have the potential to provide solutions to some of these problems.

This study was undertaken in collaboration with Whitmire Micro-Gen (Saint-Louis, Missouri). This private enterprise specializes in a wide variety of pyrethrin-based insecticide formulations with an emphasis on highly efficient insecticide-delivery formats such as crack and crevice applications and microencapsulation. Early discussions with company scientists highlighted a need for novel synergists for use in conjunction with pyrethrin. Currently, piperonyl butoxide (PBO) and N-octyl-bicycloheptene dicarboxiimide (MGK-264) are the most widely used pyrethrin synergists (Dorman, 1991). Toxicological concerns are starting to accumulate concerning these two products, in particular due to indications of hepatocarcinogenicity (Okamiya *et al.*, 1998). Botanical products were pinpointed as a potential source of such novel molecules due to simplified toxicological review standards for natural products which are classified as "reduced-risk pesticides" by the US Environmental Protection Agency (US EPA, 2003).

Botanical products derived from commercially cultivated plants were of particular interest due to the ready availability of large quantities of raw material. Such plants include Indian dill (*Anethum graveoloans*) and black pepper (*P. nigrum*), both of which contain active phytochemicals characterized by the presence of a methylenedioxyphenyl functional group associated with polysubstrate monooxygenase inhibition. *P. nigrum*

was selected for further study in this context due to the bifunctional nature of the insecticidal piperamides which have neurotoxic activity in addition to polysubstrate monooxygenase inhibition.

1.3.2 Hypotheses

In this research an investigation of a *P. nigrum* extract in terms of its insecticidal properties when used alone and its synergistic properties when used in conjunction with pyrethrin was undertaken. The first hypothesis advanced was that *P. nigrum* is a synergist for natural pyrethrin. It was predicted that the addition of a non-toxic concentration of *P. nigrum* extract to a natural pyrethrin formulation would result in increased toxicity due to the reported inhibition of detoxification enzymes and also due to the direct insecticidal activity of the extract. The second hypothesis advanced was that the physiological response of *Drosophila* to an insecticidal treatment with a botanical extract such as *P. nigrum* is complex due to the complex phytochemistry of the extract. It was predicted that the response would involve numerous detoxification genes. The third and final hypothesis was that there is a difference in the physiological response of *Drosophila* to *P. nigrum* extracts when it is used as an insecticide and when it is used as a synergist. It was predicted that gene expression profiles of *Drosophila* exposed to sub-acute doses of pyrethrin, *P. nigrum* extract or pyrethrin plus *P. nigrum* extract would differ in terms of the nature of the genes induced.

1.3.3 Objectives

There were three main objectives to be addressed in this study. The first objective was to test whether *P. nigrum* extract is an effective synergist when used in conjunction with pyrethrin against two test insects, *Musca domestica* and *Drosophila melanogaster*. The second objective was to determine the effect of an insecticidal dose

of *P. nigrum* extract upon gene induction and suppression in the model insect *Drosophila melanogaster* in order to obtain novel information concerning the insect response to a *P. nigrum* extract when it is used as an insecticide. The third and final objective was to determine the effects of pyrethrin, *P. nigrum* extract and a synergistic combination of pyrethrin and *P. nigrum* extract upon gene induction and suppression in *Drosophila melanogaster* in order to obtain information concerning the insect response to the use of *P. nigrum* extract as a pyrethrin synergist compared to the response to either *P. nigrum* extract or pyrethrin used alone.

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Chapter 2: Synergy of the natural pyrethrin esters of *Chrysanthemum cinerariaefolium* by a *Piper nigrum* extract: efficacy against the Dipteran insects *Musca domestica* and *Drosophila melanogaster*

2.1 Introduction

Over the past 70 years insect pests have been controlled by insecticides optimized synthetically for photostability, resistance to degradation by polysubstrate monooxygenase activity and a high degree of efficacy. The widespread application of synthetic insecticides and synergists is currently under scrutiny in light of a growing body of evidence concerning the environmental and health concerns associated with their use. In 2001, the Supreme Court of Canada ruled that municipal governments have the right to pass by-laws regulating pesticide use (Supreme Court of Canada, 2001). As a result, many Canadian municipalities have now chosen to ban the use of synthetic insecticides for cosmetic purposes within city limits. Additionally, one of the cornerstones of the growing organic farming movement is that the use of such products should be avoided in order to develop a healthy agricultural ecosystem and maintain the long-term productivity of agricultural lands (Altieri, 1983). In light of these scientific and social developments the investigation of alternatives to synthetic insecticides is growing in importance.

Plant-derived chemicals have shorter residence times in the environment than synthetic products and as such may have reduced environmental impacts (Demoute, 1989). Botanical insecticides which have not been altered synthetically are acceptable for organic agriculture. The most important botanical insecticide in use today is pyrethrum (Casida, 1995). The insecticidal components of this product are the natural pyrethrin esters (cinerin I, cinerin II, jasmolin I, jasmolin II, pyrethrin I and pyrethrin II (figure 1.2)) extracted from the flowers of *Chrysanthemum cinerariaefolium* Benth. & Hook. (Asteraceae) (Soderlund, 1995). The pyrethrins have a neurotoxic mode of action

and their target is site 7 of the voltage-gated sodium channel (Zlotkin, 1999). Closing of sodium channels is delayed, leading to a prolonged sodium current during depolarization and repetitive neuronal discharge (Soderlund, 1995). Insect defense against pyrethrins occurs through the action of cytochrome P450 and other detoxification enzymes (Demoute, 1989) which severely limit the efficacy of pyrethrin in the absence of a synergist. Mammalian toxicity is generally low because detoxification occurs before the compounds penetrate to the central nervous system (Katsuda, 1999). A typical pyrethrum formulation is completed by the addition of a synthetic synergist such as piperonyl butoxide (PBO) or N-octyl-bicycloheptene dicarboxiimide (MGK-264) with polysubstrate monooxygenase inhibiting properties (Dorman and Beasly, 1991).

Currently there is a need for novel synergists for pyrethrin and other insecticides. The benefits of formulating an insecticide with a synergist are twofold. First, the application rate is decreased which reduces the total quantity of insecticide released in the environment and also reduces the financial cost of the operation. Second, there is evidence that the use of complex mixtures of active compounds reduces the evolution of resistance among insect populations (Feng and Isman, 1995) due to a reduced probability that resistance to compounds with different modes of action will develop simultaneously. A synergy between pyrethroid and organophosphate insecticides has been exploited for two decades in West Africa to combat vectors of malaria and curtail resistance development (Corbel *et al.*, 2003).

The majority of the new synergists investigated in recent years have been synthetic products (Pap *et al.*, 2001) and novel synergies have been pursued more extensively with regards to the pyrethroids and other synthetic insecticides than to the pyrethrins (Corbel *et al.*, 2003, Buès *et al.*, 2003). The identification of a botanical synergist for pyrethrin could lead to the formulation and registration of a purely botanical

pyrethrin-based product. In this study, black pepper (*Piper nigrum* L. (Piperaceae)), was selected as a candidate for a novel pyrethrin synergist. Plants of the genus *Piper* grow in tropical regions of the Americas, Africa, Asia and Oceania. There is a broad cultural, geographical and temporal distribution of their recorded use as spices and medicines (Parmar *et al.*, 1997). The piperamides extracted from *P. nigrum* and other members of the genus *Piper* have shown potent insecticidal activity (Su and Horvat, 1981, Bernard *et al.*, 1995). They are also characterized by a bifunctional mode of action. Molecules with the isobutylamide functional group (e.g. 4,5-dihydropiperlonguminine, piperlonguminine) are sodium channel agonists (McFerren *et al.*, 2002) that bind to site 2 of voltage-gated sodium channels leading to persistent neuronal activation (Zlotkin, 1999). The methylenedioxyphenyl functional group present on certain piperamides (figure 1.3) is well characterized for its inhibition of PSMO activity (Hodgson and Philpot, 1974) and is responsible for the synergistic properties of PBO (Dalvi and Dalvi, 1991) (figure 1.4). Recent research has demonstrated that piperamides do, in fact, inhibit PSMO activity (Bhardwaj *et al.*, 2002, Scott *et al.*, 2003).

The goal of this study was to investigate the efficacy of a *P. nigrum* extract as a synergist for the natural pyrethrins. It was hypothesized that the synergy between these two compounds is strong based on the known PSMO inhibition of the piperamides and the two distinct neurotoxic effects exhibited by pyrethrin and *P. nigrum* caused by binding to separate sites of the voltage-gated sodium channel. Two Dipteran insect species were used as test subjects: *Musca domestica* L. (Diptera: Muscidae) and *Drosophila melanogaster* (Diptera: Drosophilidae). Efficacy was determined by calculating the LC_{50} values for *P. nigrum*, pyrethrin and for pyrethrin supplemented with a non-toxic concentration of *P. nigrum*.

2.2 Materials and Methods

2.2.1 Insect cultures

A culture of *Musca domestica* was maintained at room temperature (20-25°C). Adults aged 7 to 14 days were used in all trials. An Oregon-R strain of *D. melanogaster* was maintained on instant *Drosophila* medium, formula 4-24 (Carolina Biological Supply, Burlington, North Carolina) at a temperature of 23°C, relative humidity of 60% and light: dark cycle of 16:8 hours. Adult female flies aged between 3 and 14 days were used in all experiments. In all cases age was calculated as the number of days after egg hatch.

2.2.2 Botanicals materials

An extraction was performed on *Piper nigrum* seeds (peppercorns) obtained from Country Bulk (London, Ontario). Voucher specimens were retained at the University of Ottawa. For each 200 g of plant material a 300 mL volume of ethylacetate as used as a solvent and a 300 mL volume of water was used as a wash. The organic fraction was separated and washed a second time with another 300 mL of water. Excess water was removed using anhydrous sodium sulfate and the solvent was removed using a rotary evaporator at 30°C under reduced pressure until a resinous material was obtained. A standardized commercial pyrethrin solution with a 51% concentration of the 6 naturally occurring pyrethrins (pyrethrin I and II, cinerin I and II and jasmolin I and II) was obtained from Whitmire Micro-Gen (St-Louis, Missouri). For *M. domestica* assays, stock solutions of 1% (w/w) *P. nigrum* extract and pyrethrin were prepared in 99% ethanol. For *D. melanogaster* assays, stock solutions of 20 mg/mL *P. nigrum* extract and pyrethrin were prepared in 99% ethanol.

2.2.3 LC_{50} determination

Musca domestica: Flies were anaesthetized using CO_2 and maintained on ice prior to use. Twenty flies were placed on a filter paper in a 9 cm Pyrex Petri dish and sprayed with 2 mL of *P. nigrum* extract, pyrethrin or pyrethrin supplemented with *P. nigrum* extract using a Potter's tower at a pressure of 5 psi (34.5 KPa). Nitrogen was used as the carrier gas. For *P. nigrum* extract the range of concentrations assayed were: (0.1, 0.2, 0.4 and 0.8%), for pyrethrin they were: (0.04, 0.08, 0.16, 0.32 and 0.64%) and for pyrethrin plus *P. nigrum* extract they were: (0.01, 0.02, 0.04 and 0.08% with each concentration containing 0.1% *P. nigrum* extract). Flies were transferred to a mesh-covered cage and provided with a 5% sucrose solution. Mortality was scored after twenty-four hours and used for the calculation of LC_{50} values via Probit analysis (Hubert and Carter, 1990a). For LC_{50} calculations a total of 190 flies were used for *P. nigrum* extract, 343 for pyrethrin and 197 for pyrethrin plus *P. nigrum* extract. The LC_{50} for *P. nigrum* extract employed alone was determined and an approximately ten-fold lower value (0.1%) was used in combination with pyrethrin for the synergy assays. All assays included a solvent control and the synergy assay also included a 0.1% *P. nigrum* extract control and controls for the tested range of synergized pyrethrin concentrations (0.01, 0.02, 0.04 and 0.08 %) without the addition of *P. nigrum* extract. LC_{50} values were compared for significant differences using a Chi-squared test followed by pairwise comparisons using a Z-test and the slopes of the regression lines were compared using a Chi-squared test followed by pairwise comparisons using Scheffe's test (Hubert and Carter, 1990b). Synergist ratios for *P. nigrum* utilized as a synergist for pyrethrin were calculated using the formula:

Synergist ratio = LC_{50} for pyrethrin / LC_{50} for pyrethrin plus *P. nigrum*

D. melanogaster. Female flies were anaesthetized on ice prior to use. Twenty flies were placed on a 10 cm Petri dish and sprayed with 2 mL of *P. nigrum* extract, pyrethrin or *P. nigrum* extract and pyrethrin combined using a Potter's tower at an air pressure of 5 psi (34.5 KPa). Nitrogen was used as the carrier gas. For *P. nigrum* extract the range of concentrations assayed were: (0.4, 0.6, 0.8, 1.0, 1.2 mg/mL), for pyrethrin they were: (0.2, 0.4, 0.6, 0.8 mg/mL) and for pyrethrin plus 0.1 mg/mL *P. nigrum* extract they were: (0.01, 0.02, 0.04, 0.08, 0.16 mg/mL with each concentration containing 0.1mg/mL *P. nigrum* extract). Flies were subsequently placed in 3 dram glass vials plugged with cotton wool soaked in a 5% sucrose solution and mortality was scored after 24 hours. For the calculation of LC_{50} values a total of 697 flies were used for *P. nigrum* extract, 462 for pyrethrin and 596 for pyrethrin plus *P. nigrum* extract. The LC_{50} for *P. nigrum* extract employed alone was determined and a value approximately ten-fold lower (0.1 mg/mL) was used in combination with pyrethrin to investigate synergy. All bioassays included a solvent control and the synergy assay also included a 0.1 mg/mL *P. nigrum* extract control. All statistical calculations were identical to those used for *M. domestica*.

2.2.4 Phytochemical analysis and Potter's tower calibration

The concentrations of piperamides in the solutions of *P.* nigrum used to determine LC_{50} values were quantified. To do this, dilutions of the *P. nigrum* stock solution were prepared to give concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL in 99% ethanol in order to represent the range which was used to evaluate LC_{50} values for *D. melanogaster*. A 1 mL sample of each concentration was filtered for HPLC analysis and concentrations of three characteristic piperamides (piperine, 4,5-dihydropiperine and piperlonguminine) were evaluated using HPLC (Agilent, 1100 series reverse phase

LC/MSD) according to the method of Scott *et al.* (2002). This data is shown in appendix I.

The quantity of the marker compound piperine which reached the Petri plates after spray application of the *P. nigrum* extract solution using the Potter's tower was determined. The quantities of the other potential marker compounds, 4,5-dihydropiperine and piperlonguminine, were too low to permit accurate measurement. Empty 9 cm pyrex Petri plates were sprayed with 2 mL the of *P. nigrum* solutions with concentrations of 0.2, 0.4, 0.8 and 1.0 mg/mL. Three plates were sprayed for each concentration. The inside walls of plates were cleaned with a kimwipe dipped in 99% ethanol and the *P. nigrum* extract present on the bottom of the plate was recovered using 3 mL of 99% EtOH. A 1 mL aliquot of this solution was filtered and piperine concentration was analyzed by HPLC as previously described. The total quantity of piperine on the Petri plate and the application rate of piperine per square centimeter were calculated. A simple linear regression was performed to determine the predictive value of the initial concentration of the *P. nigrum* stock solution in relation to the application rate of piperine using the Potter's tower. This data is shown in appendix II.

The concentrations of piperine, 4,5-dihydropiperine and piperlonguminine in solutions showed a linear relationship to the concentration of *P. nigrum* extract in the solution with r^2 values higher than 0.99 (see appendix I). This indicates that the extract is homogeneous and that the different treatment levels show consistent differences in the quantities of these active compounds. The application rate of piperine to Petri plates showed a linear relationship to the concentration of *P. nigrum* in the solution used to spray the plate (R^2=0.63, p=0.002, df=1) (see appendix II). This validates the use of this methodology and confirms that the insect treatments are replicable.

2.3 Results

2.3.1 *M. domestica* LC_{50}

Mortality was not present in the *P. nigrum* controls (0.1%) nor were the pyrethrin concentrations of 0.01, 0.02, 0.04 and 0.08 % found to have any toxicity in the absence of 0.1% *P. nigrum* (data not shown). Probit regression lines were used to determine LC_{50} values for the three treatments (figure 2.1). These three values were significantly different according to a Chi-square test (χ^2=474.87, χ^2_{crit}=5.99, df=2, $p<0.001$). Pairwise comparisons using a Z-test determined all three LC_{50} values to be significantly different ($p<0.05$). A synergist ratio of 13.9 was obtained with the addition of 0.1% *P. nigrum* to pyrethrin. The slopes of the three regression lines were not significantly different (F=4.26, F_{crit}=4.74, $df_{n,d}$= (2,7), $p>0.05$).

2.3.2 *D. melanogaster* LC_{50}

Mortality was not present in solvent controls or in the *P. nigrum* 0.1 mg/mL control (data not shown). Probit regression lines were used to determine LC_{50} values for the three treatments (figure 2.2). These three values were significantly different according to a Chi-squared test (χ^2=2809.01, χ^2_{crit}=5.99, df=2, $p<0.001$). Pairwise comparisons using a Z-test determined all three LC_{50} values to be significantly different ($p<0.05$). The slopes of the regression lines for *P. nigrum* and pyrethrin were not significantly different using Scheffe's test to perform multiple pairwise comparisons (S=2.06, S_{crit}=2.99, $p>0.05$). The slopes for *P. nigrum* and pyrethrin + *P. nigrum* were significantly different (S=5.59, S_{crit}=2.99, $p<0.05$) as were those for pyrethrin and pyrethrin + *P. nigrum* (S=3.62, S_{crit}=2.99, $p<0.05$). A synergist ratio of 11.6 was obtained with the addition of 0.1 mg/mL *P. nigrum* to pyrethrin.

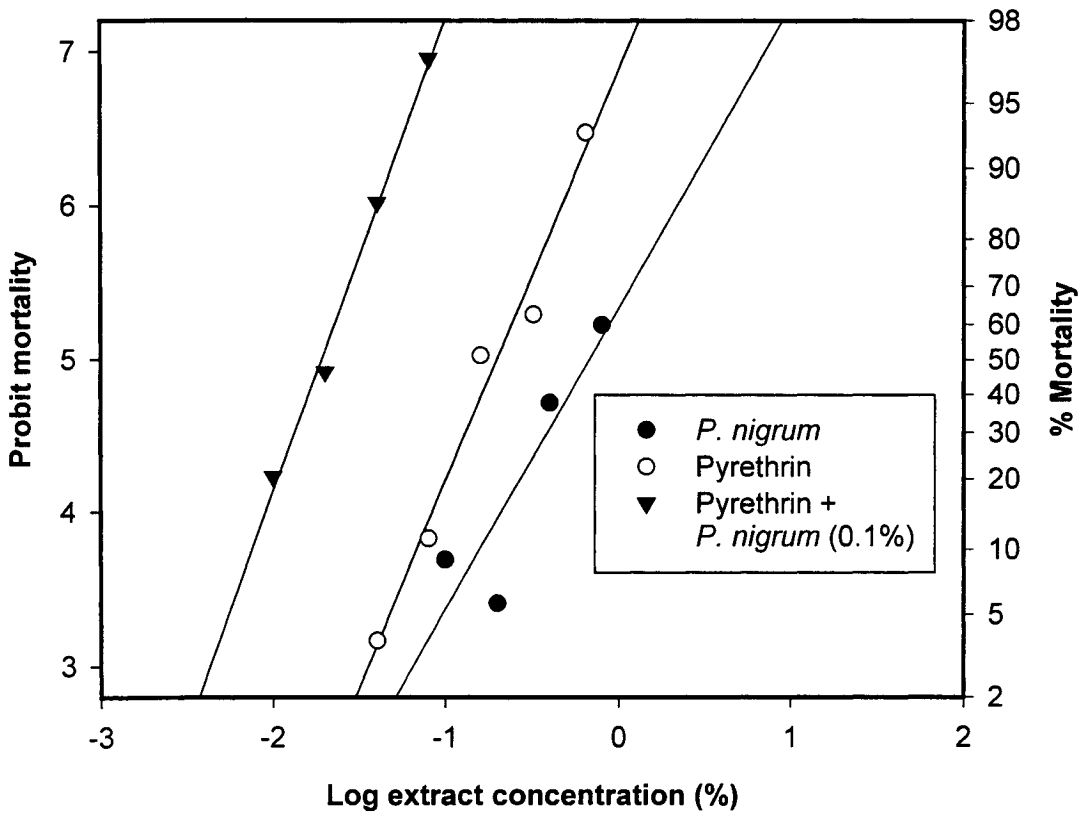

Figure 2.1: Probit regression lines used for calculating the LC_{50} values of *P. nigrum*, pyrethrin and pyrethrin supplemented with 0.1% *P. nigrum* for *M. domestica*. LC_{50} values (95% confidence intervals) are 0.8212 (0.5737, 1.6214), 0.2203 (0.1872, 0.2619) and 0.0159 (0.0124, 0.0192) respectively. The synergist ratio for *P. nigrum* is 13.9.

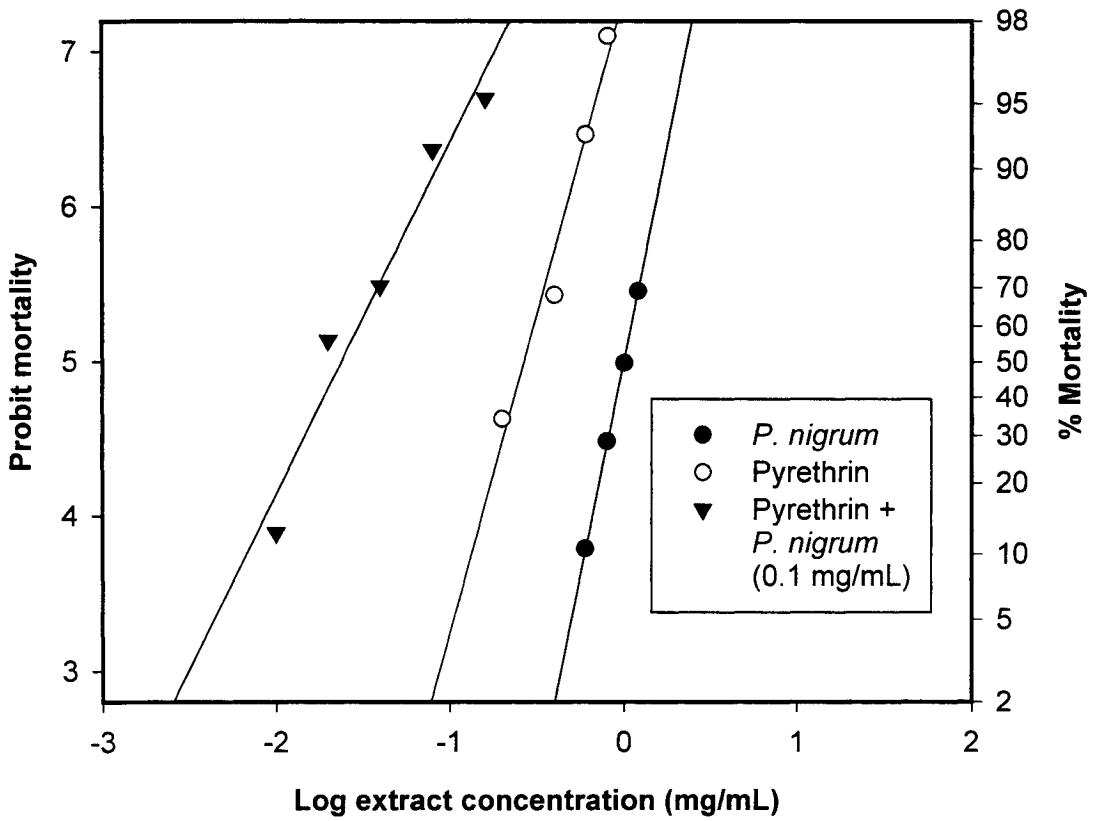

Figure 2.2: Probit regression lines used for calculating the LC_{50} values of *P. nigrum*, pyrethrin and pyrethrin supplemented with *P. nigrum* for *D. melanogaster*. LC_{50} values (95% confidence intervals) are 0.997 (0.947, 1.060), 0.267 (0.238, 0.293) and 0.023 (0.020, 0.026) respectively. The synergist ratio for *P. nigrum* is 11.6.

2.4 Discussion

High synergistic activity was obtained with the addition of *P. nigrum* extract to pyrethrin as is witnessed by the synergist ratios of 13.9 and 11.6 obtained with M. *Domestica* and *D. melanogaster*, respectively (figures 2.1 and 2.2). This synergy was observed with the use of a concentration of *P. nigrum* extract that had no toxicity when used alone, confirming that *P. nigrum* is acting as a synergist. The findings of the current study are consistent with early work that demonstrated a synergist ratio of 14 between pyrethrin and piperettine (a piperamide) using *M. domestica* as a test insect (Gersdorff and Piquett, 1957).

The synergist ratios described in this study present extremely high values for a mixture of botanical products. In contrast, the combination of pyrenol (a pyrethrum product) with neem oil or with a capsicanoid formulation (derived from capsicum extracted from chili peppers) resulted in synergist ratios of 2.7 and 2.6 respectively (Edelson *et al.*, 2002). It should be noted, however, that the pyrethrum formulation used in the aforementioned study already contained PBO which may have limited the potential for further synergistic interactions.

It was predicted that the *P. nigrum* extract would have synergistic properties similar to piperonyl butoxide due to the presence of the polysubstrate monooxygenase inhibiting MDP functional group. However, in some cases *P. nigrum* appears to be more effective than PBO. In a study where PBO was assayed in combination with the botanical products *Azadirachta indica* oil and pure azadirachtin against the mollusk *Lymnaea acuminata* the resulting synergist ratios ranged between 1.96 and 3.88 (Singh *et al.*, 1998). When PBO is used in conjunction with pyrethrin against the test insect *Musca domestica* the synergist ratio has been calculated to be anywhere between 5 and 15 (Incho and Greenberg, 1952, Nash, 1954). The high synergist ratio obtained

between *P. nigrum* and pyrethrin can most likely be attributed to the dual mode of action of the piperamides. *P. nigrum* is acting as a classic synergist by inhibiting PSMO activity and slowing detoxification (Dalvi and Dalvi, 1991). In addition, the piperamides and pyrethrins both act upon different sodium channel receptors (site 2 and 7) and their neurotoxic effects should therefore be additive or synergistic, rather than antagonistic or competitive (Zlotkin, 1999).

It is likely that the use of a *P. nigrum* extract, rather than a pure compound, may have contributed to the synergy observed. Due to the presence of analogue synergism it has previously been found that tertiary mixtures of piperamides show synergy (Scott *et al.*, 2002). The use of *P. nigrum* extract as a synergist could potentially be of use against insects with an evolved resistance to pyrethrum and pyrethroids. An extract of *P. tuberculatum* was effective against a strain of the Colorado potato beetle (*Leptinotarsa decemlineata*) with multiple insecticide resistance and a resistance ratio of 22 to cypermethrin (a pyrethroid) (Scott *et al.*, 2003). The development of resistance to the combination of pyrethrin and *P. nigrum* would probably require a substantial increase in PSMO expression and/or mutations of 2 different sodium channel receptor sites.

The *Piper nigrum* extract answers many of the qualifications of a good synergist. It has low mammalian toxicity, possesses an extremely high synergist ratio with pyrethrins and is rapidly degraded under natural conditions (Scott *et al.*, 2003). An additional benefit lies in the numerous structurally related compounds which are synergistic amongst themselves such as 4,5-dihydropiperlonguminine, piperlonguminine, 4,5-dihydropiperine and piperine (Scott *et al.*, 2002). This active mixture combined with pyrethrin may have the potential to slow or prevent the development of resistance in target insects.

2.5 Conclusion

This study has demonstrated the presence of a strong synergy between an extract of *P. nigrum* and pyrethrin. This is likely due to the inhibition of PSMO activity by *P. nigrum* and the effects of *P. nigrum* and pyrethrin upon different binding sites of voltage-gated sodium channels. These results suggest that, although there is usually some degree of synergy present between most botanical products, the choice of two candidates whose modes of action are complementary should lead to better than average synergist ratios. Future work will involve the use of cDNA microarrays to investigate the effects of *P. nigrum* and pyrethrin upon gene expression profiles in *Drosophila melanogaster.*

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Chapter 3: Gene expression profiles of *Drosophila melanogaster* exposed to insecticidal extracts of *P. nigrum*: identification of a novel *Cyp 9* gene associated with insect defense.

3.1 Introduction

Specific piperamides synthesized by plants of the genus *Piper* possess a cytochrome P450 inhibiting methylenedioxyphenyl group (Bernard *et al.*, 1995) and in some cases are also characterized by a neurotoxic isobutylamide group (Hatakoshi, 1984). Their strong insecticidal and synergistic properties make them good candidates as novel insect control products. Black pepper (*P. nigrum*) has previously been studied with regards to its acute toxicity towards a number of insects (Park *et al.*, 2002, Scott *et al.*, 2004), its phytochemistry (Su and Horvat, 1981) and its neurological activity (Hatakoshi *et al.*, 1984, McFerren *et al.*, 2002). The piperamides piperine, 4,5-dihydropiperine, piperlonguminine and 4,5-dihydropiperlonguminine present in *P. tuberculatum* demonstrate analogue synergism when used in tertiary mixtures (Scott *et al.*, 2002). Furthermore, cross-resistance was not observed when a strain of the Colorado potato beetle (*Leptinotarsa decemlineata*) with evolved resistance to multiple classes of insecticides was treated with a *P. tuberculatum* extract (Scott *et al.*, 2003). Treatment of housefly microsomes with piperine causes an inhibition of PSMO activity (Scott et al., 2003) and the previously mentioned lack of cross-resistance has been attributed to this property. In rats, piperine from *P. nigrum* inhibits cytochrome P450 enzyme activity within 1 hour of administration (Dalvi and Dalvi, 1991). In rat hepatoma cells piperine causes a biphasic response in terms of cytochrome P450 mediated arylhydrocarbon hydroxylase activity with an initial inhibition followed by induction (Singh and Reen, 1994). This may be due to a feedback mechanism whereby an upregulation of transcription of cytochrome P450 genes occurs in response to the inhibition of

cytochrome P450 enzymes. This has been shown to be the case for CYP 1A1 in the rat hepatoma 5L cell line where initial inhibition of the enzyme by piperine is followed by an increase in mRNA transcription and protein levels (Reen et al., 1996).

There is currently very little information concerning the effect of *Piper* extracts and pure compounds upon gene expression. Previous research has found that a compound from *P. nigrum* inhibits the transcription of aflatoxin biosynthetic genes in *Aspergillus parasiticus* (Annis *et al.*, 2000). The lignan piperlactum S isolated from *Piper kadsura* has also been found to suppress the synthesis of RNA and proteins and to stop the cell cycle progression in human T lymphocytes (Kuo *et al.*, 2000). In insects, the accumulated evidence concerning the insecticidal activities and physiological effects of phytochemicals obtained from *Piper* species suggests that they are likely to also cause significant perturbations in gene expression.

The purpose of this study was to investigate the effects of a *Piper nigrum* extract upon the target insect *Drosophila* at the level of gene expression. This research was also intended as in initial foray into the use of cDNA microarrays as a novel experimental platform to elucidate the possible mode of action of botanical insecticides and the insect response to these products. In this study, cDNA microarrays containing 7380 of the 14 000 *Drosophila melanogaster* genes were used to investigate the expressed transcriptome of this model insect in response to an acute exposure to *P. nigrum*. It was predicted that exposure to *P. nigrum* extracts would lead to the upregulation of a number of cytochrome P450 genes due to the biphasic nature of cytochrome 450 inhibition followed by induction exhibited by synergists with the MDP functional group such as piperonyl butoxide (Dalvi and Dalvi, 1991). Other detoxification genes such as glutathione-S-transferases and ABC-transporters were also expected to be upregulated due to the complex nature of the mixture. Genes related to the nervous system were

also predicted to be differentially expressed in response to the neurotoxic activity of the isobutylamides.

3.2 Materials and Methods

3.2.1 Experimental Design

Three comparisons were performed for this study (table 3.1). The first was a comparison between the expression profiles of *D. melanogaster* adults 6 hours after being exposed to ethanol (the carrier solvent) or water (assumed to have no effect). This was in order to investigate the possible effect of the solvent upon *Drosophila* gene expression and upon the results of the other experiments. The second comparison investigated the distribution of differential expression ratios observed after 6 hours between two groups of flies which had received identical treatments of ethanol. This was done in order to determine an appropriate threshold ratio for differential expression. The third comparison investigated the effects of a high concentration of *P. nigrum* (equivalent to the LC_{50} value) upon gene expression in *D. melanogaster* after a 4 hour exposure time. The experimental design used was a direct comparison between RNA extracted from treated and untreated *Drosophila*. The comparison of interest occurred between the two channels (represented by the fluorescent dyes Cy3 and Cy5) present on the same microarray slide (Bowtell and Sambrook, 2003). Each independent replicate consisted of a sample of RNA from a treated or a control pool of insects (1 sample=240 whole *Drosophila*).

3.2.2 Insect culture

An Oregon-R strain of *D. melanogaster* was maintained on instant *Drosophila* medium, formula 4-24 (Carolina Biological Supply) at a temperature of 23°C, relative humidity of 60% and a light : dark cycle of 16:8 hours. Adult female flies were used for all experiments and age was calculated as the number of days after egg hatch. This was in order to minimize variations in gene expression among samples due to sex

Table 3.1: Summary of the microarray hybridizations analyzed and discussed in this study. Adult female *Drosophila melanogaster* with a maximum range of ages of 10 days were used in all experiments. One sample represents a pool of 240 insects. Dye reversal hybridizations represent hybridizations with the same RNA samples but with the labeling dye (Cy3 or Cy5) reversed between samples.

Hybridization group	Control	Treatment	Exposure time (hrs.)	Effect evaluated	n	Dye reversals
1	Water	Ethanol	6	Solvent effect	2	0
2	Ethanol	Ethanol	6	Random variation	4	4
3	Ethanol	*P. nigrum* 0.9 mg/mL	4	Insecticide effect	2	2

(Kasai and Tomita, 2003). For the ethanol versus water treatment flies aged 4-7 days were used, for the ethanol versus ethanol treatment flies aged 6-9 days were used and for the *P. nigrum* versus ethanol treatment flies aged 10-20 days were used. This was in order to minimize variations in gene expression among samples due to the presence of extremely young or extremely old flies (Jin *et al.*, 2001). The Oregon-R strain was selected for study because it is not resistant to any insecticides and therefore does not constitutively overexpress specific *Cyp* or other detoxification genes. This could potentially limit further induction of these genes and thereby mask certain effects of the treatment (Brandt *et al.*, 2002).

3.2.3 *P. nigrum* extract preparation

An extraction was performed on *Piper nigrum* seeds (peppercorns) using a 300 mL volume of ethylacetate as a solvent and a 300 mL water wash per 200 g of plant material. The organic fraction was separated and was extracted a second time with another 300 mL of water. Excess water was removed using anhydrous sodium sulfate and the solvent was removed using a rotary evaporator at 30°C under reduced pressure until a resinous material was obtained. Voucher specimens of *P. nigrum* seeds were retained at the University of Ottawa. For microarray treatments a 0.9 mg/mL solution of *P. nigrum* extract was formulated in 99% ethanol. This concentration of *P. nigrum* extract is equivalent to the LC_{50} value for adult *D. melanogaster* females (see section 2.3.2) and therefore represents a treatment with a marked physiological effect.

3.2.4 Insect treatments

This experiment sought to mimic the field application of an insecticide as closely as was reasonably possible. The day of the experiment, female insects were placed in 5 dram glass vials (60 insects/vial) plugged with cotton wool soaked in a 5% sucrose

solution and subsequently returned to the growth chamber. Four hours later the flies were anaesthetized on ice, placed on a 90 mm diameter Whatman filter paper in a 90 mm Petri plate and sprayed with a total of 2 mL of the selected treatment (water, 99% ethanol or a 0.9 mg/mL *P.* nigrum solution). This was done using a Potter's tower set to a pressure of 5 psi (34.5 KPa) with nitrogen serving as the carrier gas. Flies were returned to the vials and placed in the growth chamber for four or six hours (depending on the hybridization group). This exposure time was chosen based on a prior study in which insects were exposed to immune response inducers and which found maximum gene expression to peak between 4 and 6 hours (Irving *et al.*, 2001). At 17:00 all dead flies were removed from each sample and the remaining flies were anaesthetized, flash frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. Flies from four vials were pooled to give samples of approximately 240 whole insects to ensure a sufficient yield of RNA. Samples to be pooled received the same treatment at the same time to minimize variation.

3.2.5 Potter's tower calibration and extract characterization using phytochemical analysis

After the insect treatments were completed, 3 blank Petri plates were sprayed with 2 mL of 0.9 mg/mL of *P. nigrum* in order to quantify the application rate of the extract. The blank plates treated with *P. nigrum* were rinsed with 4 mL of 99% EtOH in order to recover the extract residues. Samples were prepared and analyzed for the presence of the marker compound piperine using HPLC as described in chapter two (section 2.2.4). The quantity of piperine on the Petri plates for the *P. nigrum* treatment was 0.67 $\mu g/cm^2$ with a standard error of 0.13 (n=3) (appendix III). The *P. nigrum* stock solution used was characterized in terms of the concentration of four insecticidal piperamides: 4,5-dihydropiperlonguminine, piperlonguminine, 4,5-dihydropiperine and

piperine (appendix III). Overall, the phytochemical data demonstrated that the applied concentration of the marker compound piperine using the Potter's tower was consistent.

3.2.6 RNA extraction

Total RNA was isolated using the acid guanidium thiocyanate-phenol-choloroform extraction method (Chomczynski and Sacchi, 1987). Samples were initially homogenized in 200 µL of solution D (73 mL DEPC H_2O, 62.6 g solid GITC, 4.4 mL sodium citrate, 6.6 mL of 10% sarkosyl and 0.3 mL β-mercaptoethanol) using a Kontes pestle (VWR) and further homogenized with 3 short sonicator pulses after adding an additional 800 µL solution D. Insoluble material was removed from samples by centrifuging at 16 000 x g for 3 minutes and transferring the supernatant to new microtubes. RNA was separated from DNA and protein fraction after the addition of sodium acetate, phenol and chloroform and was precipitated using isopropanol. Three washes with cold 80% EtOH were performed to ensure the removal of red coloured eye pigments and organic contaminants from the RNA pellet. RNA was resuspended in a 50 µL volume of DEPC treated H_2O to ensure a minimum RNA concentration of 4 µg/µL. RNA concentration and purity were assessed by diluting 1 µL RNA into 99 µL 0.1X TE buffer and measuring the ratio of absorbencies at 260 and 280 nm (A_{260}/A_{280}). RNA samples with a ratio between 1.8 and 2.01 were considered to be suitable for microarray analysis. RNA was stored at -80°C prior to being shipped on dry ice to the Canadian *Drosophila* Microarray Center (CDMC (www.flyarrays.com)) for further processing.

3.2.7 Microarray hybridization

Microarray slides were purchased from the CDMC. The *Drosophila* array version 7K3 was used in all experiments. This array contains 5756 target cDNAs from the Berkeley *Drosophila* Genome Project, 1078 cDNAs from the National Institutes of Health

Drosophila testis cDNA library, and 546 gene fragments amplified from genomic DNA (Neal *et al.*, 2003). The arrays were laid out in 32 subarrays, each with 22 rows and 24 columns with two replicates of each gene for a total of 16 896 spots (Neal *et al.*, 2003). Replicate spots were located adjacent to each other on the slides (Neal *et al.*, 2003). Direct labeling of RNA and subsequent hybridization to microarray slides and scanning using the ScanArray 4000 XL (GSI Lumonics/Packard Biochips) was performed at the CDMC. Protocol details concerning labeled cDNA synthesis, hybridizations reactions and scanner settings are described in Neal *et al.* (2003). For each sample, 80 µg of total RNA was labeled with cyanine 3 or cyanine 5 (Cy3 or Cy5) dye. Dye-reversal hybridizations were performed for each sample pair (with the exception of the ethanol versus water hybridizations) in order to correct for differences in dye incorporation and fluorescence.

3.2.8 Analysis of microarray data

Quantarray Microarray Analysis Software version 3.0 (Packard BioScience, copyright 2001) was used to quantify the raw images resulting from scans of the microarray slides. Images from the Cy3 and Cy5 channels of the same slide were superimposed, spots were located and those with visually obvious defects were manually flagged for exclusion from analysis. Spot and background intensity were measured using the adaptive circle quantification method which statistically determines the threshold of significant spot fluorescence relative to background fluorescence (Packard BioScience, 2001). The resulting data files were exported to Gene Traffic™ Duo, version 2.8 (Iobion Informatics, copyright 2002) for further analysis.

Data normalization was performed on background subtracted spots on a subgrid basis using the Locally Weighted Scatter Plot Smoother (LOWESS) algorithm with a smoothing factor of 20. This procedure normalizes the intensity value for each spot

based upon the data distribution for a subset of the intensity range, centered on the spots' intensity value (Leung and Cavalieri, 2003). This prevents the normalization of spots from being affected by extreme intensity values. Spots with intensity values less than 100 units and spots with intensity values below the average background intensity value and/or below the local background intensity value were excluded from normalization and analysis. Data was filtered so as to exclude all genes with less than two-thirds of spots being usable (less than 3 spots for hybridization group 1, less than 11 spots for hybridization group 2 and less than 5 spots for hybridization group 3) as defined by quality filters and by a mean differential expression ratio with a coefficient of variance higher than 100%.

To determine an initial set of genes of interest for further study $\log_2$ ratio values of 1 and -1 (see appendix IV for a table of $\log_2$ ratio conversions) were chosen as cutoff values indicating downregulation and upregulation, respectively. These values correspond to a two-fold change in gene expression which is a common reference point in the microarray literature (Bowtell and Sambrook, 2003). Hybridization 2, which compared two samples that received identical treatments, was used to determine an alternative threshold to identify additional differentially expressed genes in the context of this specific microarray platform and experimental design. To do this, differential expression ratios were determined for all genes and the number of genes with a given $\log_2$ ratio were plotted as a function of the range of $\log_2$ ratios observed. The quality and range of the data distribution was assessed graphically. The selected cutoff $\log_2$ ratios for differential expression for this microarray platform were equal to 0.5 and -0.5, equal to a 1.41-fold change in expression. The range between these two values encompassed over 99% of the differential expression ratios for the like-like hybridization. This was therefore considered to be an appropriate threshold value for differential expression. Differentially expressed genes were tabulated using the *Drosophila* genome annotation

available in flybase (http://flybase.bio.indiana.edu/). Genes were presented in tables classified by their molecular function and biological process as listed in the gene ontology (GO) page of Flybase. Both upregulated and downregulated genes were presented in the same table in order to show genes with the same molecular function grouped together. See appendix V for information concerning the fold-change, $\log_2$ ratio and standard deviation associated with each differentially expressed gene. Genes were subsequently divided into categories representing the known or inferred role they play in the organism based on the molecular function and/or biological process data and presented in figures to give an overall view of the pattern of gene induction and suppression. All microarray data will be submitted to the Gene Expression Omnibus (GEO) database and become part of the public domain upon publication of this research in a peer-reviewed journal.

3.2.9 Northern analysis

Northern blot analysis was used to confirm the expression patterns of genes selected from the microarray experimental data. The three genes chosen for study were two upregulated genes, *Cyp 6a8*, *Cyp 9b2*, as well as a control gene, CG2196, which was not differentially expressed. The cDNA clones GH05558 (*Cyp 6a8*), GH08116 (*Cyp 9b2*) and GH19680 (CG2196) from the *Drosophila* Genome Collection release 1 (Stapleton *et al.*, 2002) were obtained as agar stab cultures from the CDMC. These were used to produce streak plates on agar containing 25 µg chloramphenicol /mL. Individual colonies were selected from the streak plates and subsequently cultured overnight (approximately 16 hours) at 37°C with vigorous shaking (200 r/minute) in 10 mL Luria-Bertani medium supplemented with 25 µg/mL of chloramphenicol. Plasmid DNA was isolated from 6 mL of this culture using Wizard® Plus SV Miniprep columns (Promega) and sent for sequencing to Canadian Molecular Research Services Inc.

(Ottawa, Canada) or to the Core DNA Sequencing and Synthesizing Facility at the University of Ottawa Biotechnology Research Institute (Ottawa, Canada). Sequences were verified using NCBI BLASTn (Altschul, 1990). For long-term storage of the clones, solutions consisting of 750 µL of overnight culture and 150 µL of sterile glycerol were prepared for each clone and stored at -80°C.

Oligonucleotide primers specific to the pOT2a cloning vector (Stapleton *et al.*, 2002) were synthesized (Invitrogen) and used in PCR to amplify DNA from the cDNA clones. Sense and anti-sense strand primer sequences were (5'-AATGCAGGTTAACCTGGCTTATCG-3') and (5'-AACGCGGCTACAATTAATACATAACC-3') resulting in PCR product sizes of 1508 bp, 1887 bp and 2120 bp for *Cyp 6a8*, *Cyp 9b2* and CG2196 respectively. PCR reactions were carried out in 100 µL volumes with each reaction consisting of: 0.5 µL template DNA (overnight bacterial culture), 10 µL 10X PCR buffer (Invitrogen), 2 µL of a 10 mM solution of each dNTP, 3 µL of 50 mM $MgCl_2$ (Invitrogen), 0.5 µL *Taq* DNA polymerase (Invitrogen), 5 µL of each primer (from a 10 µM stock) and 68 µL DEPC treated H_2O. Thermal cycler settings consisted of an initial 3 minute denaturation at 94°C, followed by 35 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 60°C and extension for 60 s at 72°C and concluding the PCR with a 5 minute final extension step at 72°C. PCR products were purified using Microspin S-300 HR columns (Amersham Biosciences) and product size and purity was verified by running 3 µL of PCR product on a 1% agarose gel. DNA concentration was determined using a Pharmacia Gene Quant DNA/RNA calculator spectrophotometer to analyze 5 µL of PCR product diluted in 95 µL of 0.1X TE buffer (Tris-EDTA (Bio Basic Inc.)).

For the Northern blots a second cohort of adult female *D. melanogaster* was treated with 0.9 mg/mL of *P. nigrum* followed by total RNA extraction as described in section 3.2.5 so as to replicate the experimental conditions of the microarray experiment.

Three replicates of total RNA from EtOH treated and *P. nigrum* treated flies were loaded in volumes of 10 µg on a 1.2% denaturing agarose gel and run at 90 volts for 2.5 hours. RNA was transferred overnight (approximately 18 hours) to a Hybond N+ nylon membrane (Amersham Biosciences) using 20X SSC buffer to provide capillary action. Post-transfer, RNA was bound to the membrane in a CL-1000 ultraviolet crosslinker (UVP) using a total 254 nm UV exposure of 0.12 J/cm^2 and stored with a damp filter paper in a sealed plastic pouch at 4°C prior to use. Three replicate membranes were made (one for each gene to be tested) so as to avoid repeated stripping and probing of the membranes.

Membranes were placed in glass vials and pre-hybridized at 65°C for 4 hours in hybridization solution (4 mL dextran sulfate, 1 mL 10% SDS, 3 mL 20X SSC, 0.1 mL of salmon sperm DNA (from a 10 mg/mL stock)), 1 mL Denhardt's solution (50X stock) and 0.9 mL DEPC H_2O per membrane). During this time the radioactive probe was synthesized from the previously obtained PCR product. A 25 ng aliquot of the appropriate probe DNA was suspended in 1X TE buffer and denatured at 100°C for 5 minutes. Labeling the probe with [α^{32}P]dCTP (Amersham Biosciences) was carried out using the Rediprime II random prime DNA labeling system (Amersham Biosciences). Unbound nucleotides were removed from the probe using a Probe Quant G-50 Micro Column (Amersham Biosciences). When pre-hybridization was complete the pre-hybridization solution was replaced with an equal volume of fresh hybridization solution and the radiolabeled probes were added. Hybridization was carried out overnight (approximately 16 hours) at 65°C.

Membrane washing was carried out at 65°C under medium stringency conditions with two 15 minute washes in 2X SSC and two 30 minute washes in 1XSSC, 0.1% SDS. Membranes were then placed on a moist Whatman filter paper, sealed with plastic wrap and exposed to a Kodak K phosphor screen for 2 hours. A Bio-Rad Molecular Imager®

FX was used to scan the phosphor screens and Quantity One (version 4.4.1, Bio-Rad, copyright 1998) was used to quantify band intensities. Ribosomal RNA was visualized by ethidium bromide staining and was used to normalize for RNA loading volumes on each membrane. Densitometric data for *Cyp 6a8*, *Cyp 9b2* and CG2196 was normalized to rRNA bands. Then, for each membrane, the expression ratio of the tested gene in the *P. nigrum* treatment was calculated as a fold-change value relative to the control treatment (counts x mm^2 for a given band / mean counts x mm^2 for all control bands). For each gene a two-sample t-test with pooled variances was used to compare expression ratios between the control treatment and the *P. nigrum* treatment using SYSTAT software (version 10, SPSS Inc., copyright 2000).

3.3 Results

3.3.1 Microarrays

Three genes were found to be differentially expressed (two-fold or greater change in expression) in response to the ethanol treatment versus a water control (table 3.2). One gene was upregulated and two were downregulated. The ethanol versus ethanol hybridization showed very little differential expression occurring above a 1.41-fold change in expression and no differential expression occurring above a 2-fold ratio (figure 3.1). This 1.41-fold ratio (log_2 ratio greater than or equal to 0.5 or less than or equal to –0.5, see appendix IV for a table of log_2 ratio conversions) was therefore selected as a minimum threshold for determining differential expression. In response to the *P. nigrum* extract treatment fourteen genes showed differential expression according to the literature cutoff value of a 2-fold or more change. Of these, 12 genes were upregulated and 2 genes were downregulated (table 3.3). The twelve upregulated genes included the cytochrome P450 genes *Cyp6a8*, *Cyp9b2*, *Cyp12d1* as well as glutathione-S-transferase S1 while the two downregulated genes were of unknown

Table 3.2: Differentially expressed genes (as defined by a fold-change in expression of at least 2) identified in adult female *D. melanogaster* treated with 99% ethanol and compared to a control treatment of H2O. Mean fold-change in expression is reported along with gene ontology (GO) annotation for the molecular function and biological process associated with each gene.

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated genes						
unfolded protein binding	GO:0051082	defense response	GO:0006952	*Hsc70-2*	CG7756	2.16
Downregulated genes						
nucleic acid binding	GO:0003676	protein biosynthesis	GO:0006412, GO:0006416, GO:0006453	*mRpL13*	CG10603	-2.81
ATPase activity, coupled to transmembrane movement of substances	GO:0042626	transport	GO:0006810	--	CG1703	-2.27

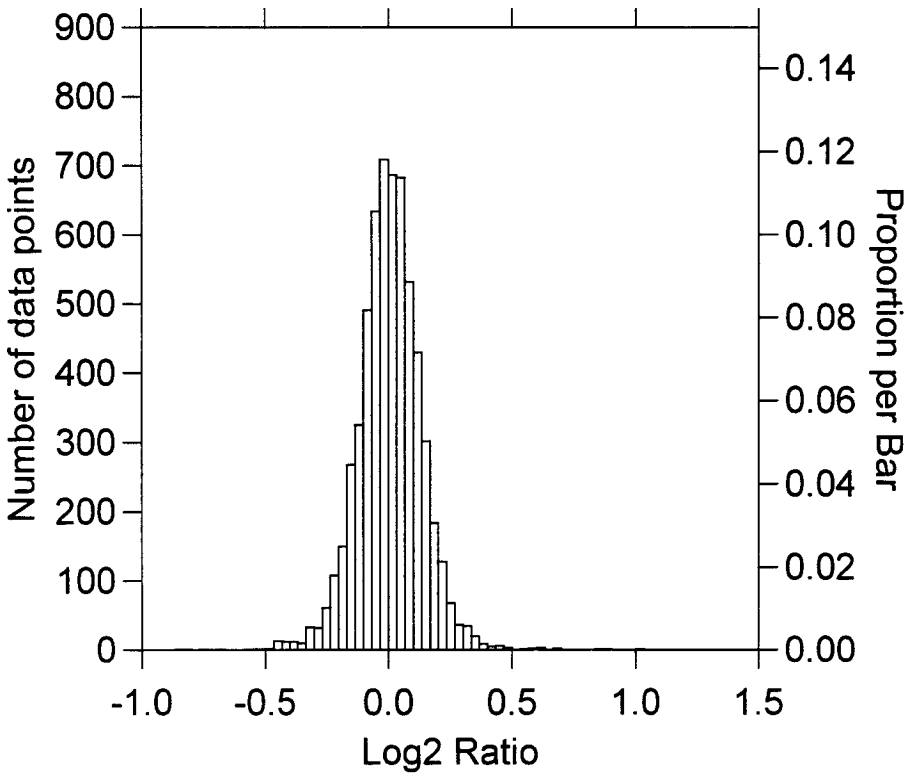

Figure 3.1: The distribution of differential expression ratios of genes on the Drosophila 7K3 cDNA microarray from the Canadian Drosophila Microarray Center. Sample pools of *D. melanogaster* adult females that received identical 99% ethanol treatments were compared after a 6 hour incubation period. Sample size was 4 with 4 dye reversal hybridizations. A $\log_2$ ratio of 0.5 or -0.5 is equal to a fold-change in gene expression of 1.41 and a $\log_2$ ratio of 1.0 or –1.0 is equal to a fold-change in gene expression of 2.

Table 3.3: Differentially expressed genes with a fold-change value of 2 or more identified by cDNA microarray analysis in adult female *D. melanogaster* treated with a 0.9 mg/mL concentration of *P. nigrum* compared to a control of 99% EtOH. Mean fold-change in expression is reported along with gene ontology (GO) annotation for the molecular function and biological process associated with each gene.

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated genes						
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp9b2*	CG4486	3.12
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp6a8*	CG10248	3.53
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp12d1*	CG18240	2.51
glutathione transferase activity	GO:0004364	response to toxin	GO:0009636	*GstS1*	CG8938	2.22
hydrolase activity	GO:0016787	--	--	--	CG5707	2.13
RNA binding	GO:0003723	transcription from Pol II promoter	GO:0006366	*Aly*	CG1101	2.03
phosphoserine phosphatase activity	GO:0004647	L-serine biosynthesis	GO:0006564	*aay*	CG3705	2.04
structural constituent of cytoskeleton	GO:0005200	cytoskeleton organization and biogenesis	GO:0007010	*Act88F*	CG5178	2.19
--	--	response to cold	GO:0009409	*Fst*	CG9434	2.55
--	--	--	--	--	CG31904	2.00
--	--	--	--	*fln*	CG7445	2.36
--	--	--	--	--	CG11893	2.13
Downregulated genes						
--	--	--	--	--	CG11892	-2.79
--	--	--	--	--	CG5107	-2.08

function. Using the 1.41-fold change in expression as a cutoff value based on the data from the ethanol versus ethanol hybridization resulted in the identification of an additional 51 upregulated and 7 downregulated genes. These included 3 additional *Cyp 6* genes (*Cyp 6d4*, *Cyp 6d5* and *Cyp 6w1*) and glutathione-S-transferase E7 (table 3.4).

All upregulated genes with a fold-change in expression greater than or equal to 1.41 were classified by their molecular function and/or the biological process to which they are associated (figure 3.2). The categories with the greatest number of associated genes were phase I and phase II metabolism (figure 3.3), muscular/cytoskeleton (figure 3.4) and stress/defense response (figure 3.5). All downregulated genes with a fold-change in expression less than or equal to -1.41 were also classified by their molecular function and/or biological process (figure 3.6). The most important category for the downregulated genes was proteolysis and peptidolysis.

3.3.2 Northern analysis

The results of two-sample t-tests used to analyze the Northern blot data revealed a significant 2-fold upregulation of mRNA expression for *Cyp 6a8* ($p=0.003$, $df=4$, $n=6$) and a significant 2.9-fold upregulation of mRNA expression for *Cyp 9b2* ($p=0.004$, $df=4$, $n=6$) but no significant change in CG2196 mRNA expression level ($p=0.399$, $df=4$, $n=6$) (figure 3.7).

Table 3.4: Differentially expressed genes with a fold-change value greater or equal to 1.41 and less than 2 identified by cDNA microarray analysis in adult female *D. melanogaster* treated with a 0.9 mg/mL concentration of *P. nigrum* compared to a control of 99% EtOH. Mean fold-change in expression is reported along with gene ontology (GO) annotation for the molecular function and biological process associated with each gene.

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated and downregulated genes of known function						
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp6d5*	CG3050	1.74
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp6w1*	CG8345	1.80
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp6d4*	CG12800	1.44
glutathione transferase activity	GO:0004364	response to toxin	GO:0009636	*GstE7*	CG17531	1.41
actin binding	GO:0003779	--	--	*Keap1*	CG3962	1.41
actin binding	GO:0003779	muscle contraction	GO:0006936	*wupA*	CG7178	1.43
structural constituent of cytoskeleton	GO:0005200	cytoskeleton organization and biogenesis	GO:0007010	*Act57B*	CG10067	1.61
structural constituent of cytoskeleton	GO:0005200	cytoskeleton organization and biogenesis	GO:0007010	--	CG7940	-1.52
structural constituent of cuticle (sensu Insecta)	GO:0005214	--	--	--	CG1919	1.54
structural constituent of cuticle (sensu Insecta)	GO:0005214	--	--	--	CG8505	1.59
calmodulin binding	GO:0005516	calcium ion transport	GO:0006816	--	CG9297	1.52
calmodulin binding	GO:0005516	muscle contraction	GO:0006936	*TpnC41C*	CG2981	1.67
calcium ion binding	GO:0005509	lipid metabolism	GO:0006629	--	CG1583	1.53
magnesium ion binding	GO:0000287	amino acid biosynthesis	GO:0008652	*Nmdmc*	CG18466	1.74
ATP binding	GO:0005524	striated muscle contraction	GO:0006941	*Mhc*	CG17927	1.79

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated and downregulated genes of known function (cont.)						
RNA binding	GO:0003723	RNA elongation from Pol II promoter	GO:0006368	*Zn72D*	CG5215	-1.55
RNA polymerase II transcription mediator activity	GO:0016455	regulation of transcription from Pol II promoter	GO:0006357	*Trap25*	CG17183	1.45
nucleic acid binding	GO:0003676	mitosis	GO:0007067	*Enc*	CG10847	1.75
chitin binding	GO:0008061	chitin metabolism	GO:0006030	--	CG8756	1.43
fatty acid binding	GO:0005504	lipid transport	GO:0006869	*RfaBp*	CG11064	-1.43
hydrogen-transporting ATP synthase activity, rotational mechanism	GO:0046933	ATP biosynthesis	GO:0006754, GO:0006758	*blw*	CG3612	1.41
oxidoreductase activity	GO:0016491	electron transport	GO:0006118	--	CG5653	1.41
4-hydroxyphenylpyruvate dioxygenase activity	GO:0003868	amino acid catabolism	GO:0009063	--	CG11796	1.42
receptor activity	GO:0004872	transmission of nerve impulse	GO:0019226	--	CG7896	1.44
succinate-CoA ligase (ADP-forming) activity	GO:0004775	tricarboxylic acid cycle	GO:0006099	--	CG11963	1.46
transferase activity	GO:0016740	fatty acid metabolism	GO:0006631	--	CG30008	1.46
citrate (Si)-synthase activity	GO:0004108	tricarboxylic acid cycle	GO:0006099	*l(1)G0030*	CG3861	1.48
ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	GO:0015662	cation transport	GO:0006812, GO:0006819	--	CG6263	1.51
electron transporter activity	GO:0005489	oxidative phosphorylation	GO:0006119	*Cyt-c-p*	CG17903	1.52

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated and downregulated genes of known function (cont.)						
N-acetylglucosamine-6-sulfatase activity	GO:0008449	N-acetylglucosamine metabolism	GO:0006044	--	CG30059	1.57
transcription factor activity	GO:0003700, GO:0000130	regulation of transcription, DNA-dependent	GO:0006355	--	CG9404	1.57
epoxide hydrolase activity	GO:0004301	response to toxin	GO:0009636	*Jheh1*	CG15101	1.58
eukaryotic initiation factor 4E binding	GO:0008190	response to stress	GO:0006950	*Thor*	CG8846	1.62
triacylglycerol lipase activity	GO:0004806	lipid metabolism	GO:0006629	--	CG5966	1.87
serine-type endopeptidase activity	GO:0004252	proteolysis and peptidolysis	GO:0006508	--	CG11911	-1.44
chymotrypsin activity	GO:0004263	proteolysis and peptidolysis	GO:0006508	--	CG18180	-1.53
ornithine-oxo-acid transaminase activity	GO:0004587	amino acid metabolism	GO:0006520	--	CG8745	-1.66
adenosine deaminase activity	GO:0004000	purine ribonucleoside monophosphate biosynthesis	GO:0009168	*Adgf-E*	CG10143	1.73
IMP cyclohydrolase activity	GO:0003937	purine nucleotide biosynthesis	GO:0006164	--	CG11089	1.74
--	--	defense response	GO:0006952	*l(2)efl*	CG4533	1.79
Upregulated genes of unknown function						
--	--	--	--	--	CG1572	1.45
--	--	--	--	--	CG2471	1.51
--	--	--	--	--	CG3630	1.59
--	--	--	--	--	CG7214	1.80
--	--	--	--	*CheA75a*	CG7313	1.51
--	--	--	--	--	CG10126	1.41

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated genes of unknown function (cont.)						
--	--	--	--	--	CG10383	1.73
--	--	--	--	--	CG10407	1.65
--	--	--	--	--	CG10550	1.44
--	--	--	--	--	CG10562	1.78
--	--	--	--	--	CG13335	1.48
--	--	--	--	--	CG13868	1.64
--	--	--	--	--	CG14207	1.52
--	--	--	--	--	CG15825	1.42
--	--	--	--	--	CG16884	1.80
--	--	--	--	--	CG16926	1.54
--	--	--	--	*CG32041 gene cassette*	CG32041	1.58
Downregulated genes of unknown function						
--	--	--	--	*Tsp42Eb*	CG18816	-1.46

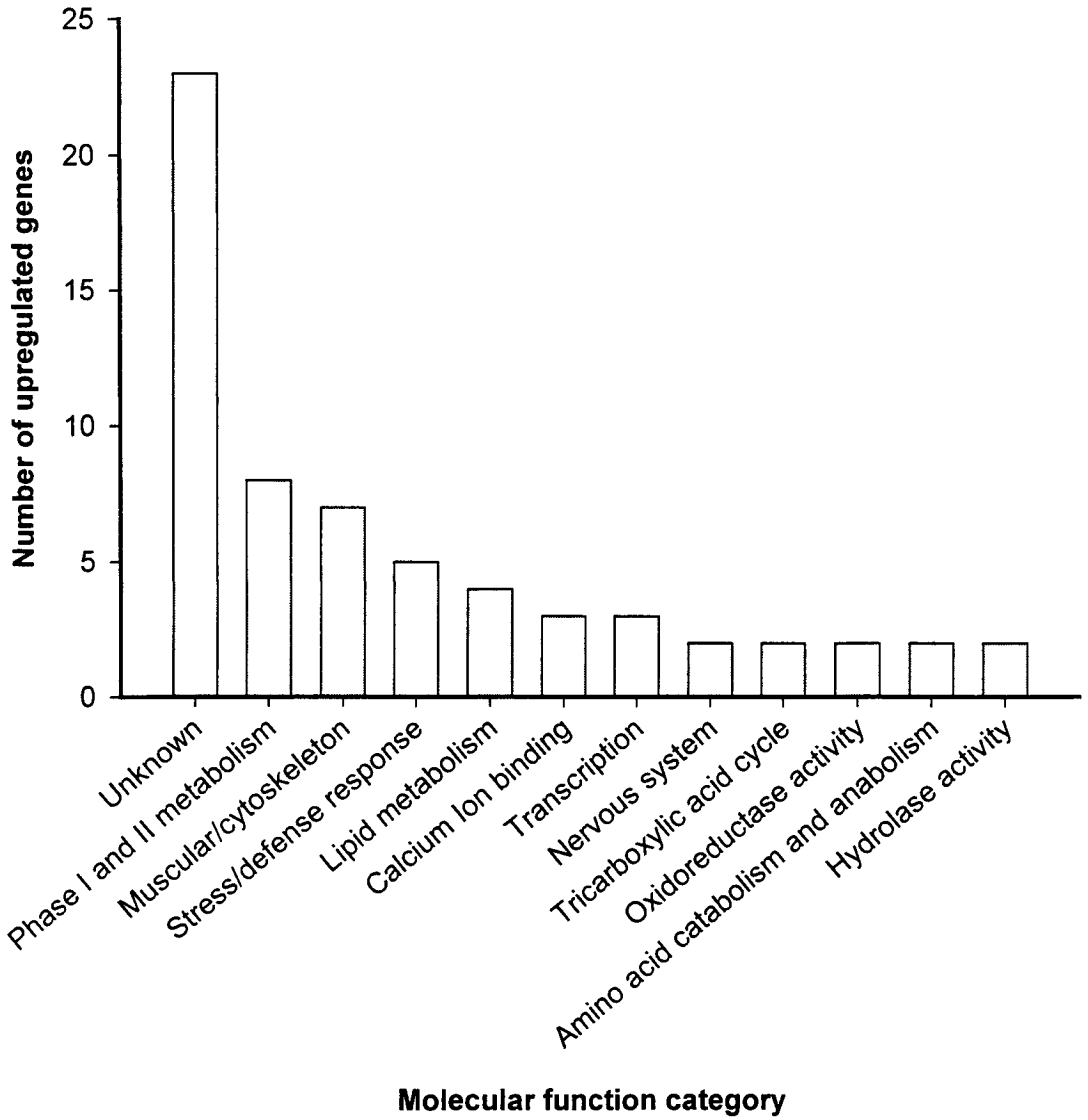

Figure 3.2: All *D. melanogaster* genes upregulated 1.41-fold or more by a treatment of 0.9 mg/mL *P. nigrum* as determined by cDNA microarray analysis. Genes were assigned categories according to their molecular function and associated biological process. A total of 63 genes were found to be upregulated.

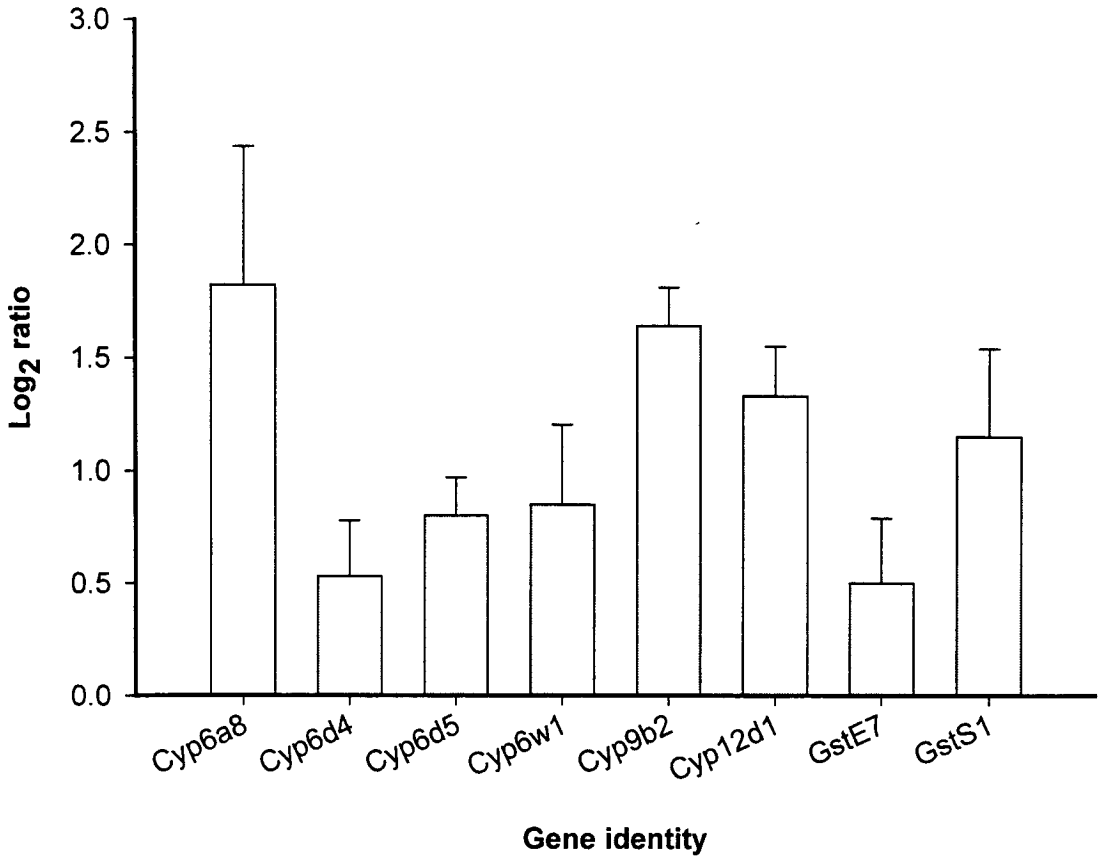

Figure 3.3: Identities and differential expression ratios of *D. melanogaster* genes implicated in phase I and phase II metabolism of toxins. These genes were upregulated four hours after insects were treated with 0.9 mg/mL of *P. nigrum*. The cytochrome P450 genes are associated with phase I metabolism and the glutathione S transferase genes are associated with phase II metabolism. Displayed are the mean (+SEM) $\log_2$ expression ratio values (based on two independent samples) for all valid microarray spots associated with a given gene.

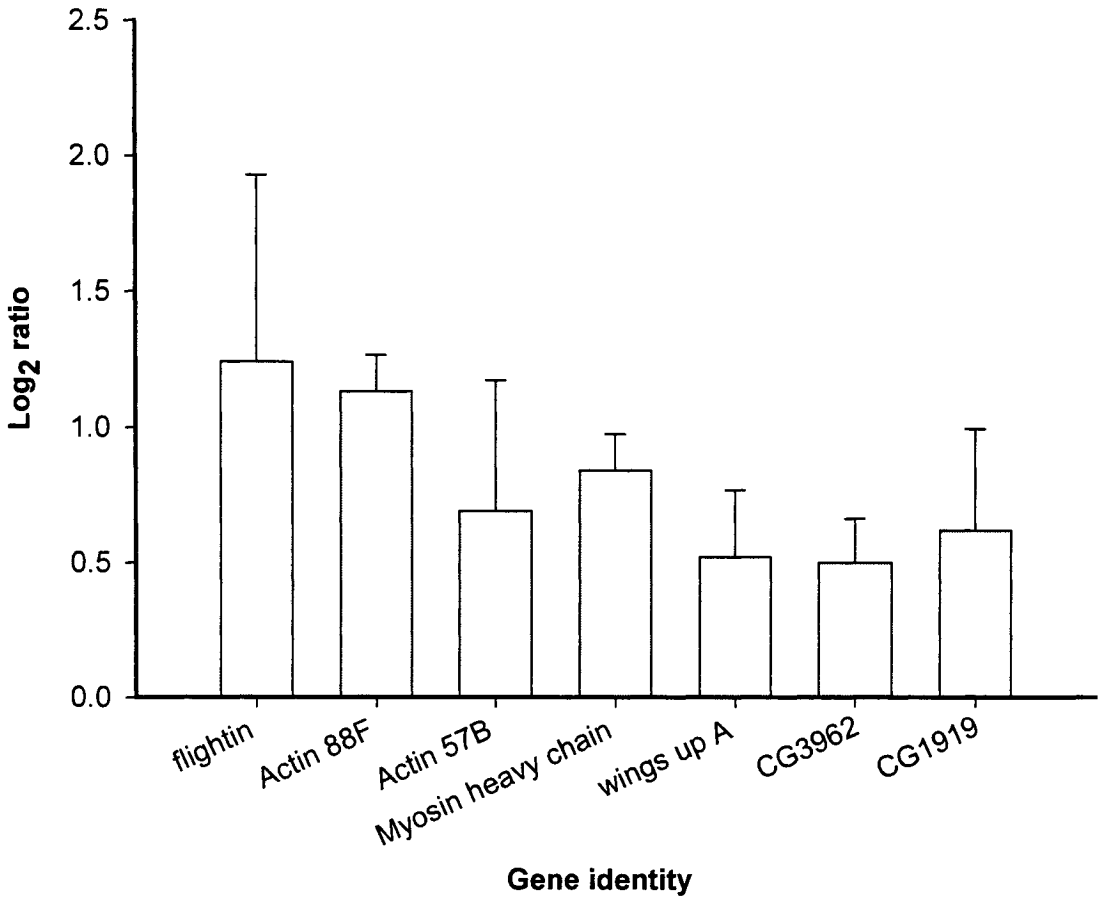

Figure 3.4: Identities and differential expression ratios of *D. melanogaster* genes related to muscles and cytoskeleton structure. These genes were all upregulated four hours after insects were treated with 0.9 mg/mL *P. nigrum*. Displayed are the mean (+SEM) $\log_2$ expression ratio values (based on two independent samples) for all valid microarray spots associated with a given gene.

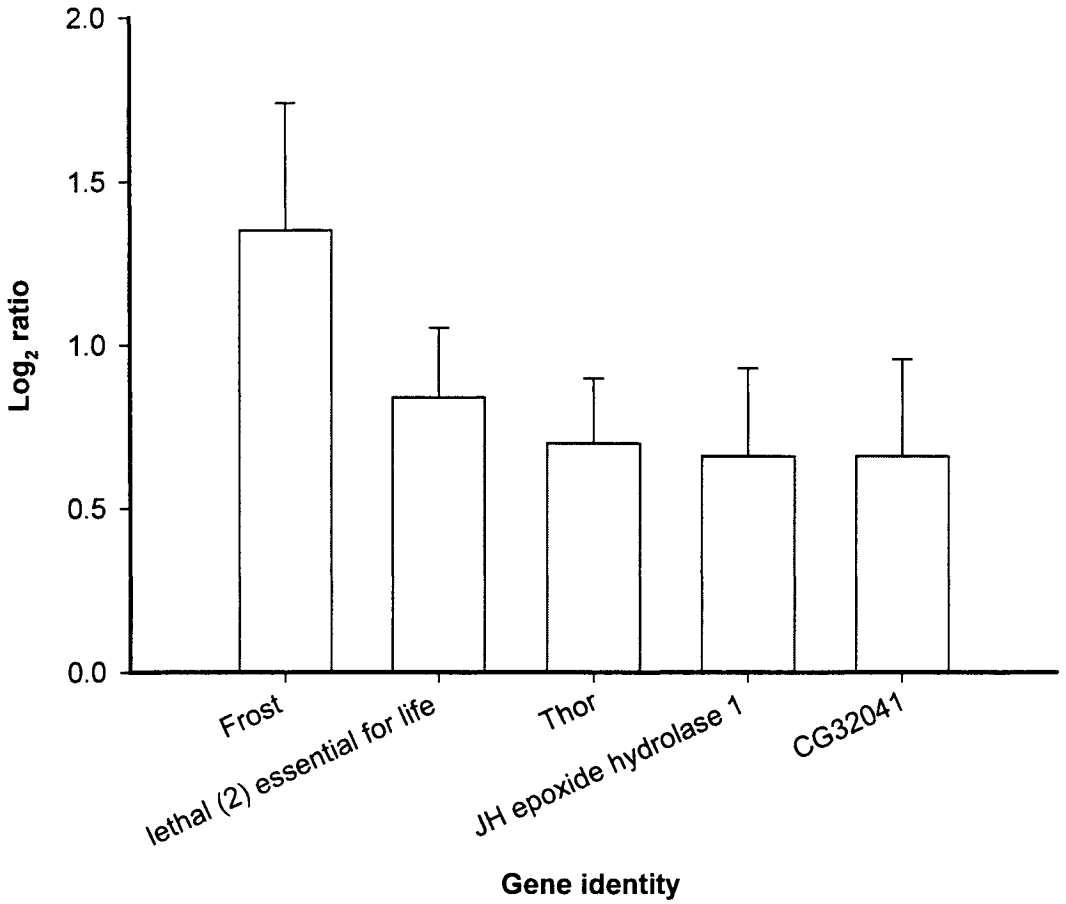

Figure 3.5: Identities and differential expression ratios of *D. melanogaster* genes implicated in the response to stress. These genes were all upregulated four hours after insects were treated with *P. nigrum*. Displayed are the mean (+SEM) $\log_2$ expression ratio values (based on two independent samples) for all valid microarray spots associated with a given gene.

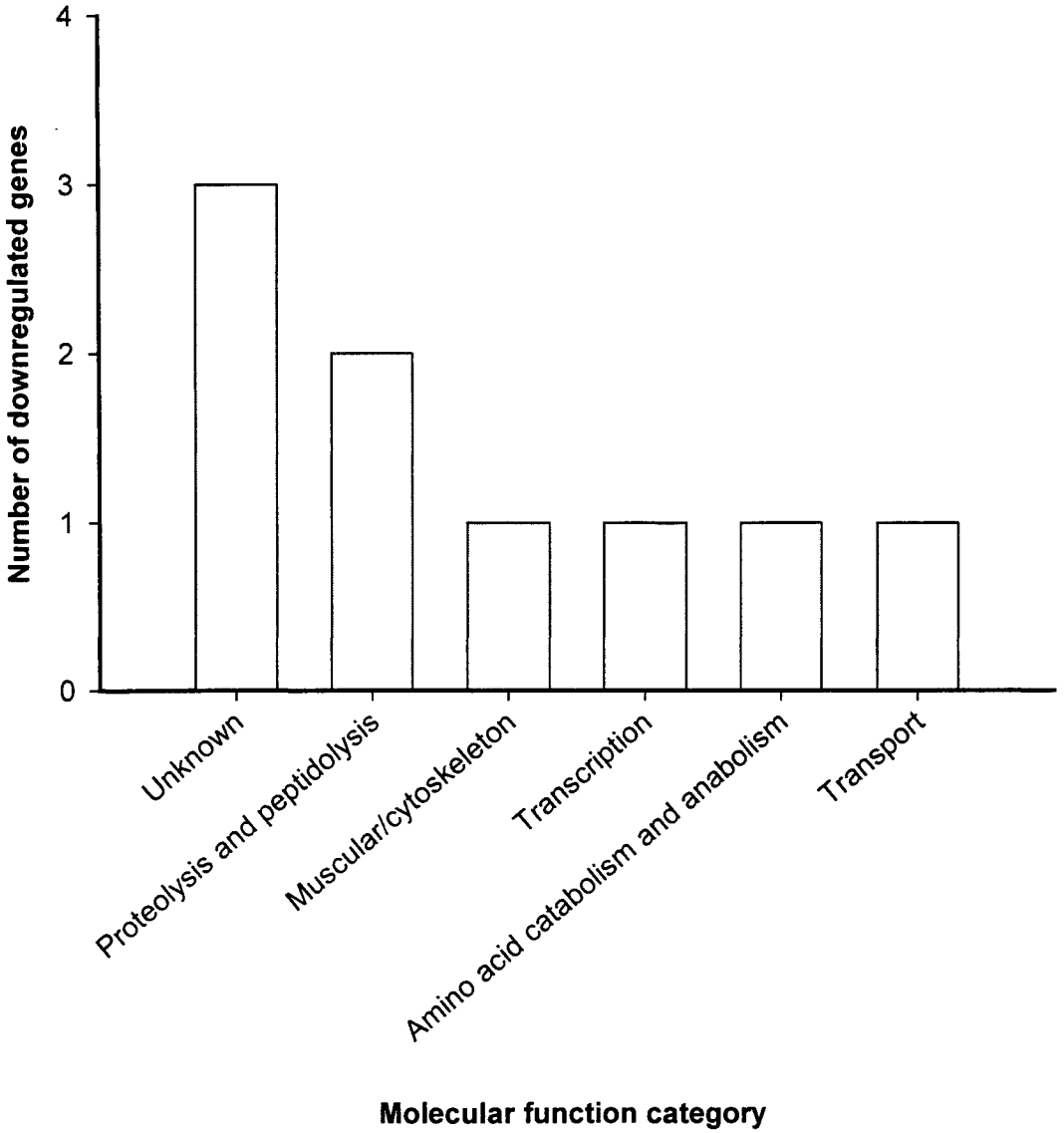

Figure 3.6: All *D. melanogaster* genes downregulated 1.41-fold or more by a treatment of 0.9 mg/mL *P. nigrum* as determined by cDNA microarray analysis. Genes were assigned categories according to their molecular function and associated biological process. A total of 9 genes were found to be downregulated.

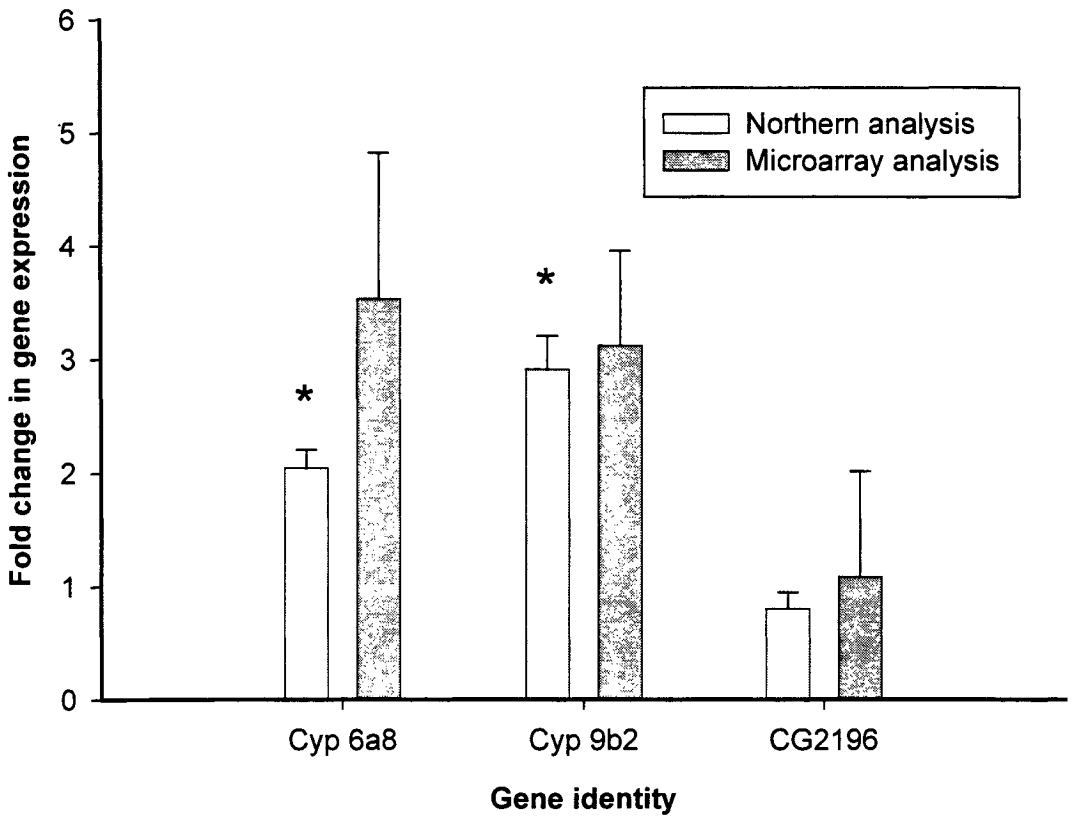

Figure 3.7: Relative mRNA expression levels represented as the fold-change from the control value of *Cyp 9b2*, *Cyp 6a8* and CG2196 in *D. melanogaster* adult females treated with an ethanol control or a 0.9 mg/mL *P. nigrum* extract and evaluated using Northern analysis and microarray analysis. In the Northern analysis separate membranes were used to evaluate each gene and each membrane was loaded with three RNA samples from the control treatment and three RNA samples from the *P. nigrum* treatment. Values from the Northern analysis marked with an asterisk are significantly different from the control value for the associated gene ($p<0.05$) in a 2-sample t-test (n=3, d.f.=4).

3.4 Discussion

This study investigated the effect of an insecticidal concentration of *P. nigrum* extract upon gene expression in *Drosophila melanogaster* with a particular emphasis upon genes involved in toxin metabolism. The main concern with the use of ethanol as a carrier solvent was that the strong induction of detoxification genes related to ethanol might mask or confound any gene induction related to *P. nigrum*. However, only three genes were found to be highly differentially expressed when test insects were treated with ethanol compared to water (table 3.2). These genes were excluded from the analysis of the other microarray experiments. It was concluded that the effect of the carrier solvent upon gene expression profiles induced by *P. nigrum* and other products was not an important confounding variable. This is probably because the exposure route is through contact rather than through dietary exposure and the majority of the ethanol is likely to evaporate rapidly after the treatment, thereby minimizing the effective dose. In the case of long-term dietary exposure to ethanol, cytochrome P450 enzymes have been induced (Scott *et al.*, 1996) but in the context of the current study this does not appear to be the case.

The *Hsc70-2* gene was upregulated in the EtOH treatment. It is a heat shock protein with chaperone activity associated with the mitochondrion. Previous research has found this gene to be ethanol induced in *Drosophila* neural cells (Wilke *et al.*, 1994). It is believed that this chaperone mediates the effects of ethanol on the central nervous system through the depletion of the calcium stores of the endoplasmic reticulum and glycoprotein processing (Wilke *et al.*, 1994).

There were two downregulated genes in the ethanol treatment. The m*RpL13* gene is a mitochondrial ribosomal protein and CG1703 is associated with the ATPase-coupled transmembrane transport function of ATP binding cassette (ABC) activity (The

Flybase Consortium, 2003). ABC proteins have two transmembrane domains and two cytosolic ATP binding domains which harness the energy necessary for the transport of substances from the cytoplasm to cellular compartments or into extracellular space (Lodish *et al.*, 2000). As such these proteins can be involved in defense mechanisms against toxins (Dean *et al.*, 2001). The presence of ABC transporter proteins has been linked to nicotine detoxification processes at the blood-brain barrier in the tobacco hornworm (Murray *et al.*, 1994). Interestingly, the downregulated CG1703 gene belongs to the ABCE subfamily which does not possess transmembrane domains and is not involved in transport in humans (Dean *et al.*, 2001). Due to strong homology between human and *Drosophila* genes of the ABCE family (Dean *et al.*, 2001) it is possible that this lack of true ABC function is also applicable to *Drosophila.* Further work would be required to elucidate the function of this gene. The differential expression of two genes of mitochondrial origin (*Hsc70-2* was upregulated and *mRpL13* was downregulated) suggests an effect of ethanol upon energy metabolism.

The gene expression profile exhibited by *D. melanogaster* in response to *P. nigrum* extract is indicative both of generalized organismal stress and of a specific response to a toxin (table 3.3, figure 3.2). The detoxification response includes six upregulated *Cyp* genes involved in phase I metabolism and 2 upregulated *Gst* genes involved in phase II metabolism (figure 3.3). The three *Cyp* genes with the highest differential expression ratios are *Cyp 6a8*, *9b2*, and *12d1.* The upregulation of *Cyp* genes is consistent with the biphasic effect of compounds with a MDP group such as piperonyl butoxide which causes an initial inhibition of CYP enzymes followed by induction (Dalvi and Dalvi, 1991). This second step of enzyme induction by piperine has been linked to an increase in RNA transcription in rat liver cells (Reen et al., 1996). The results of the current study suggest that this is also likely to be the case for the response of *D. melanogaster* to *P. nigrum* extract.

The CYP 6 family plays an important role in insecticide resistance. The *Cyp 6a8* gene which was upregulated in this study is characteristic of detoxification processes in insects and its constitutive overexpression in *D. melanogaster* confers DDT resistance (Maitra *et al.*, 1996). DDT-resistant strains of *D. melanogaster* have also been found to overexpress *Cyp 6a2* (Dombrowski *et al.*, 1998, Pedra *et al.*, 2004) and *Cyp 6g1* (Daborn *et al.*, 2001, Brandt *et al.*, 2002). These enzymes confer cross-resistance to other insecticides and share sequence similarities with the drug metabolizing CYP 3 family in humans (Lewis, 2001). Enzymes of the CYP 6 family are also implicated in insect defense against plant secondary metabolites. The *Cyp 6b1* and *Cyp 6b3* genes are induced in the gut tissues of the black swallowtail butterfly *Papilio polyxenes* after exposure to furanocoumarins (phototoxins present in the Apiaceae and Rutaceae plant families) (Petersen *et al.*, 2001). The implication of CYP 6 in the detoxification of synthetic products such as DDT and in the induced response to two different classes of plant defense chemicals (furanocoumarins and piperamides) denotes a response to a range of substrates. The evolved overexpression of CYP 6 enzymes in insecticide-resistant insects has been attributed to this broad range of substrates which can confer a higher degree of cross-resistance to other classes of insecticide. Of the CYP 6 family, CYP 6g1 has so far been thought to confer the broadest cross-resistance to synthetic insecticides (Le Goff *et al.*, 2003).

The upregulated *Cyp 9b2* gene encodes a microsomal enzyme with electron transporter activity. The CYP 9 family of enzymes is exclusive to arthropods (Lewis, 2001) and has not been studied as extensively as the *Cyp 6* family but there is still strong evidence that these enzymes play a role in detoxification processes. *Cyp 9a1* shares sequence similarities to the mammalian *Cyp 3* enzymes involved in drug metabolism and also to the insect *Cyp 6* enzymes involved in insecticide metabolism (Rose *et al.*, 1997). The constitutive overexpression of the *Cyp 9a1* gene in the tobacco

budworm *Heliothis virescens* is correlated with resistance to the carbamate insecticide thiodicarb (Rose et al, 1997). There is also some evidence that the CYP 9 family is involved in insect defense against plant toxins. Previous work has found that *Cyp 9a2* is induced in *Manduca sexta* after dietary exposure to 2-undecanone, a toxin derived from the wild tomato (Stevens *et al.*, 2000). In the current study *Cyp 9b2* is upregulated after *Drosophila* exposure to piperamides, reinforcing the link between the CYP 9 family and the metabolism of plant toxins. This is the first evidence linking *Cyp 9b2* to a specific biological response.

The upregulation of *Cyp 12d1* in this study suggests that this gene may also play a role in the detoxification of the *P. nigrum* extract. Prior studies have demonstrated that *Cyp 12d1* is constitutively overexpressed in two DDT-resistant strains of *Drosophila* and that in these strains the exposure to DDT results in a further induction of *Cyp 12d1* transcripts (Festucci-Buselli *et al.*, 2005). A genome-wide microarray analysis of DDT-resistant *Drosophila* strains has also identified *Cyp 12d1* as an important gene in insecticide resistance (Pedra *et al.*, 2004). The data presented in this study suggest that CYP 12d1 may also be involved in the detoxification of *P. nigrum* compounds and possibly other plant toxins and possess broad substrate binding capacities similar to the CYP 6 enzymes. There is a bias in the literature towards the study of these *Cyp* genes in relation to their constitutive overexpression in cases of insect resistance to DDT and other synthetic insecticides. This study highlights that *Cyp 6a8, 9b2* and *12d1* should be selected for further study with respect to their functions in the inducible detoxification processes of insects in relation to plant secondary metabolites.

The upregulated *Gst-S1* and *Gst-E7* genes are associated with glutathione S transferase activity or possibly glutathione peroxidase activity. GST enzymes catalyze the conjugation of glutathione to lipophilic compounds in order to increase their solubility and facilitate their excretion from the cell (Vontas *et al.*, 2002). Glutathione S transferase

activity is associated with phase II metabolism of toxins through conjugation (Syvanen *et al.*, 1994) while glutathione peroxidase activity is associated with physiological defenses against oxidative stress by conjugation of the products of lipid peroxidation (Singh *et al.*, 2001). Resistance to organophosphate and organochlorine insecticides has classically been attributed to constitutive overexpression of GST enzymes and recently some cases of pyrethroid resistance have also been linked to this same adaptation (Kostaropoulos *et al.*, 2001). The upregulation of these two *Gst* genes is probably linked to an increase in phase II metabolism activity to facilitate the elimination of the products of phase I metabolism associated with the upregulation of *Cyp* genes.

The upregulation of six different *Cyp* genes as well as two *Gst* genes indicates a classical detoxification response involving both phase I and II metabolism. It also indicates that the effect of the *P. nigrum* extract upon *Drosophila* is complex and may mean that at least eight different phase I and II metabolism enzymes are required in the detoxification process. If this is the case then it is possible that the evolution of resistance may require constitutive overexpression or modification of more than one detoxification gene thereby reducing the probability of the occurrence of resistance. This is in contrast to synthetic molecules to which insects can acquire resistance by overexpressing a single gene (Le Goff *et al.*, 2003). These results suggest that the development of resistance to a *P. nigrum*-based insecticide could be reduced relative to a classical insecticide composed of a single molecule although further work would be required to confirm this.

A second biological process category with a large number of upregulated genes was muscle function and cytoskeleton structure (figure 3.4). This is probably related to the observed knockdown effect of *P. nigrum* which causes uncoordinated movements which is, in turn, related to the neurotoxicity of the piperamides. A third biological process category with a large number of upregulated genes related to the response to

physiological stress (figure 3.5) and comprises genes with chaperone activity and oxidoreductase activity. This is to be expected considering the overall increase in transcription activity and also the acute toxic effects which may lead to oxidative stress. Both of these could lead to the need for chaperone molecules to mediate the proper folding of proteins and the repair of damaged proteins.

One upregulated gene named *Frost* has been tentatively associated with physiological stress (figure 3.5). The *Frost* gene identified has previously been induced in response to cold shock but not to heat shock in *Drosophila melanogaster* (Goto, 2001). The expression of this gene in response to *P. nigrum* may indicate that it is not induced solely by cold but by a broader range of stressors. Two other upregulated genes, CG7896 and *Astray,* were identified as being linked to the nervous system. The gene identified as CG7896 has receptor activity linked to the transmission of nerve impulses. The *Astray* gene has phosphoserine phosphatase activity which catalyzes the ATP driven final step of biosynthesis of L-serine from glycine (Voet *et al.*, 1999). In mammalian systems these reactions occur primarily in brain synapses (Wood *et al.*, 1996) indicating the probable occurrence of amino acid synthesis in neurons. The upregulation of these two nervous system genes is likely correlated with the neurotoxic activity of the amide moiety of the active *P. nigrum* chemicals (Hatakoshi *et al.*, 1984).

The upregulated *Aly* gene encodes a product with RNA binding and transcription coactivator activities. This is indicative of the increased synthesis of proteins that is occurring in response to the treatment. Three genes of unknown molecular function were also identified as being strongly upregulated and merit further investigation as to their functions.

The downregulated genes were fewer in number than the upregulated genes (table 3.2, figure 3.6). Limited information is available concerning the two most strongly downregulated genes with a fold-change in expression greater than 2 (table 3.2). The

gene identified as CG11892 which was downregulated in the *P. nigrum* treatment has also been found to be downregulated after four hours in *Drosophila* larvae in response to starvation (Zinke *et al.*, 2002). No further experiments were performed and the gene has not been characterized. The only unifying element between this study and the starvation study is the fact that in both cases this gene was downregulated when the organism was subjected to a stress. The second downregulated gene, CG5107, is expressed during the embryonic development of *Drosophila melanogaster* (Tomancak *et al.*, 2002). No function has been proposed for this gene. The expression of CG5107 in adult female insects exposed to *P. nigrum*, if corroborated by further study, may be indicative of a function in the organism that is not limited to development. The molecular function category associated with the largest number of downregulated genes was proteolysis and peptidolysis (figure 3.4) which probably indicates a shift in the protein synthetic machinery of the insects in order to account for the changes in gene expression occurring. This downregulation of proteolysis and peptidolysis associated genes may also be a part of the toxicity mechanism of the *P. nigrum* extract.

Northern blot analysis confirmed the upregulation of *Cyp 6a8* and *Cyp 9b2* in response to *P. nigrum* (figure 3.7). This makes these two genes especially interesting candidates for further study of their role in the interaction between *D. melanogaster* and *P. nigrum*. Because the Northern blot analysis was conducted with RNA samples collected from a different sample of *Drosophila* than the microarray analysis the upregulation of these two genes was definitely confirmed. Furthermore, the known detoxification functions of *Cyp 6a8* and glutathione S transferase S1 support the validity of the results obtained from this study. It can be stated with a reasonable degree of confidence that the genes of unknown function may also have roles in insecticide metabolism or be indicative of the specific response of *D. melanogaster* to *P. nigrum*.

3.5 Conclusion

The results of this study indicate that the use of cDNA microarrays is promising for identifying novel genes induced by natural products. Furthermore, many of the identified genes have previously been associated with exposure or resistance to synthetic pesticides but this data suggests that they are probably also important in the detoxification of plant secondary metabolites and that further study of these genes in the context of plant-insect interactions would be beneficial. The presence of genes previously associated in other studies with ethanol exposure, stress and insecticide metabolism provides a confirmation of the validity of the microarray platform for correctly identifying differentially expressed genes. The six *Cyp* genes identified (particularly *Cyp 6a8*, *9b2* and *12d1*) warrant further study of their role in metabolizing piperamides and in plant-insect interactions in general. The *Cyp 9b2* gene in particular has never been studied specifically in any biological context. The current study indicates that further study of the *Cyp 9b2* gene and the associated protein would be of interest in the context of insecticide metabolism and resistance. This study confirms previous work linking CYP 9 enzymes to insect defense against phytochemicals (Stevens *et al.*, 2000). The presence of two genes associated with neuron activity corroborates the neurotoxic activity of the piperamides. A novel biological process involving the defense response has been associated with the *frost* gene which has previously been associated only with cold stress. Three unknown genes were strongly upregulated and two were strongly downregulated in response to *P. nigrum*. Further study of these genes may lead to novel insights concerning physiological responses to stress and defense responses to plant secondary metabolites.

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Chapter 4: The effects of sublethal and synergistic doses of a *P. nigrum* extract used in conjunction with pyrethrin upon gene expression in *Drosophila melanogaster*

4.1 Introduction

Previous research has demonstrated that *P. nigrum* extract is an effective botanical insecticide against an array of economically important insects (Scott *et al.*, 2004). *Piper tuberculatum,* another insecticidal species, is not affected by previously evolved cross-resistance to multiple classes of insecticide in the Colorado potato beetle, *Leptinotarsa decemlineata* (Scott *et al.*, 2003). In the two previous chapters of this thesis it was demonstrated that *P. nigrum* extract is an effective synergist for pyrethrin and that it leads to the upregulation of phase I and II metabolism genes in *D. melanogaster.*

Natural pyrethrin is generally used in conjunction with the synthetic synergist piperonyl butoxide to increase its toxicity. At the physiological level, the effect of a synergist is often related to an interaction with transport and/or metabolism mechanisms or to a complementary physiological effect (Berenbaum, 1989). Piperonyl butoxide possesses both of these properties, leading to an inhibition of cytochrome P450 enzymes (Corbel *et al.*, 2002) and also to a potentiating effect on ATPase activity in neural cells (Kakko *et al.*, 2000). Piperine extracted from *P. nigrum* also demonstrates effects on toxin metabolism through the inhibition of cytochrome P450 enzymes (Bhardwaj *et al.*, 2002, Scott *et al.*, 2003) and on the nervous system by interacting with voltage-gated sodium channels (Hatakoshi *et al.*, 1984, McFerren *et al.*, 2002).

It was hypothesized that the synergy between *P. nigrum* extracts and pyrethrin and the effect of this synergy on the target insect is underscored at the molecular level by a characteristic differential gene expression profile distinct from that of either substance used individually. It is predicted that the synergistic mixture will induce a

gene expression profile in *Drosophila* of greater complexity than that of either product used alone involving an array of detoxification genes and also a greater number of genes specific to the neurotoxic properties of *P. nigrum* and pyrethrin. In this study the gene expression profiles of *D. melanogaster* exposed to sublethal concentrations of *P. nigrum* extract, pyrethrin or *P. nigrum* extract plus pyrethrin were compared in order to characterize the transient response of the expressed transcriptome of an insecticide-susceptible strain of *Drosophila* to these botanical insecticides and synergistic combinations. The data obtained is of interest in investigating the possible nature of the synergy.

4.2 Materials and Methods

4.2.1 Experimental Design

Microarray analysis was used in order to investigate the effects of sublethal concentrations of *P. nigrum* extract, pyrethrin and pyrethrin plus *P. nigrum* extract (equivalent to 1/5 of the LC_{50} value as previously determined in chapter two) upon gene expression in *D. melanogaster* after a 6 hour exposure time. The experimental design used was a direct comparison between treated and untreated RNA extracted from whole *Drosophila* adult females. The comparisons of interest occurred between the two channels (represented by the two fluorescent dyes, Cy3 and Cy5) present on the same microarray slide (Bowtell *et al.*, 2003) as well as between the individual experiments (*P. nigrum*, pyrethrin and pyrethrin plus *P. nigrum*). Replication was via independent samples of pooled adult female *Drosophila* (one sample=240 insects) which received either an ethanol control or a botanical product treatment. Sample size and the number of dye reversal hybridizations performed for each experiment are shown in table 4.1.

4.2.2 Insect culture

The *D. melanogaster* culture was maintained as described in chapter two (see section 2.2.1). Adult female flies aged 4-10 days were used in all experiments with no more than a five day range of ages for any given trial. Age was calculated as the number of days after egg hatch.

4.2.3 Natural products: *P. nigrum* and pyrethrin

P. nigrum and pyrethrin were prepared as described in chapter two (see section 2.2.2). For treatments all extracts were formulated in 99% ethanol. Final extract concentrations were as follows: *P. nigrum* 0.2 mg/mL, pyrethrin 0.04 mg/mL and

Table 4.1: Summary of the cDNA microarray hybridization groups along with treatments and dye labels analyzed and discussed in this study.

Treatment evaluated	Treatment (mg/mL)	Treatment label	Control	Control label	Time (Hrs)	N	Dye swaps
P. nigrum	0.2	Cy3	EtOH (99%)	Cy5	6	4	4
Pyrethrin	0.04	Cy3	EtOH (99%)	Cy5	6	4	0
Pyrethrin + *P. nigrum*	0.004 + 0.1	Cy3	EtOH (99%)	Cy5	6	4	4

pyrethrin 0.004 mg/mL plus *P. nigrum* 0.1 mg/mL. These concentrations were equal to 1/5 of the LC_{50} value of these extracts or extract combinations for adult *D. melanogaster* females (see chapter 2, section 2.3.2).

4.2.4 Insect treatments

The morning of the day prior to the experiment (between 9:00h and 12:00h), female insects were placed in 5 dram glass vials (60 insects/vial) plugged with cotton wool soaked in a 5% sucrose solution. At approximately 9:30h the next morning the flies were anaesthetized on ice, placed on a 90 mm diameter Whatman filter paper in a 90 mm Petri plate and sprayed with a total of 2 mL of the appropriate extract solution or of a 99% ethanol control. This was done using a Potter's tower set to a pressure of 5 psi (34.5 KPa) with nitrogen serving as the carrier gas.

Flies were returned to the vials and placed in the growth chamber for six hours. At this time (approximately 15:30h) any dead flies were removed from the samples and the remaining flies were anaesthetized, flash frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. Flies from four vials were pooled to give samples of approximately 240 flies to ensure a sufficient yield of RNA. Samples to be pooled received the same treatment at the same time to minimize variation.

4.2.5 Potter's tower calibration and extract characterization using phytochemical analysis

After the insect treatments were completed, six blank plates were sprayed with 2 mL of 0.2 mg/mL of *P. nigrum* or with 0.004 mg/mL pyrethrin plus 0.1 mg/mL *P. nigrum* in order to determine the application rate of the extract used. These plates were rinsed with 2 mL of 99% ethanol in order to recover the extract residues. Samples were prepared and analyzed for the presence of the marker compound piperine as described in chapter two (section 2.2.4). The quantity of piperine on the Petri plates for the *P.*

nigrum treatment was 0.236 µg/cm^2 with a standard error of 0.014 (n=6) and for the pyrethrin plus *P. nigrum* treatment the quantity of piperine on the Petri plates was 0.112 µg/cm^2 with a standard error of 0.006 (n=6) (appendix III). The *P. nigrum* stock solution used in both treatments was characterized in terms of the concentration of four insecticidal piperamides: 4,5-dihydropiperlonguminine, piperlonguminine, 4,5-dihydropiperine and piperine (appendix III). Overall, the phytochemical data demonstrated that the applied concentration of the marker compound piperine using the Potter's tower was replicable and that there was consistency between the piperamides present in the stock solutions for the two different treatments.

4.2.6 RNA extraction

Total RNA was isolated using the acid guanidium thiocyanate-phenol-choloroform extraction method (Chomczynski and Sacchi, 1987) as described in chapter 3 (section 3.2.4) and stored at -80°C prior to being shipped on dry ice to the Canadian *Drosophila* Microarray Center (CDMC) for further processing.

4.2.7 cDNA microarrays

Microarray slides were purchased from the CDMC (www.flyarrays.com). The *Drosophila* array version 7K3 was used in all experiments. This array contains 5756 target cDNAs from the Berkeley *Drosophila* Genome Project, 1078 cDNAs from the National Institutes of Health *Drosophila* testis cDNA library, and 546 gene fragments amplified from genomic DNA (Neal *et al.*, 2003). The arrays were laid out in 32 subarrays, each with 22 rows and 24 columns with two replicates of each gene for a total of 16 896 spots (Neal *et al.*, 2003). Replicate spots were located adjacent to each other on the slides (Neal *et al.*, 2003). Direct labeling of RNA and subsequent hybridization to microarray slides and scanning using the ScanArray 4000 XL (GSI Lumonics/Packard

Biochips) was performed at the CDMC. Protocol details concerning labeled cDNA synthesis, hybridizations reactions and scanner settings are described in Neal *et al.* (2003). For each sample, 80 µg of total RNA was labeled with either cyanine 3 or cyanine 5 (Cy3 or Cy5) dye. Dye-reversal hybridizations were performed for each sample pair (with the exception of the pyrethrin experiment) in order to test for differences in dye binding and fluorescence.

4.2.8 Analysis of microarray data

Quantarray Microarray Analysis Software version 3.0 (Packard BioScience, copyright 2001) was used to quantify the raw images resulting from scans of the microarray slides. Images from the Cy3 and Cy5 channels of the same slide were superimposed, spots were located and those with visually obvious defects were manually flagged for exclusion from analysis. Spot and background intensity were measured using the adaptive circle quantification method which statistically determines the threshold of significant spot fluorescence relative to background fluorescence (Packard BioScience, 2001). The resulting data files were exported to Gene Traffic™ Duo, version 2.8 (Iobion Informatics, copyright 2002) for further analysis.

Data normalization was performed on background subtracted spots on a subgrid basis using the Locally Weighted Scatter Plot Smoother (LOWESS) algorithm with a smoothing factor of 20. This procedure normalizes the intensity value for each spot based upon the data distribution for a subset of the intensity range, centered on the spots' intensity value (Leung *et al.*, 2003). This prevents the normalization of spots from being affected by extreme intensity values. Spots with intensity values less than 100 units and spots with intensity values below the average background intensity value and/or below the local background intensity value were excluded from normalization and analysis. Data was filtered so as to exclude all genes with less than two-thirds of spots

being usable (i.e. less than 11 valid spots for *P. nigrum* and pyrethrin plus *P. nigrum* and less than 6 valid spots for pyrethrin) as defined by quality filters and those whose mean differential expression ratio had a coefficient of variance higher than 100%.

The ethanol versus ethanol (like-like) hybridization described in chapter 3 (section 3.2.1) which compared two samples that received identical treatments was used to select cutoff values to identify differentially expressed genes in the context of this specific microarray platform and experimental design. The selected cutoff $\log_2$ ratios for differential expression for this microarray platform were equal to 0.5 and -0.5, equal to a 1.41-fold change in expression. See appendix IV for a table of $\log_2$ ratio conversions. The range between these two values encompassed over 99% of the differential expression ratios for the like-like hybridization. This was therefore considered to be an appropriate threshold value for differential expression.

The data was examined first for all three treatments separately (i.e. a comparison between the two channels present on a single slide) to examine the effect of each separate product. Genes were tabulated using the *Drosophila* genome annotation available in flybase (http://flybase.bio.indiana.edu/). Genes were presented in tables classified by their molecular function and biological process as listed in the gene ontology (GO) page of Flybase. For the pyrethrin treatment a large number of genes were differentially expressed and both downregulated and upregulated genes were presented together in order to show genes with the same molecular function grouped together. For the *P. nigrum* and the pyrethrin plus *P. nigrum* treatments there was a small number of differentially expressed genes and they were categorized as either upregulated or downregulated for presentation in tables. For information concerning the fold-change, $\log_2$ ratio and standard deviation associated with each differentially expressed gene see appendix V. For the pyrethrin treatment genes were subsequently divided into categories representing the known or inferred (based on sequence

similarity) role they play in the organism based on the molecular function and/or biological process data. Gene expression profiles between the individual experiments (*P. nigrum*, pyrethrin and pyrethrin plus *P. nigrum*) were compared among each other to identify common differentially expressed genes. Cluster analysis was not considered appropriate for this data set. All microarray data will be submitted to the Gene Expression Omnibus (GEO) database and become part of the public domain upon publication of this research in a peer-reviewed journal.

4.2.9 Quantitative RT-PCR

Real-time quantitative polymerase chain reaction (RT-QPCR) was used to confirm the expression patterns of sample genes found to be upregulated or downregulated in the microarray experiments. For each of the three microarray experiments 2 genes were selected for study (1 upregulated and 1 downregulated). Ribosomal protein L32 (*RpL32*) was used as an internal control to normalize results. See table 4.2 for a list of the genes tested for each experiment. A second cohort of adult *Drosophila* females were treated so as to replicate the 0.2 mg/mL *P. nigrum*, 0.004 mg/mL pyrethrin and 0.04 mg/mL pyrethrin plus 0.1 mg/mL *P. nigrum* treatments used for the microarray experiments. Total RNA was isolated as previously described.

The total RNA samples were DNase digested to remove any possible contamination of the samples with genomic DNA. Reactions were prepared in 600 µL PCR tubes (25 µg RNA, 25 uL RNase-free DNase (Promega), 10 µL of 10X reaction buffer (Promega) and DEPC-treated H_2O for a total volume of 100 µL) and incubated for 30 minutes at 37°C. A 10 µL volume of Stop Solution (Promega) was then added to the reactions followed by a 10 minute incubation at 65°C. Incubations were carried out using a Hybaid thermal cycler. The DNase was removed from the reactions using the RNeasy Mini Kit (QIAGEN) as per the instructions of the RNA cleanup protocol. Briefly,

Table 4.2: List of the *D. melanogaster* genes selected for RT-QPCR confirmation of the upregulation or downregulation trends identified by cDNA microarray analysis in response to treatment of adult females with *P. nigrum*, pyrethrin or pyrethrin + *P. nigrum*. Genes are identified by their Flybase Curated Gene number (CG) and their abbreviation when available.

Experiment	Upregulated genes	Downregulated gene	Internal control gene
P. nigrum	CG13091	CG5185 (*Tom*)	CG7939 (*RpL32*)
Pyrethrin	CG13091 CG4533 (*l(2)efl*)	CG5185 (*Tom*)	CG7939 (*RpL32*)
Pyrethrin + *P. nigrum*	CG4533 (*l(2)efl*)	CG5185 (*Tom*)	CG7939 (*RpL32*)

the 110 µL samples resulting from the DNase treatment were added to 350 µL of Buffer RLT (QIAGEN) containing 10 µL/mL β-mercaptoethanol. A 240 µL volume of 99% ethanol was added and samples were mixed and applied to RNeasy mini columns which were centrifuged at 8000 x g for 15 seconds. A 500 µL volume of Buffer RPE (QIAGEN) diluted 1:4 with 99% ethanol was applied to each column and centrifuged at 8000 x g for 15 seconds. A second 500 µL volume of Buffer RPE was applied to the columns followed by centrifugation at 8000 x g for 2 minutes. RNA was eluted from the RNeasy column using 30 µL of RNase-free H_2O and was quantified using a Pharmacia Gene Quant DNA/RNA calculator spectrophotometer as described in chapter 3 (section 3.2.4).

The resulting RNA was used as a template to synthesize cDNA. Reactions were prepared in PCR tubes and consisted of 1 µg RNA, 1 µL random hexamers (200 ng/µL, Invitrogen), and DEPC-treated H_2O to a final volume of 10 µL. Reactions were incubated in a Hybaid thermal cycler for 10 minutes at 70°C and the temperature was subsequently lowered to 4°C. At this time the following reagents were added: 4 µL of 5X First Strand Buffer (Invitrogen), 2 µL of 0.1M DTT (Invitrogen), 1 µL of a 10 mM dNTP mix (Invitrogen) and 1 µL of RNAse inhibitor (1:5 dilution, Invitrogen). Reactions were incubated at 42°C for 2 minutes, 2 µL of Superscript II reverse transcriptase (Invitrogen) was added and the reaction was incubated at 42°C for 50 minutes and inactivated by heating at 70°C for 15 minutes. Selected control samples were incubated in the absence of the reverse transcriptase enzyme in order to verify the absence of DNA contamination. The cDNA obtained was used as a template in RT-QPCR.

Primers for CG13091, CG5185 and CG4533 were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) for optimal use with SYBRgreen fluorescent dye technology (product size ranging from 150-400 bp, primers 15-30 bp in length, 40-60% GC content, melting temperature 55-60°C). Oligo Analyzer software (http://molbiol-tools.ca/molecular_biology_freeware.htm) was used to choose

primer pairs with a minimum of secondary structure and low potential for self-annealing and formation of primer-dimers. Primers sequences designed for the quantification of *RpL32* using RT-QPCR were obtained from a published study (Gabbler *et al.*, 2005). Primer sequences and amplicon sizes are listed in table 4.3. Primers were synthesized and HPLC purified by Invitrogen. All primers were tested in standard PCR reactions prior to use in RT-QPCR. PCR reactions were carried out in 25 µL volumes with each reaction consisting of: 1 µL template DNA (cDNA), 2.5 µL 10X PCR buffer (Invitrogen), 0.5 µL of a 10 mM solution of each dNTP, 0.75 µL of 50 mM $MgCl_2$ (Invitrogen), 0.2 µL *Taq* DNA polymerase (Invitrogen), 1 µL of each primer (from a 10 µM stock) and 16.5 µL DEPC treated H_2O. Thermal cycler settings consisted of an initial 3 minute denaturation at 94°C, followed by 35 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 60°Cand extension for 30 s at 72°C and concluding the PCR with a 5 minute final extension step at 72°C. PCR products were verified for purity and size by running on a 1% agarose gel at 100 volts and visualizing with ethidium bromide. Remaining PCR products were purified using Microspin S-300 HR columns (Amersham Biosciences) and sent for sequencing to the CDSSF. Sequences were verified using NCBI BLASTn (Altschul, 1990) and were all found to have greater than 97% identity with their target amplicons.

Real-time QPCR was used to quantify the relative copy number of the four selected genes in cDNA samples. The Brilliant® SYBR® Green QPCR Master Mix kit (Stratagene) was used for all RT-QPCR experiments. Reactions were carried out in 25 µL volumes with each reaction consisting of: 2 µL template DNA (cDNA), 0.38 µL each of forward and reverse primers (from a 10 µM stock, final concentration of 150 nM each), 12.5 µL of Brilliant® SYBR® Green QPCR Master Mix, 0.38 µL of Reference dye (1:500 dilution) and 9.38 µL of autoclaved DDH_2O (Invitrogen). Thermal cycling and dye detection were carried out using an Mx4000® instrument (Stratagene). Basic run

Table 4.3: List of the primers used in RT-QPCR analysis of selected genes. Primer sequences are listed in 5'-3' order.

Gene	Forward primer	Reverse primer	Product (bp)
CG13091	CCCTTCAACTTGTACTTTGC	ACGCACGAGCATATAACTTT	250
CG4533	CGTAGTGCCACTGATGTTC	ATCCAGAATGACCTCGAACT	247
CG5185	CTACCAACTCTCAACCGAGT	GTTGACTGCATTTTGGTTCT	256
CG7939	TGTCCTTCCAGCTTCAAGATGACCATC	CTTGGGCTTGCGCCATTTGTG	132

parameters consisted of an initial 10 minute denaturation at 95°C, followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 60 s at 53°Cand extension for 60 s at 72°C and concluding the run with a dissociation curve run on a gradient between 53°C and 95°C. Fluorescence detection and reporting occurred during the annealing step of the cycle for all genes with the exception of CG5185 where an additional 8 s detection step was added at 79.5°C. Products were verified for purity using dissociation curve analysis and also by running them on a 1% agarose gel at 100 volts and visualizing with ethidium bromide.

A standard curve for each gene was established using a composite cDNA sample from the 6 different RNA pools (3 controls and 3 treatments). A 2-fold dilution of the original sample was followed by five-fold serial dilutions to give relative concentrations of 25, 5, 1, 0.2, 0.04, and 0.008 ng/ µL of cDNA (based on the original quantity of RNA used to synthesize the cDNA). These samples were analyzed in replicates of 3 in the RT-QPCR programs described above for each primer set and used to create a standard curve for each gene. Quality criteria for the standard curve were to have an R^2 value greater than or equal to 0.985 and reaction efficiency between 80% and 120%. These were the recommended standard curve parameters for the Brilliant® SYBR® Green QPCR Master Mix kit (Stratagene). The cDNA samples were analyzed for all applicable genes in the RT-QPCR programs along with no-template and no reverse-transcriptase controls to monitor for contamination from external sources and genomic DNA. See appendix VI for an example of RT-QPCR data.

The RT-QPCR data was normalized and processed prior to statistical analysis. First, for each sample, the relative quantity of cDNA for the gene of interest was normalized to the control gene [cDNA quantity (gene of interest) / cDNA quantity (*RpL32)*]. Second, the average cDNA quantity of the test gene for the control samples was determined. Third, the normalized value of the test gene for each sample was

divided by the average value of the control group to determine the fold-change in cDNA quantity. Finally, the average fold-change and standard deviation was determined for each control and treatment group. Statistical analysis was performed using SPSS software (version 12.0, SPSS Inc., copyright 2003). Control values were all identical due to the normalization procedure (normalized to equal 1) and were pooled to increase power. For each gene a one-way ANOVA was performed comparing fold-change in gene expression across the relevant treatments followed by Dunnett`s one-sided pairwise comparisons. Dunnett's test was used because it is appropriate in cases where the comparisons of interest are limited to the value for each treatment compared to the control.

4.3 Results

4.3.1 Microarray results

Differential expression was defined as a 1.41-fold change in expression (this translates to a $\log_2$ ratio greater than or equal to 0.5 or less than or equal to –0.5, see appendix IV for a table of $\log_2$ ratio conversions). This was based on the results of an experiment comparing the differential expression ratios between samples which had received identical treatments. It was demonstrated that above a threshold fold-change value of 1.41 there were no changes in gene expression due to random variation (see sections 3.2.1, 3.3.1 and 3.4.) The exposure of *D. melanogaster* to *P. nigrum* alone resulted in the upregulation of 3 genes including CG13091 which has oxidoreductase activity (table 4.4). The only gene downregulated in the *P. nigrum* treatment was the *Tom* gene of unknown molecular function involved in the Notch signaling pathway table 4.4). Exposure to *P. nigrum* plus pyrethrin resulted in the upregulation of 5 genes including *lethal (2)essential for life* (*l(2)efl*) which encodes a product with a heat shock protein 20 domain involved in defense responses (table 4.5). The two downregulated genes in this treatment included the *Tom* gene which was also downregulated in the *P. nigrum* treatment (table 4.5).

Exposure to pyrethrin alone resulted in the upregulation of 70 genes and the downregulation of 25 genes (table 4.6). The most important categories of upregulated genes in the pyrethrin treatment (other than genes of unknown function) were: stress/defense response, proteolysis and peptidolysis, muscular/cytoskeleton, transport, RNA metabolism and catabolism and phase I and II metabolism (figure 4.1). Genes of note involved in the stress/defense response include five upregulated genes associated with heat shock proteins: *heat shock protein 68* (CG5436), the *heat shock factor* (CG5748) that functions as a transcription activator, *heat shock protein cognate 2*

Table 4.4: *D. melanogaster* genes upregulated or downregulated in adult females treated with 0.2 mg/mL *P. nigrum* as determined by cDNA microarray analysis. Mean fold-change in expression is reported along with gene ontology information for the molecular function and biological process of each gene.

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated genes						
oxidoreductase activity	GO:0016491	--	--	--	CG13091	1.73
pre-mRNA splicing factor activity	GO:0008248	nuclear mRNA splicing, via spliceosome	GO:0000398	*snRNP70K*	CG8749	1.60
nicotinic acetylcholine-activated cation-selective channel activity	GO:0004889, GO:0016904	ion transport	GO:0006811	*nAcRβ-21C*	CG11822	1.44
Downregulated genes						
--	--	Notch signaling pathway	GO:0007219, GO:0030179	*Tom*	CG5185	-1.58

Table 4.5: *D. melanogaster* genes upregulated or downregulated in adult females treated with 0.1 mg/mL *P. nigrum* plus 0.004 mg/mL pyrethrin as determined by cDNA microarray analysis. Mean fold-change in expression is reported along with gene ontology information for the molecular function and biological process of each gene.

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated genes						
transporter activity	GO:0005215	transport	GO:0006810	--	CG6640	1.77
glucose transporter activity	GO:0005355	carbohydrate transport	GO:0008643, GO:0006861, GO:0008644	--	CG6484	1.52
Structural molecule activity	GO:0005198	development	GO:0007275	--	CG2330	1.59
--	--	defense response	GO:0006952	*l(2)efl*	CG4533	1.68
--	--	--	--	--	CG10383	1.69
Downregulated genes						
--	--	Notch signaling pathway	GO:0007219, GO:0030179	*Tom*	CG5185	-1.48
--	--	--	--	--	CG13333	-1.45

Table 4.6: *D. melanogaster* genes upregulated or downregulated in adult females treated with 0.04 mg/mL pyrethrin as determined by cDNA microarray analysis. Mean fold-change in expression is reported along with gene ontology information for the molecular function and biological process of each gene.

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated and downregulated genes of known function						
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp 9f2*	CG11466	1.46
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp 12d1*	CG18240	1.53
oxidoreductase activity	GO:0016491	--	--	--	CG13091	1.61
calcium ion binding	GO:0005509	protein folding	GO:0006457	--	CG1924	1.55
calcium ion binding	GO:0005509	defense response	GO:0006952	*AnnX*	CG9579	1.52
calcium ion binding	GO:0005509	protein folding	GO:0006457	--	CG9906	1.78
calmodulin binding	GO:0005516	muscle contraction	GO:0006936	*TpnC41C*	CG2981	-1.78
actin binding	GO:0003779	muscle contraction	GO:0006936	*Tm2*	CG4843	1.61
tubulin binding	GO:0015631	microtubule-based movement	GO:0007018	*αTub67E*	CG8308	2.20
tubulin binding	GO:0015631	microtubule-based movement	GO:0007018	*αTub85E*	CG9476	2.22
unfolded protein binding	GO:0051082	defense response	GO:0006952	*Hsp68*	CG5436	2.39
unfolded protein binding	GO:0051082	defense response	GO:0006952	*Hsc70-2*	CG7756	2.14
protein binding	GO:0005515, GO:0045308	mitosis	GO:0007067	*Smt3*	CG4494	-1.53
ATP binding	GO:0005524	defense response	GO:0006952	--	CG2918	1.49
ATP binding	GO:0005524	intracellular signaling cascade	GO:0007242	--	CG7156	1.44
GTP binding	GO:0005525	cell proliferation	GO:0008283	--	CG8801	1.65
specific RNA polymerase II transcription factor activity	GO:0003704	defense response	GO:0006952	--	CG5748	1.43

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated and downregulated genes of known function (cont.)						
RNA polymerase II transcription factor activity	GO:0003702	regulation of transcription, DNA-dependent	GO:0006355	*Cf2*	CG11924	-1.41
transcription regulator activity	GO:0030528	transcription from Pol II promoter	GO:0006366	--	CG8165	1.54
serine-type endopeptidase inhibitor activity	GO:0004867	response to pest, pathogen or parasite	GO:0009613	*Spn27A*	CG11331	1.75
superoxide dismutase activity	GO:0004784	superoxide free radical metabolism	GO:0006801	--	CG9027	1.57
serine-type endopeptidase activity	GO:0004252	proteolysis and peptidolysis	GO:0006508	--	CG9631	1.44
serine-type endopeptidase activity	GO:0004252	proteolysis and peptidolysis	GO:0006508	--	CG17012	-1.66
endopeptidase activity	GO:0004175, GO:0016809	proteolysis and peptidolysis	GO:0006508	*Dox-A2*	CG10484	1.61
cathepsin K activity	GO:0004216	proteolysis and peptidolysis	GO:0006508	--	CG4847	1.46
cathepsin B activity	GO:0004213	proteolysis and peptidolysis	GO:0006508	--	CG10992	1.54
dipeptidyl-peptidase III activity	GO:0017039	proteolysis and peptidolysis	GO:0006508	*DppIII*	CG7415	1.47
GABA-A receptor activity	GO:0004890	nerve-nerve synaptic transmission	GO:0007270	*Rdl*	CG10537	1.45
structural molecule activity	GO:0005198	transmission of nerve impulse	GO:0019226	*NetB*	CG10521	1.55
monoamine transporter activity	GO:0008504, GO:0015201	neurotransmitter transport	GO:0006836	--	CG6119	2.38
t-SNARE activity	GO:0005486	neurotransmitter secretion	GO:0007269	*Syx17*	CG7452	1.49

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated and downregulated genes of known function (cont.)						
hydrogen-transporting ATPase activity, rotational mechanism	GO:0046961	ATP synthesis coupled proton transport	GO:0015986	--	CG6687	1.82
oligopeptidase A activity	GO:0008944	proteolysis and peptidolysis	GO:0006508	--	CG11771	1.43
structural constituent of cytoskeleton	GO:0005200	cytoskeleton organization and biogenesis	GO:0007010	*Act5C*	CG4027	2.62
structural constituent of cytoskeleton	GO:0005200	protein metabolism	GO:0019538, GO:0006411	--	CG8918	1.52
structural constituent of cytoskeleton	GO:0005200	cytoskeleton organization and biogenesis	GO:0007010	*Act57B*	CG10067	1.43
structural constituent of cuticle (sensu Insecta)	GO:0005214	--	--	--	CG8505	-1.65
hydrolase activity	GO:0016787	polysaccharide metabolism	GO:0005976	--	CG30059	1.88
lipase activity	GO:0016298	lipid metabolism	GO:0006629	--	CG18858	1.8
protein carrier activity	GO:0008320	intracellular protein transport	GO:0006886	--	CG9053	1.42
nucleocytoplasmic transporter activity	GO:0005487	nucleocytoplasmic transport	GO:0006913, GO:0000063	--	CG8831	1.43
succinate-CoA ligase (GDP-forming) activity	GO:0004776	tricarboxylic acid cycle	GO:0006099	--	CG6255	1.55
succinate-semialdehyde dehydrogenase activity	GO:0004777, GO:0008952	amino acid catabolism	GO:0009063	--	CG4685	1.82
hydrolase activity, hydrolyzing N-glycosyl compounds	GO:0016799	signal transduction	GO:0007165	*Idgf3*	CG4559	1.43

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated and downregulated genes of known function (cont.)						
hydrolase activity, hydrolyzing N-glycosyl compounds	GO:0016799	protein amino acid glycosylation	GO:0006486	--	CG12582	1.65
pre-mRNA splicing factor activity	GO:0008248	nuclear mRNA splicing, via spliceosome	GO:0000398	*snRNP70K*	CG8749	1.55
nucleic acid binding	GO:0003676	rRNA processing	GO:0006364	--	CG8939	1.45
ATP-dependent RNA helicase activity	GO:0004004	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	GO:0006139	--	CG7878	1.77
pantothenate kinase activity	GO:0004594	coenzyme metabolism	GO:0006732	*Fbl*	CG5725	1.52
lysophosphatidate acyltransferase activity	GO:0004469	metabolism	GO:0008152	--	CG3209	1.52
protein serine/threonine phosphatase activity	GO:0004722	mitosis	GO:0007067	*Mts*	CG7109	1.45
chromatin binding	GO:0003682	mitosis	GO:0007067	*GM130*	CG11061	1.88
choline dehydrogenase activity	GO:0008812	electron transport	GO:0006118	--	CG9512	1.67
peptidoglycan binding	GO:0042834	detection of bacteria	GO:0016045	*PGRP-SC1b*	CG8577	-1.51
transcription regulator activity	GO:0030528	regulation of transcription from Pol II promoter	GO:0006357	*E(bx)*	CG10894	-1.42
antigen binding	GO:0003823	signal transduction	GO:0007165	*Ama*	CG2198	-1.66
DNA binding	GO:0003677	chromatin assembly or disassembly	GO:0006333	*His2Av*	CG5499	-1.41
DNA binding	GO:0003677	dosage compensation	GO:0007549	*Msl-1*	CG10385	-1.46

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated and downregulated genes of known function (cont.)						
peptidyl-prolyl cis-trans isomerase activity	GO:0003755, GO:0004752	protein folding	GO:0006457	--	CG1475	-1.47
N-acetylgalactosamine-4-sulfatase activity	GO:0003943	phospholipid metabolism	GO:0006644	--	CG7408	-1.46
receptor binding	GO:0005102	--	--	--	CG3494	-1.42
--	--	Notch signaling pathway	GO:0007219, GO:0030179	*Tom*	CG5185	-1.92
--	--	mitosis	GO:0007067	--	CG31687	-1.68
--	--	transmission of nerve impulse	GO:0019226	*fax*	CG4609	-1.42
--	--	mRNA catabolism, nonsense-mediated decay	GO:0000184	*Smg6*	CG6369	1.44
--	--	defense response	GO:0006952	*L(2)efl*	CG4533	1.79
Upregulated genes of unknown function						
--	--	--	--	--	CG1600	1.55
--	--	--	--	--	CG2207	1.91
--	--	--	--	--	CG3305	1.56
--	--	--	--	--	CG6073	1.48
--	--	--	--	--	CG6621	1.54
--	--	--	--	--	CG6640	1.79
--	--	--	--	--	CG7874	1.57
--	--	--	--	*ocn*	CG7929	1.74
--	--	--	--	--	CG8289	1.46
--	--	--	--	--	CG8949	1.49
--	--	--	--	--	CG9083	1.41
--	--	--	--	--	CG9641	1.73

Molecular function	GO code	Biological process	GO code	Gene symbol	CG identifier	Fold change
Upregulated genes of unknown function (cont.)						
--	--	--	--	--	CG9759	1.45
--	--	--	--	--	CG9978	1.61
--	--	--	--	--	CG10383	1.41
--	--	--	--	--	CG10841	1.46
--	--	--	--	--	CG14207	1.47
--	--	--	--	--	CG14998	1.44
--	--	--	--	--	CG16772	1.60
--	--	--	--	--	CG16782	1.85
Downregulated genes of unknown function						
--	--	--	--	*fln*	CG7445	-1.46
--	--	--	--	--	CG7637	-1.57
--	--	--	--	--	CG13333	-1.62
--	--	--	--	--	CG15196	-1.48
--	--	--	--	--	CG31969	-1.44
--	--	--	--	--	CG32957	-1.56
--	--	--	--	--	CG33111	-1.54
--	--	--	--	*NA*	CG3274	-1.44
--	--	--	--	*scylla*	CG7590	-1.41

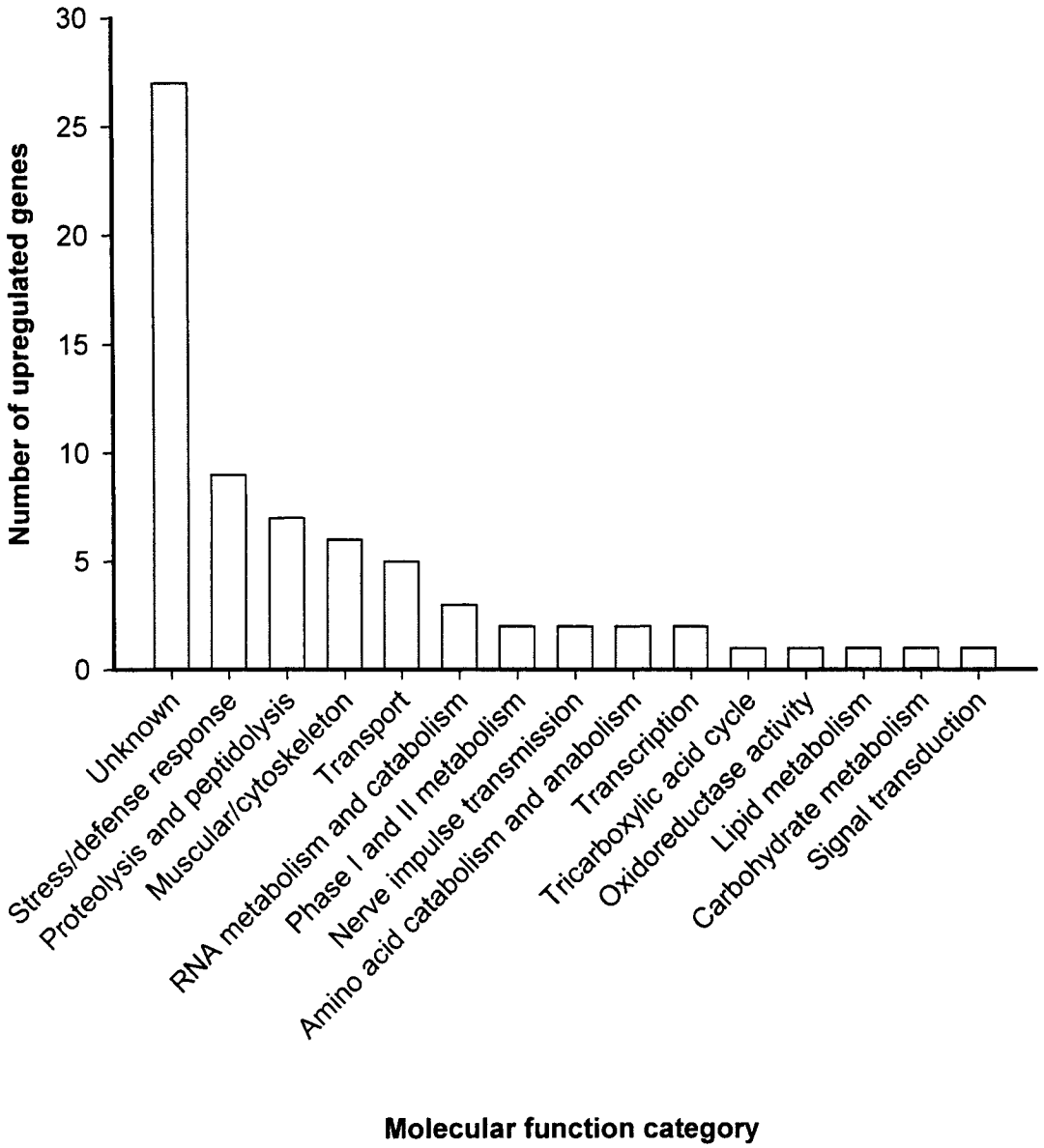

Figure 4.1: All genes upregulated 1.41-fold or more by a treatment of 0.04 mg/mL pyrethrin as determined by cDNA microarray analysis. Genes were assigned categories according to their molecular function and associated biological process based on Flybase annotation. A total of 70 genes were found to be upregulated.

(CG7756), *lethal (2) essential for life* (CG4533) which encodes a protein with a Hsp20 domain and CG2918 which encodes a protein with an Hsp70 domain (table 4.6). There were seven upregulated genes involved in proteolysis and peptidolysis. Upregulated genes associated with muscular and cytoskeleton structure included two actin and two tubulin genes (table 4.6). The *syntaxin 17* (CG7452) gene associated with intracellular protein transport and neurotransmitter secretion was upregulated (table 4.6). The most important categories of downregulated genes in the pyrethrin treatment were related to mitosis and regulation of cell cycle, transcription, and DNA binding although there were fewer genes overall in each downregulated gene category than in the upregulated gene categories (figure 4.2).

A comparison of the results from the three different treatments revealed that five genes were differentially upregulated and two genes were differentially downregulated in more than one treatment (figure 4.3). Overall, the pyrethrin treatment resulted in a much larger number of differentially expressed genes than the *P. nigrum* or the *P. nigrum* plus pyrethrin treatment. All microarray data will be submitted to the Gene Expression Omnibus (GEO) database and become part of the public domain upon publication of this research in a peer-reviewed journal.

4.3.2 RT- qPCR results

The upregulation of the heat shock protein encoding gene *l(2)efl* (CG4533) was confirmed for the pyrethrin treatment but not for the *P. nigrum* plus pyrethrin treatment (figure 4.4). The upregulation of CG13091, a gene with oxidoreductase activity, was confirmed for the pyrethrin treatment but not for the *P. nigrum* treatment (figure 4.5). The downregulation of the *Tom* gene associated with the Notch signaling pathway was confirmed for the pyrethrin and the pyrethrin + *P. nigrum* treatments but not for the *P. nigrum* treatment (figure 4.6).

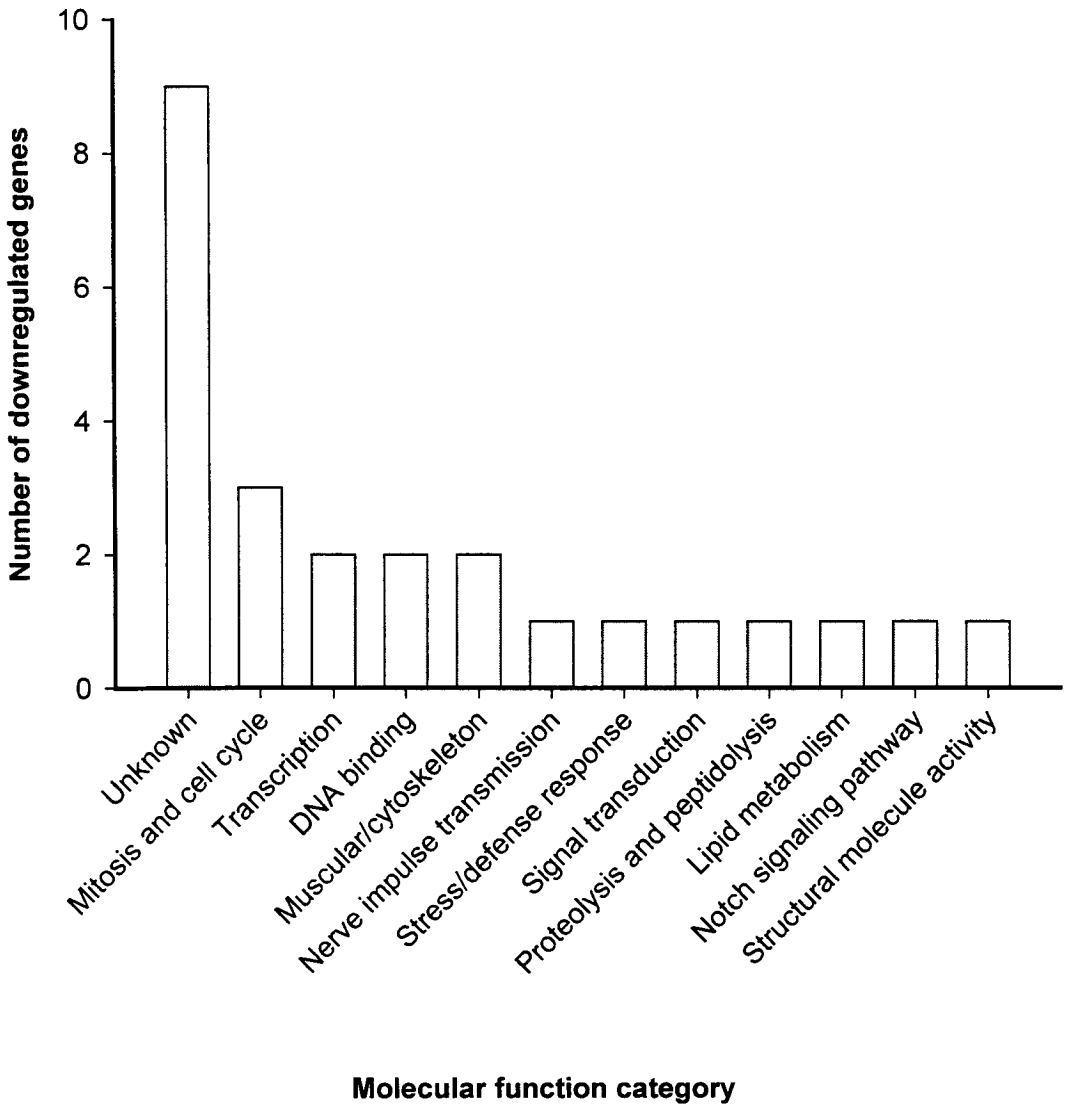

Figure 4.2: All genes downregulated 1.41-fold or more by a treatment of 0.04 mg/mL pyrethrin as determined by cDNA microarray analysis. Genes were assigned categories according to their molecular function and associated biological process based on Flybase annotation. A total of 25 genes were found to be downregulated.

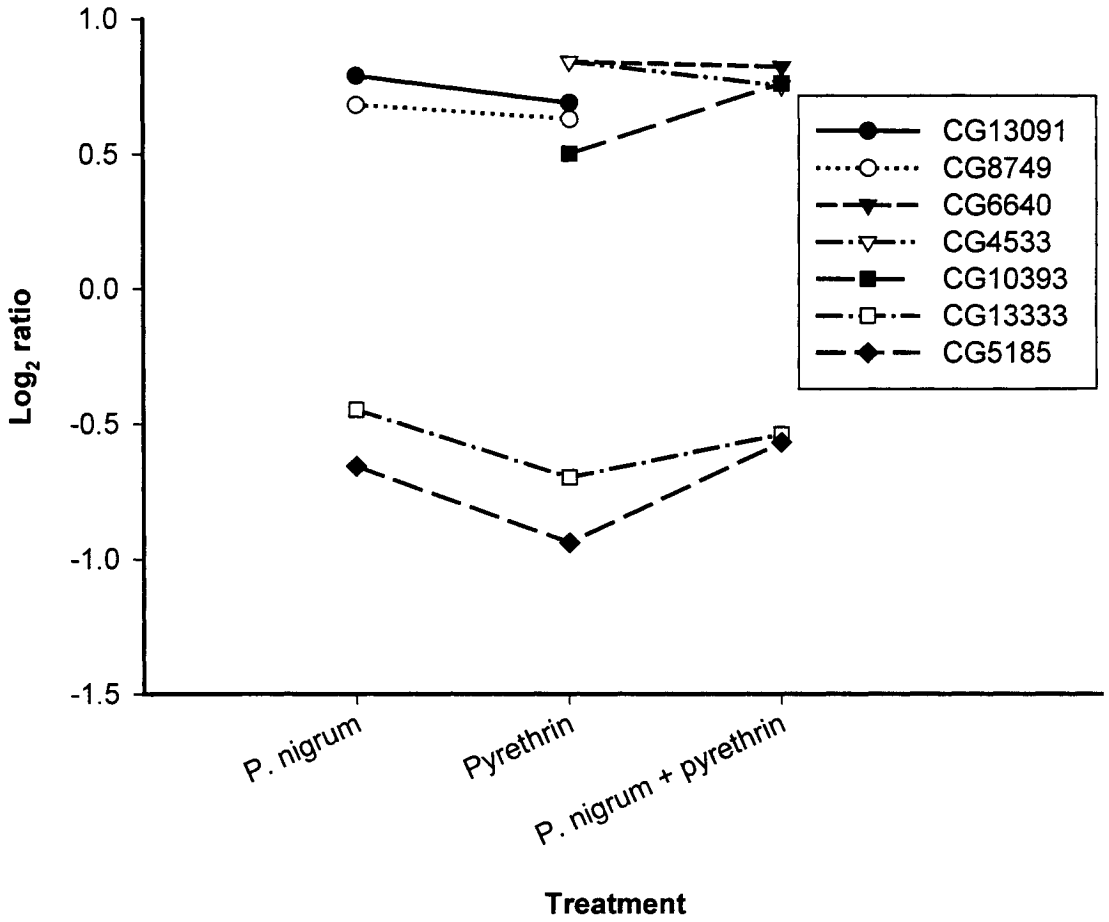

Figure 4.3: The mean differential expression ratio (expressed in $\log_2$ units) for *D. melanogaster* genes showing differential expression in microarray analysis in response to more than one of the following three treatments: *P. nigrum*, pyrethrin, or *P. nigrum* + pyrethrin. Missing values are due to data points which were filtered out of the final data set due to high variance. Lines are used to link identical genes and do not denote continuity of the data between treatments. Note that although the $\log_2$ ratio of CG13333 for the *P. nigrum* treatment is included in the figure it was not considered to be downregulated.

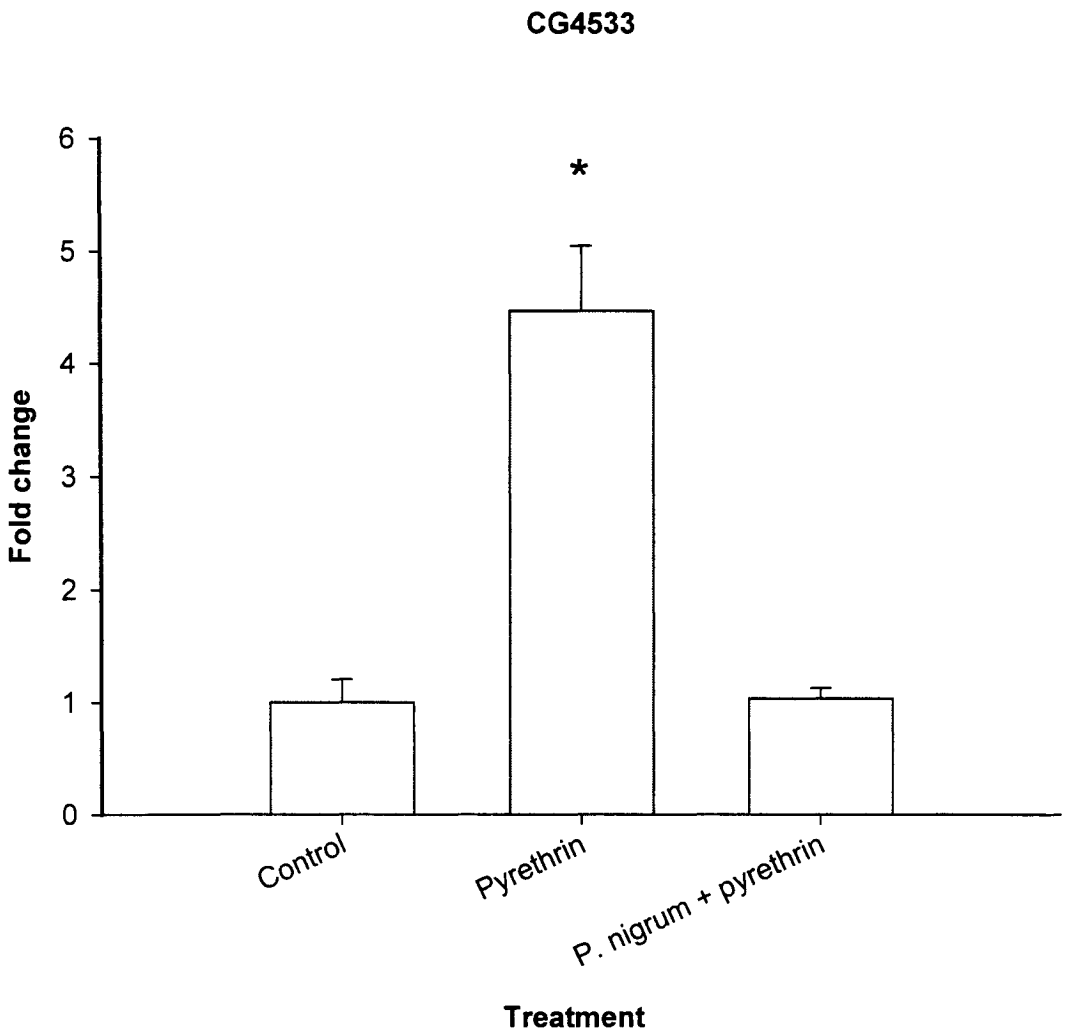

Figure 4.4: The mean fold upregulation (+SEM) of the CG4533 (or *l(2)efl*) gene in *D. melanogaster* adult females six hours after exposure to 0.04 mg/mL pyrethrin, 0.1 mg/mL *P. nigrum* + 0.004 mg/mL pyrethrin or a 99% ethanol control. Relative transcript abundance was quantified using QPCR with SYBRgreen fluorescent dye. The ribosomal protein gene *RpL32* was used to normalize results. A one-way ANOVA was performed ($p=0.000$, $F(2,9)= 36.118$, $N=12$) followed by Dunnett's 1-sided pairwise comparisons set to a significance level of $p<0.05$. Asterisk indicates a significant difference from the control.

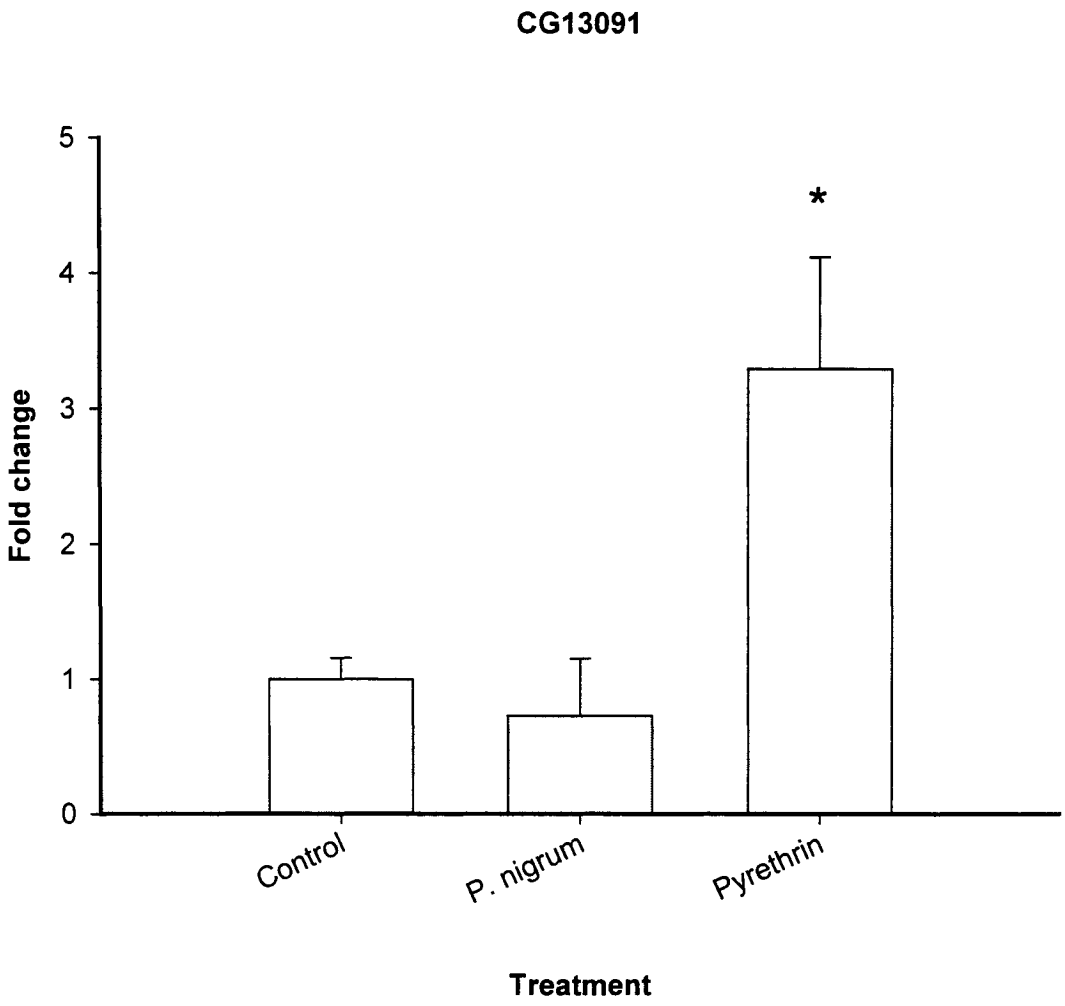

Figure 4.5: The mean fold upregulation (+SEM) of the CG13091 gene in *D. melanogaster* adult females six hours after exposure to 0.2 mg/mL *P. nigrum*, 0.04 mg/mL pyrethrin or a 99% ethanol control. Relative transcript abundance was quantified using QPCR with SYBRgreen fluorescent dye. The ribosomal protein gene *RpL32* was used to normalize results. A one-way ANOVA was performed ($p=0.005$, $F(2,9)= 9.878$, $N=12$) followed by Dunnett's 1-sided pairwise comparisons set to a significance level of $p<0.05$. Asterisk indicates a significant difference from the control.

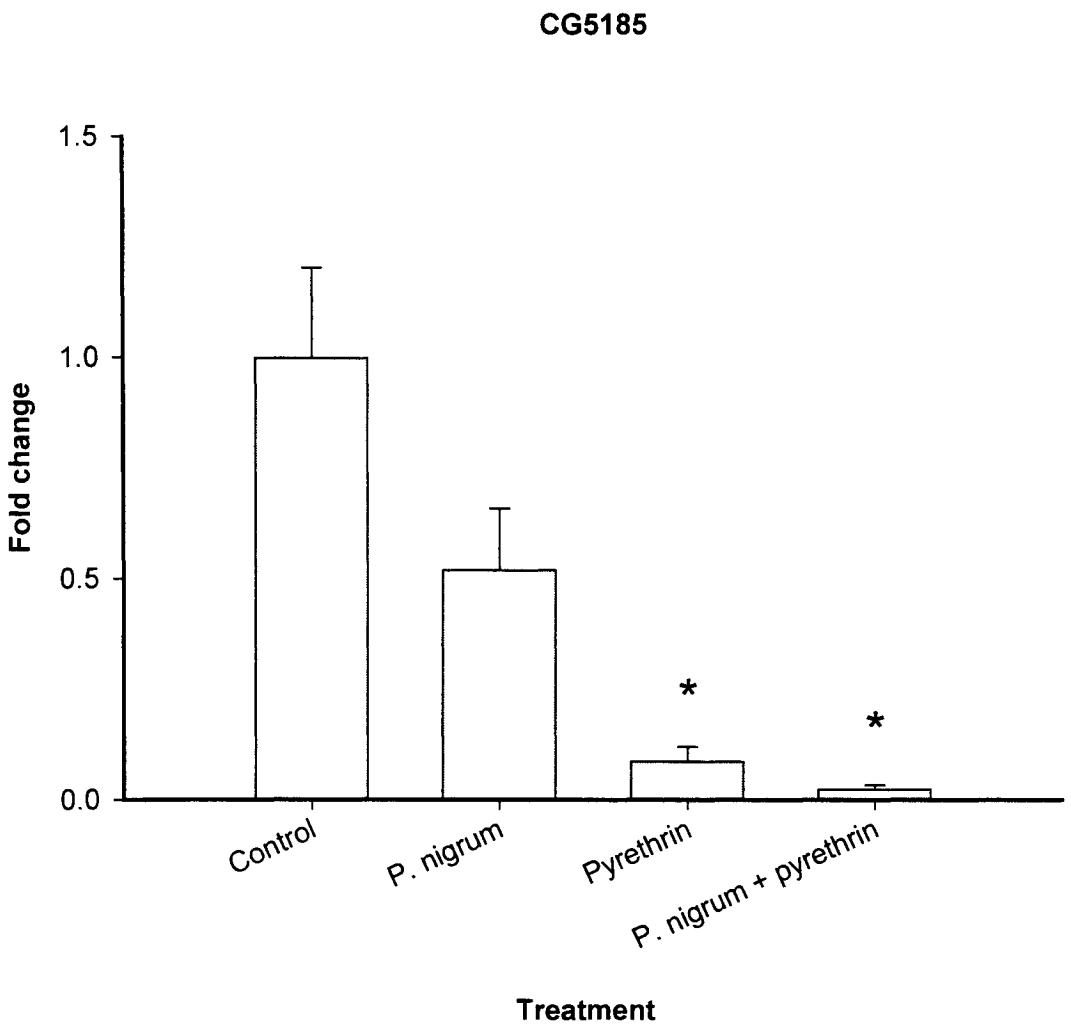

Figure 4.6: The mean fold downregulation (+SEM) of the CG5185 (or *Tom*) gene in *D. melanogaster* adult females six hours after exposure to 0.2 mg/mL *P. nigrum*, 0.04 mg/mL pyrethrin, 0.1 mg/mL *P. nigrum* + 0.004 mg/mL pyrethrin or a 99% ethanol control. Relative transcript abundance was quantified using QPCR with SYBRgreen fluorescent dye. The ribosomal protein gene *RpL32* was used to normalize results. A one-way ANOVA was performed ($p=0.016$, $F(3,14)= 4.836$, $N=18$) followed by Dunnett's 1-sided pairwise comparisons set to a significance level of $p<0.05$. Asterisk indicates a significant difference from the control.

4.4 Discussion

4.4.1 Comparison of differential expression in all treatments

This study demonstrates a marked difference between the gene expression profiles of *D. melanogaster* treated with pyrethrin alone compared to *D. melanogaster* treated with pyrethrin synergized with *P. nigrum* extract or *P. nigrum* extract alone. The addition of *P. nigrum* extract as a synergist to pyrethrin decreases the LC_{50} value by a factor of ten (see chapter two, section 2.3.2) and leads to very few differentially expressed genes six hours after the treatment. This is contrary to initial predictions that a combined treatment would result in a more complex array of genes being induced or suppressed as could be supposed due to the presence of a synergy. The synergistic properties of *P. nigrum* extract have previously been attributed to the neurotoxicity of the piperamides (Hatakoshi *et al.*, 1984, McFerren *et al.*, 2002) combined with the inhibition of detoxification enzymes by the MDP functional group (Bhardwaj *et al.*, 2002).

While the *P. nigrum* extract is an effective synergist at a concentration of 0.1 mg/mL, the *P. nigrum* treatment of 0.2 mg/mL had the lowest number of differentially expressed genes (table 4.4) and did not induce any visible change in the movement or behaviour of *D. melanogaster*. This is in contrast to the results from chapter 3 which identified a large number of up and downregulated genes in *D. melanogaster* exposed to a *P. nigrum* extract treatment equivalent to the LC_{50} value (0.9 mg/mL). There is therefore a marked relationship between the extract concentration and the resulting gene expression profile. This data indicates that at the molecular level as well at the physiological level the *P. nigrum* extract is a true synergist at a concentration of 0.1 mg/mL, insofar as it causes no mortality and has a minimal impact on gene expression and yet increases the toxicity of the pyrethrin insecticide.

Both the pyrethrin treatment and the pyrethrin plus *P. nigrum* extract treatments had similar observed effects which occurred within minutes of the treatment and consisted of strong knockdown activity and uncoordinated movements with a full recovery after six hours. The much smaller number of differentially expressed genes for the *P. nigrum* extract plus pyrethrin treatment (table 4.5) than for the pyrethrin treatment alone (table 4.6) is therefore unlikely to be linked simply to a difference in the recovery process. It is possible that because the combination treatment had rapid activity combined with a very low applied concentration that recovery occurred more rapidly due to the presence of lower quantities of extract to eliminate and this resulted in fewer differentially expressed genes. The neurotoxic properties of the piperamides are likely to be important in potentiating the pyrethrin neurotoxicity due to the different binding sites of voltage-gated sodium channels affected by the two classes of compounds (Soderlund, 1995, McFerren *et al.*, 2002). A short term inhibition of detoxification enzymes is also likely to be important. Neither of these effects was reflected in the gene expression profile of *Drosophila* treated with pyrethrin plus *P. nigrum* extract. It is likely that the concentration tested was not strong enough to lead to upregulation of said enzymes as was the case with the stronger *P. nigrum* extract treatment studied in chapter 3.

This data set was not suitable for cluster analysis due to the small number of differentially expressed genes in the *P. nigrum* and pyrethrin plus *P. nigrum* treatments, but it is important to note the presence of seven differentially expressed genes common to two or more treatments (figure 4.3) which indicate some common aspects in the response of *D. melanogaster* to the three different treatments. The downregulation of the *Tom* gene (CG5185) involved in the Notch signaling pathway is common to all 3 treatments. This gene has previously been associated with the differentiation of neural cells in embryos (Zaffran and Frasch, 2000). This study represents the first evidence of differential expression of *Tom* in adult *D. melanogaster* and these results suggest that

Tom is expressed in adult insects and may possibly be involved in processes other than development. Because many studies have focused on embryogenesis and metamorphosis in *Drosophila* and fewer studies have utilized adult insects there is a general tendency in the literature to ascribe the expression and function of many genes solely to developmental processes. It is likely, however, that a number of the genes associated with development are also active in adult insects. The CG13333 gene is downregulated in both the pyrethrin and the *P. nigrum* + pyrethrin treatments. The molecular function of this gene has not been characterized but it has been found to be expressed in neural cells, neural precursor cells and cells that line tracheal invaginations in *Drosophila* embryos (Brody *et al.*, 2002). This gene has previously been studied only with regards to its role in developmental processes. The association of this gene with the central nervous system suggests that further study of its function and expression in the CNS of adult *Drosophila* would be appropriate for further study.

Three genes (CG10383, CG6640 and CG4533) were upregulated in the pyrethrin treatment and in the pyrethrin plus *P. nigrum* treatment. CG10383 has been identified in a previous study to be upregulated in *Drosophila* larvae in response to starvation but not to sucrose (Zinke *et al.*, 2002). The upregulation of this gene in two different botanical insecticide treatments suggests a broader range of inducibility. The upregulated CG6640 gene encodes a protein domain characteristic of the major facilitator superfamily (MFS) of transporters. Integral membrane proteins of this superfamily are involved the transport of a broad selection of substrates including drugs (Paulsen *et al.*, 1996). Previous work (Zou *et al.*, 2000) found this gene to be upregulated with increasing age in fruit flies although it did not appear to be directly linked to the increased oxidative stress generated by the accumulation of free radicals during the aging process. This upregulated gene (CG6640) may be involved in detoxification processes. The upregulated CG4533 gene encodes a product homologous to the small

heat shock proteins (Kurzik-Dumke and Lohmann, 1995) associated with defense responses and chaperone activity. Overall, these common upregulated genes appear to encode products involved in the protection of the organism against these botanical insecticides by facilitating transport and also by protecting cellular components against the stresses incurred by the physiological effects of the insecticides.

The genes CG13091 and CG8749 were upregulated in both the *P. nigrum* and pyrethrin treatments. The CG13091 gene encodes a product with oxidoreductase activity. Previous gene expression profiles have found that CG13091 is downregulated in response to oxidative stress (Zou *et al.*, 2000). The associated product has a protein domain homologous to the male sterility protein in *Arabidopsis thaliana* which has the molecular function of reducing fatty acids to fatty alcohols (Aarts *et al.*, 1997). The CG8749 gene encodes a small nuclear ribonucleoprotein (snRNP70K) which has snRNA binding properties, assisting in the formation of snRNPs involved in pre-mRNA splicing. Early studies of U1 snRNPs in *Drosophila* found that their transcription was slightly increased during heat shock (Wieben and Pederson, 1982). This suggests an increase in transcription and mRNA processing occurring with exposure to the two botanical extracts, as is evidenced by the presence of differentially expressed genes.

4.4.2 Gene expression in the *P. nigrum* and pyrethrin plus *P. nigrum* treatments

Apart from the common differentially expressed genes, the *P. nigrum* treatment resulted in the upregulation of CG11822, the gene encoding the nicotinic acetylcholine receptor beta 21C (table 4.4). This is an unexpected observation because the neurological target of the *P. nigrum* isobutylamides is believed to be the voltage-gated sodium channel (McFerren *et al.*, 2002). This may indicates a compensation for voltage-gated channel inactivation or possibly the *P. nigrum* extract has a more complex neurological activity than the pure compounds which have been tested to date.

The addition of pyrethrin to *P. nigrum* resulted in the upregulation of 5 genes (table 4.5). All of these genes are different than those observed for *P. nigrum* alone but three of them are common to the pyrethrin treatment (CG6640, CG10383 and CG4533) and have been previously discussed. The upregulated gene CG6484 with glucose transporter activity may be associated with the observation that insects exposed to pyrethrin immediately increased their consumption of glucose. The upregulated gene CG2330 has structural molecule activity associated with the extracellular matrix. Biological processes previously assigned to this gene all involve developmental processes. The two downregulated genes in this treatment (CG13333 and CG5185) were discussed in the common genes section (4.4.1).

4.4.3 Gene expression in the pyrethrin treatment

The pyrethrin treatment resulted in the upregulation of 70 genes and the downregulation of 25 genes. Grouping the upregulated genes (figure 4.1) and downregulated genes (figure 4.2) by gene ontology based categories gives rise to a profile of the overall physiological response to pyrethrin exposure. Notable among the upregulated categories of genes are those involved in stress/defense response, proteolysis and peptidolysis, muscular/cytoskeleton and transport. The upregulation of these categories of genes indicates that at this level of treatment the primary response of the insect involves a reaction to physiological stress.

Downregulated categories of importance in the pyrethrin treatment were mitosis and regulation of the cell cycle, transcription, DNA binding and nerve impulse transmission. The downregulation of certain cell cycle and mitosis genes probably indicates a selective allocation of resources towards detoxification and associated activities and away from normal cellular processes. The downregulation of CG8577, a gene with peptidoglycan recognition activity involved in the detection of and defense

against bacteria may indicate the selective allocation of defense resources towards detoxification enzymes.

Other differentially expressed genes of interest in the pyrethrin treatment include two upregulated *Cyp* genes implicated in phase I metabolism, *Cyp 12d1* and *Cyp 9f2*, which indicate an upregulation of detoxification activity. The *Cyp 12d1* gene was also implicated in the response of *Drosophila* to high concentrations of *P. nigrum* (chapter 3) as was *Cyp 9b1*, a close relative of *Cyp 9f2*.

4.4.4 Validation of microarray results using RT-QPCR

Confirmation of the differentially expressed genes common to two or more treatments using RT-QPCR was entirely successful for the pyrethrin treatment (figures 4.4, 4.5 and 4.6). The upregulation of *l(2)efl* and CG13091 were significant as was the downregulation of CG5185. For the pyrethrin plus *P. nigrum* treatment the downregulation of CG5185 was also confirmed but the upregulation of *l(2)efl* was not (figures 4.4 and 4.6). Neither of the two genes tested for the *P. nigrum* treatment (CG13091 and CG5185) were confirmed although there appeared to be a downregulation occurring in the case of CG5185 (figures 4.5 and 4.6). Due to the reduced power of the statistical test associated with a sample size of three this result might have been significant with a slightly larger sample size.

In the case of the genes whose differential expression was not confirmed it is possible that this was due to the use of new RNA samples obtained from a repetition of the initial treatments. Doses received by the flies through spray application may have varied slightly or else the botanical extracts may have undergone some degradation, particularly in the case of the *P. nigrum* extract. Differential expression of each selected gene was confirmed for at least one treatment and they are all of interest for further study of their role in the short term response of *Drosophila* and other insects with

homologous genes to botanical insecticides. Furthermore, the successful confirmation of the upregulation of these genes validates the use of a differential expression ratio of less than 2-fold for the identification of differentially expressed genes. In fact, the downregulation of the *Tom* gene was confirmed and it only had a fold-change value of -1.45 in the pyrethrin plus *P. nigrum* treatment, which is very close to the selected cut-off point of -1.41.

4.5 Conclusion

This study demonstrates the complexity of the response of *D. melanogaster* to the botanical insecticides pyrethrin and *P. nigrum* as well as to the synergistic combination of the two products. Whole insects were successfully used in this study to gain an insight into the gene expression response of *Drosophila* to selected botanical extracts. Small changes measured here are likely an underestimation of the effects of *P. nigrum* extract and pyrethrin because whole organism gene expression analysis was utilized although is likely that these changes occurred in a tissue-specific manner. Therefore, future studies should analyse specific tissue responses by microdissection followed by microarray analysis. Central nervous system tissues would be of particular interest for pursuing the study of differentially expressed genes relating to nerve impulse transmission, neurotransmitter receptors and ion channels. The common genes that were differentially expressed in two or more different treatments are of interest for further study of their functions in detoxification and or stress response.

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Chapter 5: General discussion

5.1 Hypothesis validation

In this study the insecticidal properties of *Piper nigrum* extract used alone and as a synergist for natural pyrethrin were investigated. The first hypothesis advanced was that *P. nigrum* is an effective synergist for pyrethrin (chapter two). The associated objective was to assess the acute toxicity of *P. nigrum* extract, pyrethrin and pyrethrin synergized with *P. nigrum* extract to Dipteran insects in order to determine the insecticidal potential of this combination. The second hypothesis was that at high concentrations *P. nigrum* extract functions as an insecticide (chapter three). The associated objective was to determine the effect of an insecticidal concentration of *P. nigrum* extract on the gene expression profile of *D. melanogaster*. This was in order to identify the detoxification genes involved in the response of the insect to this botanical insecticide. The third hypothesis was that at low concentrations *P. nigrum* extract functions as a synergist for pyrethrin and is not directly toxic but causes a physiological reaction distinct from that incurred by either pyrethrin or *P. nigrum* extract employed separately (chapter four). The associated objective was to determine the effect of sublethal concentrations of *P. nigrum* extract, pyrethrin and pyrethrin synergized with *P. nigrum* extract upon gene expression profiles in *Drosophila*. This was in order to identify genes whose expression is specifically related to the synergistic combination of pyrethrin and *P. nigrum* extract.

It was determined that *P. nigrum* is an effective synergist for pyrethrin. The toxicity of the combined pyrethrin and *P. nigrum* treatment was between 10 and 14 times higher than the toxicity of pyrethrin used alone. It was determined that treating *D. melanogaster* with an insecticidal concentration of *P. nigrum* leads to a strong upregulation of phase I and II metabolism genes and that treating *D. melanogaster* with

a synergistic combination of *P. nigrum* and pyrethrin results in a unique transcriptional profile compared to a treatment using *P. nigrum* alone. This research should provide a strong framework for the future study of the multiple detoxification and defense mechanisms of insects and also of the mode of action of botanical insecticides.

The hypothesis that *P. nigrum* is an effective synergist for pyrethrin was advanced in chapter two based on the known inhibition of cytochrome P450 enzymes by the MDP functional group of *P. nigrum* amides such as piperine (Scott *et al.*, 2003) in a manner similar to piperonyl butoxide. An increase of the neurotoxic activity of pyrethrin in the presence of *P. nigrum* was also expected due to the complementary modes of action of the pyrethrin and *P. nigrum* which both affect different binding sites of the voltage-gated sodium channel (Soderlund, 1995, McFerren *et al.*, 2002). The synergistic properties of *P. nigrum* were confirmed in this study with the test insects *Musca domestica* and *Drosophila melanogaster*. Synergist ratios were equal to 11.6 and 13.9 for each respective insect species. A comparison of this data with a selection of published synergist ratios shows that the activity of this combination is ranked with the very best results from the literature (table 5.1). These ratios are similar to those obtained in tests with *Musca domestica* using piperonyl butoxide or piperettine (a *P. nigrum* amide) as synergists for pyrethrin (Gersdorff and Piquett, 1957, Incho and Greenberg, 1952). The natural product dillapiol is a monofunctional synergist that has a MDP functional group that causes an inhibition of polysubstrate monooxygenase activity in the same manner as the piperamides. The bifunctional piperamides appear to have enhanced synergistic activities compared to dillapiol (Bhuiyan *et al.*, 2001). This is probably because the piperamides have neurotoxic properties in addition to causing polysubstrate monooxygenase inhibition. The synergy of pyrethrin by the *P. nigrum* extract is much higher than any of the synthetic product synergies (table 5.1). Used alone, the *P. nigrum* concentration employed to synergize pyrethrin (0.1 mg/mL in the

Table 5.1: Reported synergist ratios for various combinations of natural and synthetic insecticidal products with botanical and synthetic synergists from selected literature sources.

Insecticide		Synergist[1]		Test insect	Synergist ratio	Author(s)	Year
Name	**Botanical source or chemical group[2]**	**Name**	**Botanical source or chemical group[2]**				
Natural products							
Pyrethrin	*Chrysanthemum cinerariaefolium*	*Piper nigrum* extract	*Piper nigrum*	*Musca domestica*	13.9	Current study	2005
Pyrethrin	*Chrysanthemum cinerariaefolium*	*Piper nigrum* extract	*Piper nigrum*	*Drosophila melanogaster*	11.6	Current study	2005
Pyrethrin	*Chrysanthemum cinerariaefolium*	PBO[3]	MDP[4]	*Musca domestica*	15.3	Incho & Greenberg	1952
Pyrethrin	*Chrysanthemum cinerariaefolium*	PBO	MDP	*Musca domestica*	5.2	Nash	1954
Pyrethrin	*Chrysanthemum cinerariaefolium*	Piperettine	*Piper nigrum*	*Musca domestica*	13.7	Gersdorff & Piquett	1957
Pyronyl[5]	*Chrysanthemum cinerariaefolium*	Neem	*Azadirachta indica*	Myzus persicae	2.7	Edelson *et al.*	2002
Pyrethrum[5]	*Chrysanthemum cinerariaefolium*	Dillapiol	Anethum graveoloans	*Spodoptera litura*	1.2	Bhuiyan *et al.*	2001
Piperine	*Piper nigrum*	Dillapiol	Anethum graveoloans	*Aedes atropalpus*	3.3	Belzile	1998
Piperine	*Piper nigrum*	PBO	MDP	*Aedes atropalpus*	2.0	Belzile	1998
Neem	*Azadirachta indica*	PBO	MDP	*Lymmaea acuminata*[6]	3.6	Singh *et al.*	1998
Bacillus thuringiensis[7]	--	Neem	*Azadirachta indica*	*Leptinotarsa decemlineata*	1.3	Trisyono & Whalon	1999

Insecticide		Synergist[1]					
Name	**Botanical source or chemical group[2]**	**Name**	**Botanical source or chemical group[2]**	**Test insect**	**Synergist ratio**	**Author(s)**	**Year**
Synthetic products							
Allethrin	pyrethroid	PBO	MDP	*Aedes aegypti*	2.0	Fales *et al.*	1956
Allethrin	pyrethroid	PBO	MDP	*Anopheles quadrimaculatus*	1.5	Fales *et al.*	1956
Allethrin	pyrethroid	PBO	MDP	*Musca domestica*	3.0	Gersdorff *et al.*	1957
Allethrin	pyrethroid	PBO	MDP	*Musca domestica*	4.1	Nash	1954
Cyfluthrin	pyrethroid	PBO	MDP	*Bactrocera dorsalis*	6.3	Hsu *et al.*	2004
Cypermethrin	pyrethroid	PBO	MDP	*Bactrocera dorsalis*	4.0	Hsu *et al.*	2004
Fenvalerate	pyrethroid	PBO	MDP	*Bactrocera dorsalis*	9.0	Hsu *et al.*	2004
Permethrin	pyrethroid	PBO	MDP	*Musca domestica*	4.0	Pap *et al.*	2001
Carbofuran	carbamate	PBO	MDP	*Musca domestica*	6.4	Pap *et al.*	2001
Bifenthrin	pyrethroid	Carbosulfan	carbamate	*Anopheles gambiae*	2.0	Corbel *et al.*	2002
Bifenthrin	pyrethroid	Chlorpyrifos-methyl	organophosphate	*Anopheles gambiae*	1.8	Darriet *et al.*	2003

[1] The term "synergist" is used broadly to describe both synergists and potentiators
[2] The chemical class of insecticide is used to describe the synthetic products
[3] PBO= piperonyl butoxide
[4] MDP=methylenedioxyphenyl
[5] Formulation of natural pyrethin with PBO
[6] An insecticidal bacterial toxin
[7] *Lymmaea acuminata* is a snail, not an insect

case of *Drosophila*) displayed no acute toxicity, confirming that the activity observed could truly be characterized as synergistic.

The hypothesis that at high concentrations *P. nigrum* functions as an insecticide was investigated in chapter three. It was predicted that gene expression profiles of treated insects would reveal an upregulation of detoxification genes, in particular genes encoding cytochrome P450 enzymes and ABC-transporters. This was based on the evidence that the active piperamides cause an inhibition of PSMO activity (Scott *et al.*, 2003) and ABC-transporter activity (Bhardwaj *et al.*, 2002). An initial suppression of CYP enzymatic activity has been correlated in the past with a second phase of CYP induction (Dalvi and Dalvi, 1991) which may be linked to increased *Cyp* gene transcription (Brandt *et al.*, 2002). The inhibition of specific cytochrome P450 isomers was expected to correlate with upregulation of *Cyp* genes encoding the specific isomers inhibited and/or with upregulation of *Cyp* genes with high affinity for *P. nigrum* amides as substrates. Differential expression of nervous system related genes, particularly those associated with voltage-gated sodium channels was also predicted.

Treatment of adult *D. melanogaster* females with an insecticidal concentration of *P. nigrum* resulted in the upregulation of six cytochrome P450 genes (*Cyp 6a8, Cyp 9b2, Cyp 12d1,Cyp 6d4, Cyp 6d5* and *Cyp 6w1*). This induction had been predicted to occur because an initial suppression of the enzymatic activity of CYP proteins could lead to an induction of *Cyp* gene expression. It is not known if the upregulated *Cyp* genes correspond to the specific CYP enzymes that were inhibited. In keeping with this line of reasoning, the expression patterns of glutathione-S-transferase genes were not predicted to change because *P. nigrum* phytochemicals have never been demonstrated to inhibit GST enzymes. Contrary to this prediction, two glutathione-S-transferase genes were upregulated (*GstE7* and *GstS1*). Therefore, it appears that the relationship between differential gene expression and the botanical insecticide *P. nigrum* does not

correlate to a simple upregulation of transcripts for the specific enzymes inhibited by the extract. This is further supported by the lack of evidence of differential expression of any ABC transporter genes despite the documented inhibition of these proteins by piperine (Bhardwaj *et al.*, 2002). The upregulation of genes encoding enzymes involved in both phase I and II metabolism indicates the induction of a complete detoxification pathway comprising structural modification of toxins followed by conjugation. No genes relating directly to voltage-gated sodium channels were differentially expressed, although numerous genes relating to calcium ion transport were identified, suggesting a possible perturbation of calcium-mediated precursors of nerve impulse transmission.

This data suggests that in the context of this study, the gene expression profile obtained may be a good indicator of the mechanism of detoxification induced in *Drosophila* in response to *P. nigrum*. However, it is not necessarily indicative of the enzymes which interact directly with the piperamides. This observation is of interest because it points to the possibility of deducing genes of potential importance for the evolution of resistance to a specific insecticidal product such as *P. nigrum*. Constitutive overexpression of one or more genes is one of the most common forms of insecticide resistance (ffrench-Constant *et al.*, 2004) and by studying insecticide-induced gene expression profiles in susceptible insects it may be possible to predict genes that have the potential to be involved in evolved resistance through constitutive overexpression. This theory is supported by the fact that the *Cyp 6a8* gene which was induced in response to *P. nigrum* has been identified in previous work to be constitutively overexpressed in the WIS1 strain of *D. melanogaster* selected for DDT resistance (Le Goff *et al.*, 2003). The presence of a correlation between these two separate forms of gene expression could prove to be a powerful tool in predicting molecular markers for evolved insecticide resistance to new products prior to their emergence in the field.

In chapter four, it was hypothesized that at low concentrations, *P. nigrum* functions as a synergist for pyrethrin and is not directly toxic but causes a physiological reaction distinct from that incurred by either pyrethrin or *P. nigrum* employed separately. Treatment of *D. melanogaster* adult females with a sublethal concentration of *P. nigrum*, pyrethrin or pyrethrin plus *P. nigrum* was predicted to reveal a cluster of genes differentially expressed in the synergistic treatment that were not present in either of the individual treatments. The treatment concentrations represented identical levels of acute toxicity relative to the calculated LC_{50} values (chapter two). The experiment resulted in fewer differentially expressed genes for *P. nigrum* and pyrethrin plus *P. nigrum* and a more complex expression profile for pyrethrin. There were two upregulated genes unique to the pyrethrin plus *P. nigrum* treatment involved in neurogenesis (CG2330) and transport (CG6484) which are of interest for further study.

Of even greater interest are the seven genes with similar differential expression patterns between the three treatments which may be involved in general detoxification and defense responses (figure 4.3). The *Tom* gene was downregulated in all three treatments. This gene is associated with the antagonism of the Notch signaling pathway involved in the embryonic differentiation of neural cells (Zaffran and Frasch, 2000). The data from the current study suggests that this gene is also expressed in adult *Drosophila* although its function in this context is unknown. By far the most complex gene expression profile described in this study belonged to the pyrethrin treatment. No genes encoding esterases were differentially expressed by pyrethrin treatment although these enzymes have also been implicated in pyrethrin detoxification (Anspaugh *et al.*, 1995). Genes linked to nervous system functions were also limited in number despite the neurotoxic properties of the extracts tested. This may be due to the recovery of the insects from the acute neurotoxic effects of the extracts by the time samples were collected. The regulation of genes related to neural function can occur on a very short

time scale and therefore a return to normal transcription levels may have occurred within the six hour time frame mentioned.

A comparison between the gene expression profile of *Drosophila* exposed to an insecticidal concentration of *P. nigrum* presented in chapter three and the gene expression profiles of *Drosophila* exposed to sublethal concentrations of *P. nigrum*, pyrethrin or pyrethrin plus *P. nigrum* presented in chapter four reveals nine common upregulated genes between the two experiments but no common downregulated genes (table 5.2). None of the differentially expressed genes are common to the insecticidal *P. nigrum* treatment (0.9 mg/mL, in chapter three) and the sublethal *P. nigrum* treatment (0.2 mg/mL, in chapter four). Two genes with a heat shock protein 20 domain were upregulated in pyrethrin (CG4533 and CG14207) and one of them was also upregulated in the pyrethrin plus *P. nigrum* treatment. These genes are probably responsive to a broad range of stressors. The upregulation of the *Cyp 12d1* gene in the pyrethrin and in the insecticidal *P. nigrum* treatment indicates that the associated CYP 12D1 enzyme is probably involved in the detoxification of both *P. nigrum* and pyrethrin. There is one category of genes that were upregulated in the *P. nigrum* treatment and downregulated in the pyrethrin treatment. This is an interesting trend and it is difficult to draw conclusions concerning its implications due to the lack of sufficient data concerning the functions of the genes in question. The *troponin C at 41C* gene (CG2981) has been linked to the initiation of contraction in asynchronous flight muscle (Qiu *et al.*, 2003). Because *P. nigrum* and pyrethrin have slightly different neurotoxic effects that lead to muscle spasms, it is possible that differences in the expression of this gene are linked to the different effects of the two botanicals in the central nervous system.

In chapter three, when adult *Drosophila* females were treated with an insecticidal concentration of *P. nigrum* extract there were eight upregulated genes encoding enzymes involved in phase I and II metabolism. In chapter four, when *Drosophila* were

Table 5.2: *Drosophila* genes upregulated in response to an insecticidal treatment with *P. nigrum* (0.9 mg/mL) in chapter three that are differentially expressed in at least one sublethal treatment in chapter four (pyrethrin, *P. nigrum* or pyrethrin + *P. nigrum*).

***P. nigrum* upregulated genes**	**Gene name**	**Biological process[1]**	**Protein domains[2]**	**Gene response to sublethal treatments[3]**		
				Pyrethrin	**Pyrethrin + *P. nigrum***	***P. nigrum***
CG4533	*l(2)efl*	Defense response	Heat shock protein 20	Upregulated	Upregulated	NC
CG10383	--	--	--	Upregulated	Upregulated	NC
CG10067	*Act57B*	Cytoskeleton organization and biogenesis	Actin	Upregulated	NC	NC
CG14207	--	--	Heat shock protein 20	Upregulated	NC	NC
CG18240	*Cyp 12d1*	Electron transport	Cytochrome P450	Upregulated	NC	NC
CG30059	--	N-acetylglucosamine metabolism	Sulfatase	Upregulated	NC	NC
CG2981	*TpnC41C*	Calcium-mediated signaling	Calcium-binding EF-hand	Downregulated	NC	NC
CG7445	*fln*	--	--	Downregulated	NC	NC
CG8505	--	--	Insect cuticle protein	Downregulated	NC	NC

[1] From gene ontology (QuickGO, http://www.ebi.ac.uk/ego/)

[2] From InterPro (http://www.ebi.ac.uk/interpro/)

[3] NC indicates no change in gene expression

treated with sublethal concentrations of *P. nigrum* extract, pyrethrin or pyrethrin plus *P. nigrum* extract the only upregulated genes pertaining to phase I and II metabolism were the two cytochrome P450 genes upregulated in the pyrethrin treatment. Because of the more toxic nature of the doses in chapter three it is probable that the main physiological response consisted of an upregulation of detoxification genes in order to metabolize the toxin as quickly as possible. In chapter four the gene expression profile is probably more indicative of the genes involved in the physiological recovery of the insect from the physiological effects of treatment through the expression of heat shock proteins and other defense genes. The applied doses were probably not high enough to induce a major upregulation of detoxification genes. The reason for the small number of differentially expressed genes in the pyrethrin plus *P. nigrum* treatment relative to the pyrethrin treatment is probably due to the much lower concentration of pyrethrin that was applied.

For future studies it may be preferable to work with slightly higher doses of insecticides as was done in chapter three rather than with the sublethal type of doses used in chapter four. The higher doses elicit more complex gene expression profiles which may lend themselves to cluster analysis in order to answer questions concerning the different mechanisms and biological pathways involved in the response of insects to botanical insecticides. Although numerous studies of gene expression profiles investigate a number of time points it was felt that this would not be useful in this case due to the acute nature of the toxicity of *P. nigrum* and pyrethrin. The time points investigated in this study were four hours for the investigation of the insecticidal activity of *P. nigrum* and six hours for the investigation of the synergistic activity of *P. nigrum* used with pyrethrin. These time points were based on the observations that after six hours the test insects were either dead or fully recovered and therefore unlikely to exhibit further changes in gene expression profiles. Furthermore, a similar study investigating

the effects of a single exposure of *Drosophila* to fungal and microbial agents found that differential gene expression peaked after six hours followed by a sharp decline at later time points (Irving *et al.*, 2001).

This work is likely to represent the first investigation of the gene expression response of *Drosophila melanogaster* exposed to a botanical insecticide and the first study examining the effect of any insecticidal product upon differential expression of multiple genes in *Drosophila.* Previous gene expression studies of interest with regards to the elucidation of the relationship between insects and insecticides have investigated the differences between gene expression profiles of DDT-susceptible and DDT-resistant strains of *Drosophila* (Le Goff *et al.*, 2003, Pedra *et al.*, 2004). The study by Pedra (2004) identified constitutive overexpression of the two of the same *Cyp* genes (*Cyp 6a8* and *12d1*) in DDT-resistant *Drosophila* which were found to be induced in response to a strong dose of *P. nigrum* as discussed in chapter three. *Cyp 12d1* was also induced in the pyrethrin treatment discussed in chapter four. Other *Drosophila* microarray studies of interest in relation to the current study were concerned with investigating the effects of aging and oxidative stress (Zou *et al.*, 2000) as well as nutrients and starvation (Zinke *et al.*, 2002) upon *Drosophila* gene expression profiles. According to Zou *et al.* (2000) increasing age of *Drosophila* is correlated to an upregulation of the CG6640 gene which does not have a known function. The CG6640 gene was also upregulated by pyrethrin and pyrethrin plus *P. nigrum.* The CG13091 gene which encodes a product with oxidoreductase activity was downregulated in response to oxidative stress (Zou *et al.*, 2000) but was upregulated in *P. nigrum* and pyrethrin treatments in the current study. The genes CG10383 and CG11892 are respectively upregulated and downregulated in response to starvation in *Drosophila* larvae (Zinke *et al.*, 2002). Similar patterns of expression for these two genes were observed in response to the botanical insecticides used in the current study: CG10383 was upregulated by the insecticidal dose of *P.*

nigrum as well as by the sublethal doses of pyrethrin and pyrethrin plus *P. nigrum* while CG11892 was downregulated in response to the insecticidal dose of *P. nigrum*. The *Thor* gene is upregulated by both sugar and starvation (Zinke *et al.*, 2002) and is also upregulated by the insecticidal dose of *P. nigrum*.

5.2 Future research

This study has identified numerous genes of interest for further study of their role in the physiological reaction of *Drosophila* to treatment with the botanical insecticides *P. nigrum* and pyrethrin and to the synergistic combination of pyrethrin and *P. nigrum* extracts. Further work to pursue in this direction includes an examination of tissue specific gene expression for the genes of interest in order to obtain more information concerning the involvement of these genes in the insect response to the botanical insecticides. This could be accomplished using *in situ* hybridizations. It is also possible that the tissue-specific expression patterns of certain genes may have reduced the overall signal of certain differentially expressed transcripts due to the use of whole insects in this experiment. This is particularly true for genes linked to the central nervous system. The neurotoxic activity of both *P. nigrum* and pyrethrin was expected to lead to an induction of a large number genes linked to neurological function. There were some differentially expressed genes relating to nerve impulse transmission and ion channels but it is probable that this is not wholly representative of the response to the neurotoxins. A microarray experiment using dissected nervous system tissues as samples would be much more sensitive to differential expression signals from such genes.

This research lays the foundation for future studies of the defense response and detoxification mechanisms of insects exposed to botanical insecticides These include industry research geared towards product development, fundamental phytoprotection

research aimed at understanding the link between inducible gene expression in susceptible insects and the constitutive overexpression of genes in resistant insects and fundamental genomics and transcriptomics research geared towards understanding the function of specific genes.

Prior work by a number of researchers has demonstrated that *Piper nigrum* and other plants of the genus *Piper* have potent insecticidal activity. Furthermore, the long history of traditional use of these plants for medicinal and insecticidal purposes indicates low mammalian toxicity. This work has demonstrated the presence of a strong synergy between *P. nigrum* and pyrethrin when used against two species of Dipteran insects, *Musca domestica* and *Drosophila melanogaster*. Future work in this area should include further studies of this synergy using different test species and field trials. It is also important to test the potential for the development of target species resistance to this combination over time.

One of the great proposed advantages of complex botanical extracts as insecticides is their ability to limit the development of resistance in insects due to the presence of analogue synergism (Feng and Isman, 1995) between the numerous biologically active secondary metabolites present. The gene expression profiles studied in the context of this research relate to a short term response of *D. melanogaster* to the selected botanical insecticides *P. nigrum* and pyrethrin as well as the synergistic combination of the two products. It is of interest to determine the relationship between genes upregulated transiently in response to insecticide exposure and the genes upregulated constitutively in insects with an evolved resistance to one or many classes of insecticide.

In this context, it could be hypothesized that the large number of phase I and II metabolism genes upregulated in response to *P. nigrum* in (chapter three) indicate a complex detoxification process associated with this product. Based upon the data of this

study it is not possible to determine whether one or many of these genes would be constitutively overexpressed in the case of selection for resistance. A study investigating the evolution of resistance of this strain of *Drosophila* to these botanical products in terms of constitutive overexpression of specific genes would be of interest in order to investigate the possible presence of a correlation between short term gene induction and the evolution of resistance in a population.

Finally, the gene expression data obtained in this study using the cDNA microarray platform provides insight into possible novel genes involved in detoxification and defense response mechanisms. Further study of specific genes identified in this study could lead to the identification the function of these genes and also to the identification of novel functions for genes which have previously been characterized. In particular, the study of the cytochrome P450 and glutathione-S-transferase genes identified in chapter three and the seven genes common to two or more treatments in chapter four are promising for further study. If these genes do turn out to be inhibited by piperamides they could be used as biochemical targets for improving the efficacy of insecticides.

Overall, this study has demonstrated that cDNA microarrays represent a powerful new platform for gaining insight into complex biological interactions. A comparison between the data obtained in chapters three and four suggests that in the study of insect gene expression profiles induced in response to botanical insecticides the use of stronger doses yields more powerful data. The gene expression profile described in chapter three has higher differential expression ratios for individual genes and also has a much greater number of differentially expressed genes overall. Future work using this experimental design should take this into account.

5.3 Perspectives on botanical insecticide use

In the past, a selected number of botanical insecticides such as pyrethrin (*Chrysanthemum cinerariaefolium*, Asteraceae), rotenone (*Derris elliptica*, Fabaceae) and, more recently, neem (*Azadirachta indica*, Meliaceae) have been presented as alternatives to synthetic products and achieved wide distribution. Botanical insecticides are acceptable for limited use in organic agriculture applications and in municipalities which ban the use of synthetic insecticides. The advantage of botanical products resides in their rapid biodegradation which results in decreased environmental persistence. Botanical products such as pyrethrins have low mammalian toxicity due to rapid detoxification. Botanical insecticides composed of complex mixtures of active secondary metabolites have also been demonstrated to slow the development of resistance.

In the popular perception botanical insecticides are completely safe and devoid of any of the negative side-effects associated with synthetic insecticides. This is obviously erroneous when one considers that botanical products are not so very different from their synthetic counterparts. Both classes of insecticide affect similar target sites in insects such as voltage-gated sodium channels and mitochondrial electron transport. The natural pyrethrin esters have even served as chemical templates for the synthesis of the more efficient and persistent pyrethroid insecticides. Although botanical products are unlikely to result in the type of ecological catastrophes which resulted from the indiscriminate use of second generation pesticides such as DDT it is unethical to promote such products without also discussing the potential for undesirable side-effects.

This study has proposed the use of *P. nigrum* extract as a novel botanical synergist for natural pyrethrin which can render pyrethrum a more acceptable product for organic agriculture applications by creating a product consisting purely of botanical

extracts. It has also been suggested that such a product could prove to be robust towards preventing the development of insect resistance and towards overcoming existing resistance. These recommendations are made in the context of the knowledge that the use of any insecticide, regardless of whether it is botanical or synthetic is liable to affect more than one component of the ecosystem where it is applied and may entail negative consequences.

The use of insecticides should be restricted to specific well-defined situations where it is known that insecticide application will be useful for controlling the specific pest at hand. There are many precautions which must be seen as initials steps for combating insect pest problems. These involve the selection of appropriate crop species with strong resistance to problematic insects. It is important that novel botanical insecticide products be used in the context of an integrated pest management plan and with the same spirit of caution which has been acquired through errors made in the past in the application of conventional insecticides.

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Appendix I

Piperamides in stock solutions

A *P. nigrum* extract was used to evaluate the synergistic activity between *P. nigrum* extract and pyrethrin in chapter 2 against *D. melanogaster*. The relationship between the concentration of the *P. nigrum* extract and the concentration of 3 piperamides (piperine, 4,5-dihydropiperine and piperlonguminine) is shown here to demonstrate the phytochemical consistency of the extract over the ranges of concentrations tested.

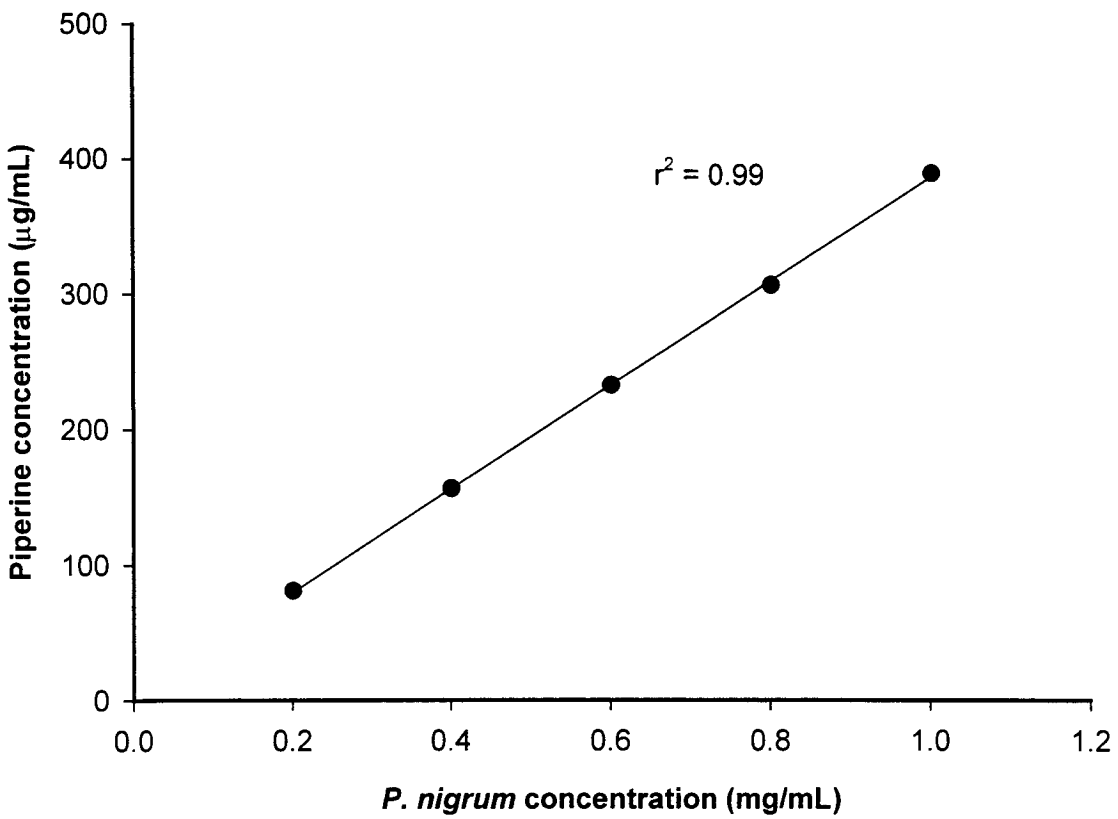

Figure 1: Relationship between the concentration of *P. nigrum* extract solutions and the concentration of piperine.

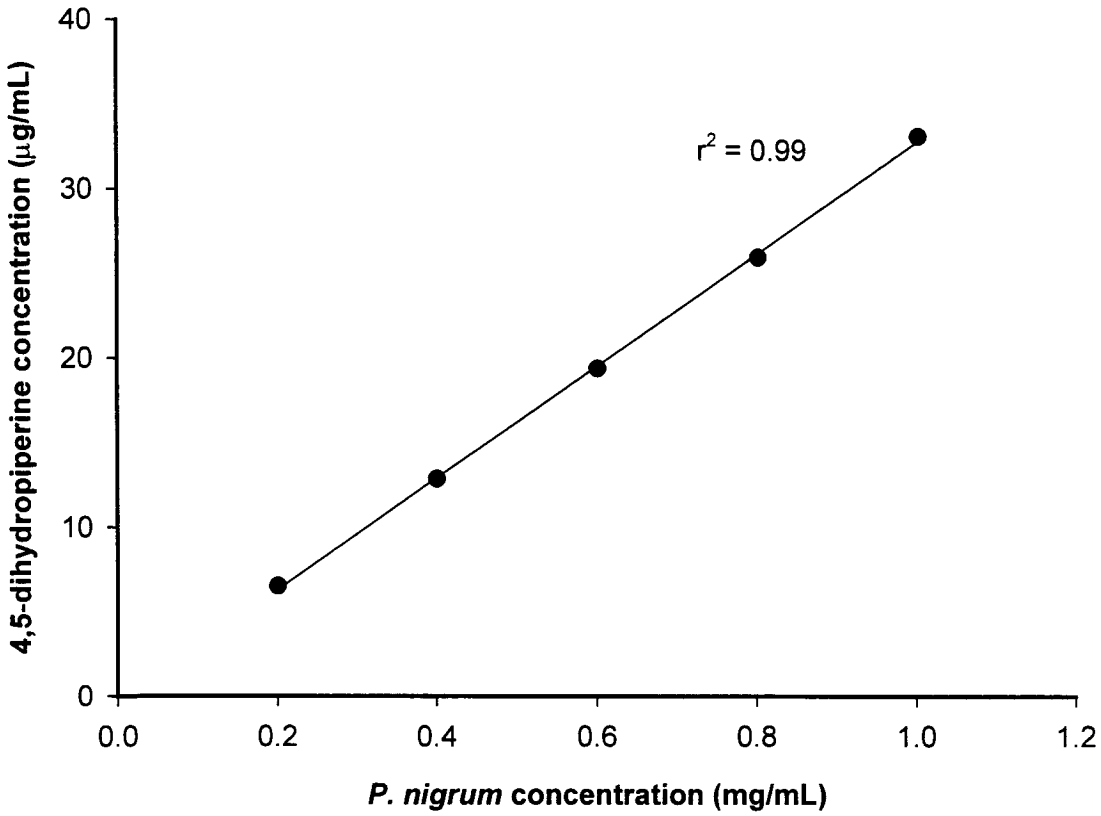

Figure 2: Relationship between the concentration of *P. nigrum* extract solutions and the concentration of 4,5-dihydropiperine.

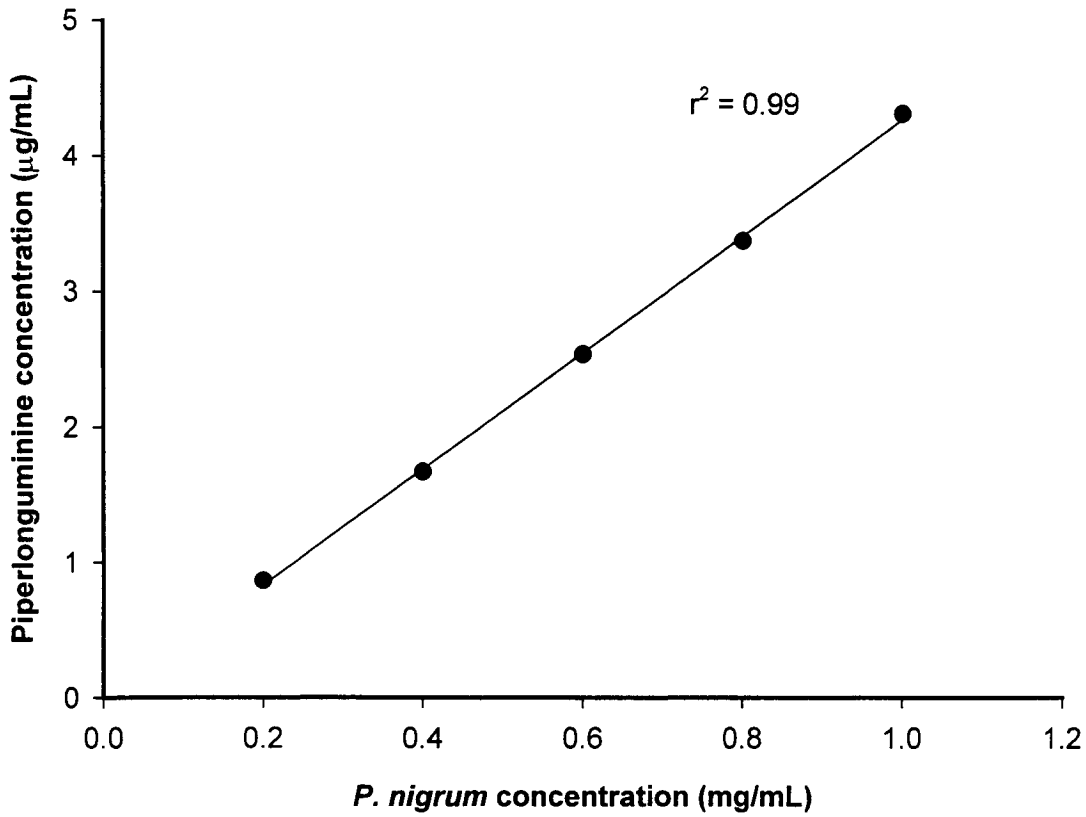

Figure 3: Relationship between the concentration of *P. nigrum* extract solutions and the concentration of piperlonguminine.

Appendix II

Piperine on Petri plates

The use of a Potter's tower to treat test insects in chapters two, three and four was validated by measuring the application rate of the marker compound piperine present in the *P. nigrum* extract. The following figure shows the relationship between the application rate of piperine and the concentration of the *P. nigrum* extract which was applied.

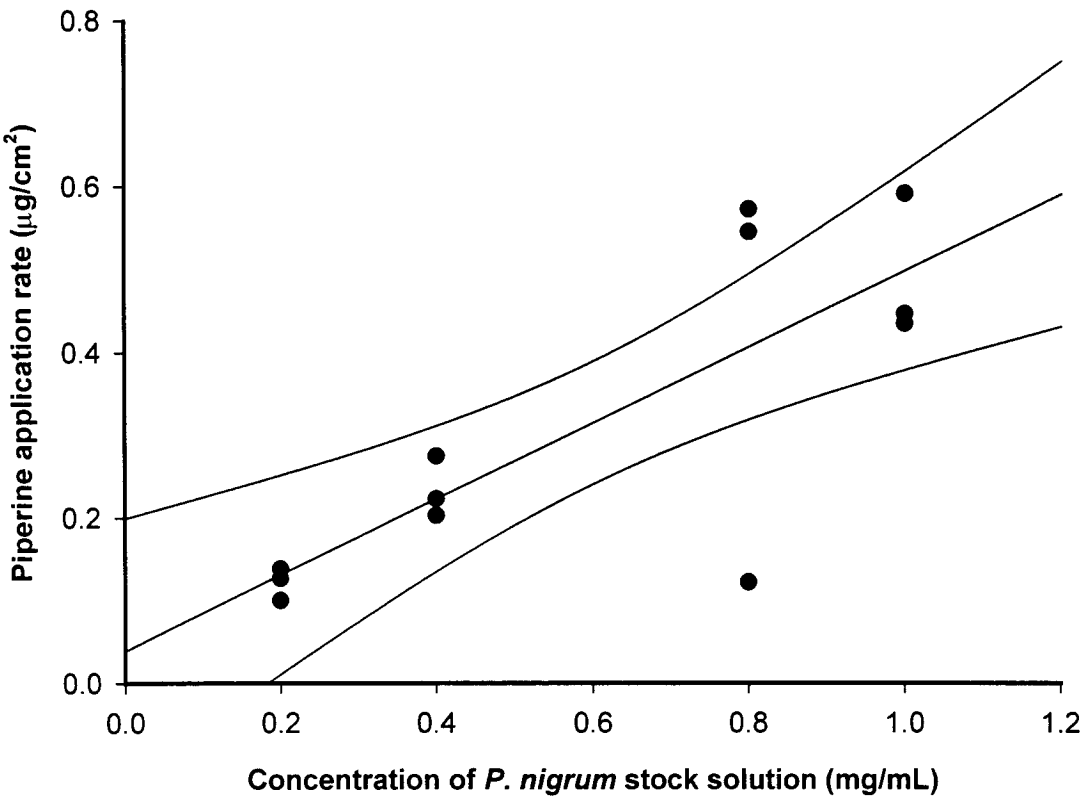

Figure 1: Linear regression and 95% confidence intervals showing the relationship between the *P. nigrum* extract concentration of the stock solution sprayed using the Potter's tower and the resulting application rate of piperine upon Petri plates. There is a significant positive relationship between these two variables (R^2=0.63, p=0.002, d.f.=1).

Appendix III

Piper extract characterization

The following tables provide phytochemical characterizations of the *P. nigrum* extracts used in the cDNA microarray experiments of chapter three and four. HPLC analysis was used to quantify the concentration of four insecticidal piperamides (4,5-dihydropiperlonguminine, piperlonguminine, 4,5-dihydropiperine and piperine) present in the stock solutions and the mean application rate of the marker compound piperine sprayed on Petri plates using the Potter's tower.

Table 1: Phytochemical characterization of the *P. nigrum* extract used in the microarray experiment described in chapter three. The mean application rate of *P. nigrum* on Petri plates using a Potter's tower and the concentrations of the piperamides 4,5-dihydropiperlonguminine, piperlonguminine, 4,5-dihydropiperine and piperine in the stock solution used to prepare the treatments for the flies are shown.

Experiment	Mean piperine on plates[1] (ug/cm^2)	Piperamides present in stock solution[2] (µg/mL)			
		4,5-dihydropiper-longuminine	piper-longuminine	4,5-dihydro-piperine	piperine
P. nigrum 0.9 mg/mL	0.674 (0.127)	5.849	27.302	208.085	2514.007

[1]Mean of 3 replicates with standard error presented in brackets.
[2] Stock solution is 9 mg/mL

Table 2: Phytochemical characterization of the *P. nigrum* extract used in the microarray experiments described in chapter four. The mean application rate of *P. nigrum* on Petri plates using a Potter's tower and the concentrations of the piperamides 4,5-dihydropiperlonguminine, piperlonguminine, 4,5-dihydropiperine and piperine in the stock solutions used to prepare the treatments for the flies are shown.

Experiment	Mean piperine on plates[1] (ug/cm^2)	Piperamides present in stock solutions[2] (µg/mL)			
		4,5-dihydropiper-longuminine	piper-longuminine	4,5-dihydro-piperine	piperine
P. nigrum 0.2 mg/mL	0.236 (0.014)	2.322	8.576	66.262	732.333
Pyrethrin 0.004 mg/mL + *P. nigrum* 0.1 mg/mL	0.112 (0.006)	Not detected	4.148	32.764	392.5177

[1]Mean of 6 replicates with standard error presented in brackets.
[2] Stock solution is 2 mg/mL for *P. nigrum* 0.2 mg/mL and 1 mg/mL for pyrethrin + *P. nigrum*.

Appendix IV

Table of log_2 ratio conversions

log_2 ratio	Fold-change in expression	Type of differential expression
2.0	4.0	Upregulation
1.5	2.8	Upregulation
1.0	2.0	Upregulation
0.5	1.4	Upregulation threshold
0	1.0 or -1.0	No change
-0.5	-1.4	Downgregulation threshold
-1.0	-2.0	Downregulation
-1.5	-2.8	Downregulation
-2.0	-4.0	Downregulation

Appendix V

Differentially expressed gene tables

The following tables provide lists of the differentially expressed genes from the microarray analysis in chapters three and four with their fold-change value of differential expression, $\log_2$ ratio of differential expression and standard deviation. Genes are listed by their curated gene (CG) number (http://flybase.bio.indiana.edu/) in increasing numerical order.

Table 1: Differentially expressed genes from the ethanol versus water hybridization (chapter three, table 3.2).

CG number	Gene symbol	Fold expression change	Mean $\log_2$ ratio	Standard deviation
Upregulated genes				
CG7756	*Hsc70-2*	2.16	1.11	0.85
Downregulated genes				
CG1703	--	-2.27	-1.18	0.87
CG10603	*mRpL13*	-2.81	-1.49	1.45

Table 2: Differentially expressed genes with a fold-change value greater than or equal to 2, or less than or equal to – 2 from the *P. nigrum* 0.9 mg/mL versus ethanol hybridization (chapter three, table 3.3).

CG number	Gene symbol	Fold expression change	Mean $\log_2$ ratio	Standard deviation
Upregulated genes				
CG1101	*Aly*	2.03	1.02	0.85
CG3705	*aay*	2.04	1.03	0.37
CG4486	*Cyp9b2*	3.12	1.64	0.24
CG5178	*Act88F*	2.19	1.13	0.19
CG5707	--	2.13	1.09	0.58
CG7445	*fln*	2.36	1.24	0.97
CG8938	*GstS1*	2.22	1.15	0.55
CG9434	*Fst*	2.55	1.35	0.55
CG10248	*Cyp6a8*	3.53	1.82	0.87
CG11893	--	2.13	1.09	0.1
CG18240	*Cyp12d1*	2.51	1.33	0.31
CG31904	--	2.00	1.00	0.26
Downregulated genes				
CG5107	--	-2.08	-1.06	0.28
CG11892	--	-2.79	-1.48	0.23

Table 3: Differentially expressed genes with a fold-change value greater than or equal to 1.41 and less than 2 or less than or equal -1.41 and greater than -2 from the *P. nigrum* 0.9 mg/mL versus ethanol hybridization (chapter three, table 3.4).

CG number	Gene symbol	Fold expression change	Mean $\log_2$ ratio	Standard deviation
Upregulated genes				
CG1572	--	1.45	0.54	0.53
CG1583	--	1.53	0.61	0.28
CG1919	--	1.54	0.62	0.53
CG2471	--	1.51	0.59	0.37
CG2981	*TpnC41C*	1.67	0.74	0.64
CG3050	*Cyp6d5*	1.74	0.8	0.24
CG3612	*blw*	1.41	0.5	0.15
CG3630	--	1.59	0.67	0.36
CG3861	*l(1)G0030*	1.48	0.57	0.17
CG3962	*Keap1*	1.41	0.5	0.23
CG4533	*l(2)efl*	1.79	0.84	0.3
CG5653	--	1.41	0.5	0.44
CG5966	--	1.87	0.9	0.3
CG6263	--	1.51	0.59	0.47
CG7178	*wupA*	1.43	0.52	0.35
CG7214	--	1.8	0.85	0.3
CG7313	*CheA75a*	1.51	0.59	0.52
CG7896	--	1.44	0.53	0.36
CG8345	*Cyp6w1*	1.8	0.85	0.5
CG8505	--	1.59	0.67	0.21
CG8756	--	1.43	0.52	0.27
CG8846	*Thor*	1.62	0.7	0.28
CG9297	--	1.52	0.6	0.25
CG9404	--	1.57	0.65	0.6
CG10067	*Act57B*	1.61	0.69	0.68
CG10126	--	1.41	0.5	0.29
CG10143	*Adgf-E*	1.73	0.79	0.71
CG10383	--	1.73	0.79	0.46
CG10407	--	1.65	0.72	0.38

CG number	Gene symbol	Fold expression change	Mean log_2 ratio	Standard deviation
Upregulated genes (cont.)				
CG10550	--	1.44	0.53	0.12
CG10562	--	1.78	0.83	0.51
CG10847	*enc*	1.75	0.81	0.39
CG11089	--	1.74	0.8	0.21
CG11796	--	1.42	0.51	0.18
CG11963	--	1.46	0.55	0.54
CG12800	*Cyp6d4*	1.44	0.53	0.35
CG13335	--	1.48	0.57	0.32
CG13868	--	1.64	0.71	0.19
CG14207	--	1.52	0.6	0.32
CG15101	*Jheh1*	1.58	0.66	0.38
CG15825	--	1.42	0.51	0.29
CG16884	--	1.8	0.85	0.54
CG16926	--	1.54	0.62	0.27
CG17183	*Trap25*	1.45	0.54	0.19
CG17531	*GstE7*	1.41	0.5	0.41
CG17903	*Cyt-c-p*	1.52	0.6	0.39
CG17927	*Mhc*	1.79	0.84	0.19
CG18466	*Nmdmc*	1.74	0.8	0.3
CG30008	--	1.46	0.55	0.28
CG30059	--	1.57	0.65	0.38
CG32041	*gene cassette*	1.58	0.66	0.42
Downregulated genes				
CG5215	*Zn72D*	-1.55	-0.63	0.3
CG7940	--	-1.52	-0.6	0.23
CG8745	--	-1.66	-0.73	0.17
CG11064	*RfaBp*	-1.43	-0.52	0.52
CG11911	--	-1.44	-0.53	0.17
CG18180	--	-1.53	-0.61	0.31
CG18816	*Tsp42Eb*	-1.46	-0.55	0.28

Table 4: Differentially expressed genes with a fold-change value greater than or equal to 1.41 or less than or equal -1.41 from the *P. nigrum* 0.2 mg/mL versus ethanol hybridization (chapter four, table 4.4).

CG number	Gene symbol	Fold expression change	Mean log_2 ratio	Standard deviation
Upregulated genes				
CG8749	*snRNP70K*	1.60	0.68	0.47
CG11822	*nAcRβ-21C*	1.44	0.53	0.42
CG13091	--	1.73	0.79	0.51
Downregulated genes				
CG5185	*Tom*	-1.58	-0.66	0.42

Table 5: Differentially expressed genes with a fold-change value greater than or equal to 1.41 or less than or equal -1.41 from the pyrethrin 0.004 mg/mL + *P. nigrum* 0.1 mg/mL versus ethanol hybridization (chapter four, table 4.5).

CG number	Gene symbol	Fold expression change	Mean log_2 ratio	Standard deviation
Upregulated genes				
CG2330	--	1.59	0.67	0.34
CG4533	l(2)efl	1.68	0.75	0.75
CG6484	--	1.52	0.6	0.55
CG6640	--	1.77	0.82	0.75
CG10383	--	1.69	0.76	0.56
Downregulated genes				
CG5185	*Tom*	-1.48	-0.57	0.52
CG13333	--	-1.45	-0.54	0.32

Table 6: Differentially expressed genes with a fold-change value greater than or equal to 1.41 or less than or equal -1.41 from the pyrethrin 0.04 mg/mL versus ethanol hybridization (chapter four, table 4.6).

CG number	Gene symbol	Fold expression change	Mean $\log_2$ ratio	Standard deviation
Upregulated genes				
CG1600	--	1.55	0.63	0.49
CG1924	--	1.55	0.63	0.21
CG2207	--	1.91	0.93	0.33
CG2918	--	1.49	0.58	0.35
CG30059	--	1.88	0.91	0.13
CG3209	--	1.52	0.6	0.4
CG3305	--	1.56	0.64	0.45
CG4027	*Act5C*	2.62	1.39	0.84
CG4533	*l(2)efl*	1.79	0.84	0.3
CG4559	*Idgf3*	1.43	0.52	0.36
CG4685	--	1.82	0.86	0.49
CG4843	*Tm2*	1.61	0.69	0.42
CG4847	--	1.46	0.55	0.32
CG5436	*Hsp68*	2.39	1.26	0.62
CG5725	*fbl*	1.52	0.6	0.28
CG5748	*Hsf*	1.43	0.52	0.31
CG6073	--	1.48	0.57	0.21
CG6119	--	2.38	1.25	0.61
CG6255	--	1.55	0.63	0.29
CG6369	*Smg6*	1.44	0.53	0.26
CG6621	--	1.54	0.62	0.19
CG6640	--	1.79	0.84	0.22
CG6687	--	1.82	0.86	0.29
CG7109	*mts*	1.45	0.54	0.39
CG7156	--	1.44	0.53	0.29
CG7415	*DppIII*	1.47	0.56	0.22
CG7452	*Syx17*	1.49	0.58	0.49
CG7756	*Hsc70-2*	2.14	1.1	0.58
CG7874	--	1.57	0.65	0.50

CG number	Gene symbol	Fold expression change	Mean log_2 ratio	Standard deviation
Upregulated genes (cont.)				
CG7878	--	1.77	0.82	0.42
CG7929	*ocn*	1.74	0.8	0.16
CG8165	--	1.54	0.62	0.30
CG8289	--	1.46	0.55	0.13
CG8308	*αTub67C*	2.2	1.14	0.31
CG8749	*snRNP70K*	1.55	0.63	0.49
CG8801	--	1.65	0.72	0.54
CG8831	--	1.43	0.52	0.41
CG8918	--	1.52	0.6	0.09
CG8939	--	1.45	0.54	0.36
CG8949	--	1.49	0.58	0.29
CG9027	--	1.57	0.65	0.19
CG9053	--	1.42	0.51	0.27
CG9083	--	1.41	0.5	0.19
CG9476	*αTub85E*	2.22	1.15	0.28
CG9512	--	1.67	0.74	0.41
CG9579	*AnnX*	1.52	0.6	0.35
CG9631	--	1.44	0.53	0.32
CG9641	--	1.73	0.79	0.34
CG9759	--	1.45	0.54	0.24
CG9906	--	1.78	0.83	0.66
CG9978	--	1.61	0.69	0.29
CG10067	*Act57B*	1.43	0.52	0.44
CG10383	--	1.41	0.5	0.49
CG10484	*Dox-A2*	1.61	0.69	0.38
CG10521	*NetB*	1.55	0.63	0.18
CG10537	--	1.45	0.54	0.16
CG10841	--	1.46	0.55	0.31
CG10992	--	1.54	0.62	0.33
CG11061	*GM130*	1.88	0.91	0.56
CG11331	*Spn27A*	1.75	0.81	0.44
CG11466	*Cyp9f2*	1.46	0.55	0.42
CG11771	--	1.43	0.52	0.41

CG number	Gene symbol	Fold expression change	Mean log_2 ratio	Standard deviation
Upregulated genes (cont.)				
CG12582	--	1.65	0.72	0.26
CG13091	--	1.61	0.69	0.48
CG14207	--	1.47	0.56	0.17
CG14998	--	1.44	0.53	0.28
CG16772	--	1.6	0.68	0.16
CG16782	--	1.85	0.89	0.74
CG18240	*Cyp12d1*	1.53	0.61	0.11
CG18858	--	1.8	0.85	0.31
Downregulated genes				
CG2198	*Ama*	-1.66	-0.73	0.26
CG2981	*TpnC41C*	-1.78	-0.83	0.2
CG3274	--	-1.44	-0.53	0.43
CG3494	--	-1.42	-0.51	0.38
CG4494	*smt3*	-1.53	-0.61	0.24
CG4609	*fax*	-1.42	-0.51	0.41
CG5185	*Tom*	-1.92	-0.94	0.53
CG5499	*His2Av*	-1.41	-0.5	0.45
CG7408	--	-1.46	-0.55	0.21
CG7445	*fln*	-1.46	-0.55	0.37
CG7590	*scylla*	-1.41	-0.5	0.16
CG7637	--	-1.57	-0.65	0.27
CG8505	--	-1.65	-0.72	0.17
CG8577	*PGRP-SC1b*	-1.51	-0.59	0.2
CG10385	*msl-1*	-1.46	-0.55	0.54
CG10894	*E(bx)*	-1.42	-0.51	0.37
CG11924	*Cf2*	-1.41	-0.5	0.25
CG13333	--	-1.62	-0.7	0.34
CG14715	--	-1.47	-0.56	0.17
CG15196	--	-1.48	-0.57	0.48
CG17012	--	-1.66	-0.73	0.26
CG31687	--	-1.68	-0.75	0.09
CG31969	*CR31969*	-1.44	-0.53	0.3
CG32957	*CR32957*	-1.56	-0.64	0.31

CG number	Gene symbol	Fold expression change	Mean $\log_2$ ratio	Standard deviation
Downregulated genes (cont.)				
CG33111	--	-1.54	-0.62	0.26

Appendix VI

Representative example of RT-QPCR analysis

Shown here are the standard curve, amplification plots and dissociation curve for the Rpl32 gene primers used in RT-QPCR.

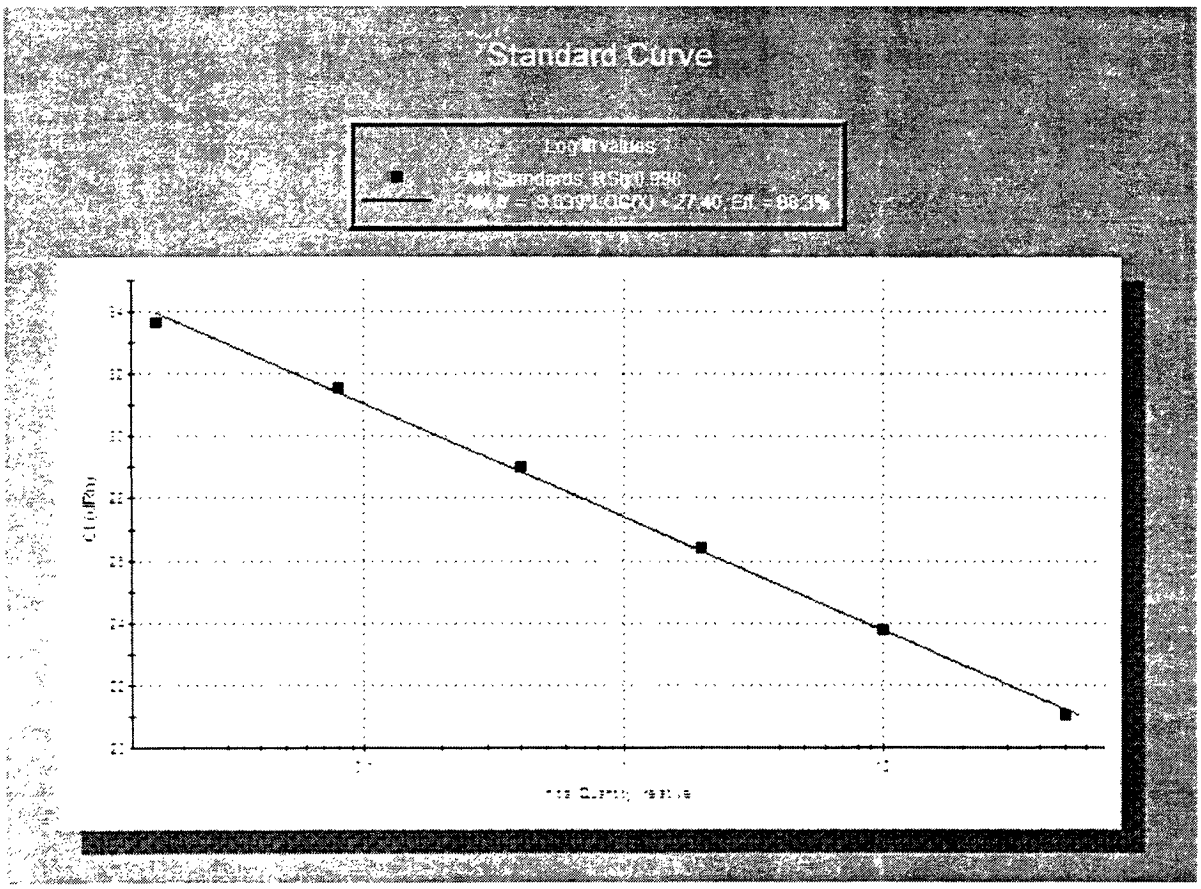

Figure 1: Standard curve for the quantification of *Rpl32* gene using RT-QPCR analysis. The R^2 value is 0.998 and the efficiency of the reaction is 88.3%.

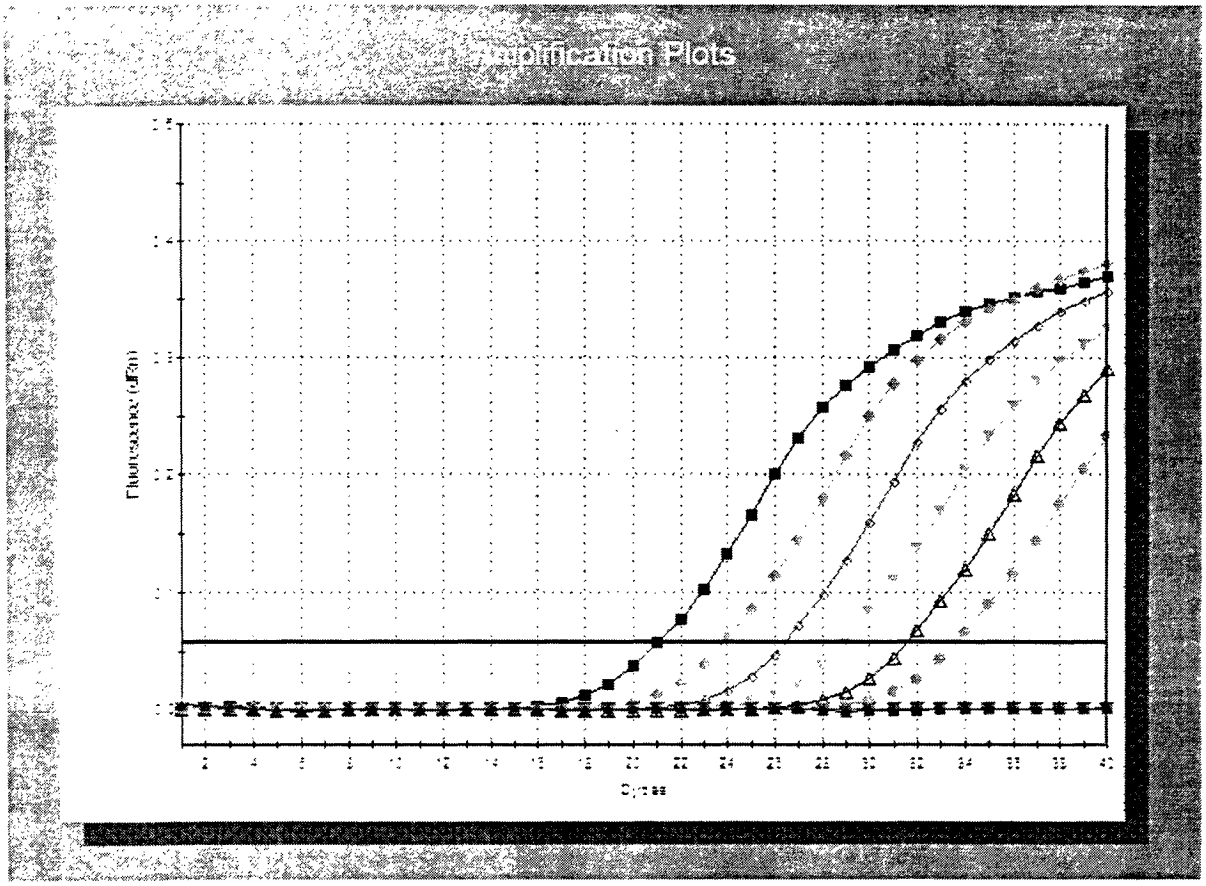

Figure 2: Amplification plots obtained for *Rpl32* in RT-QPCR.

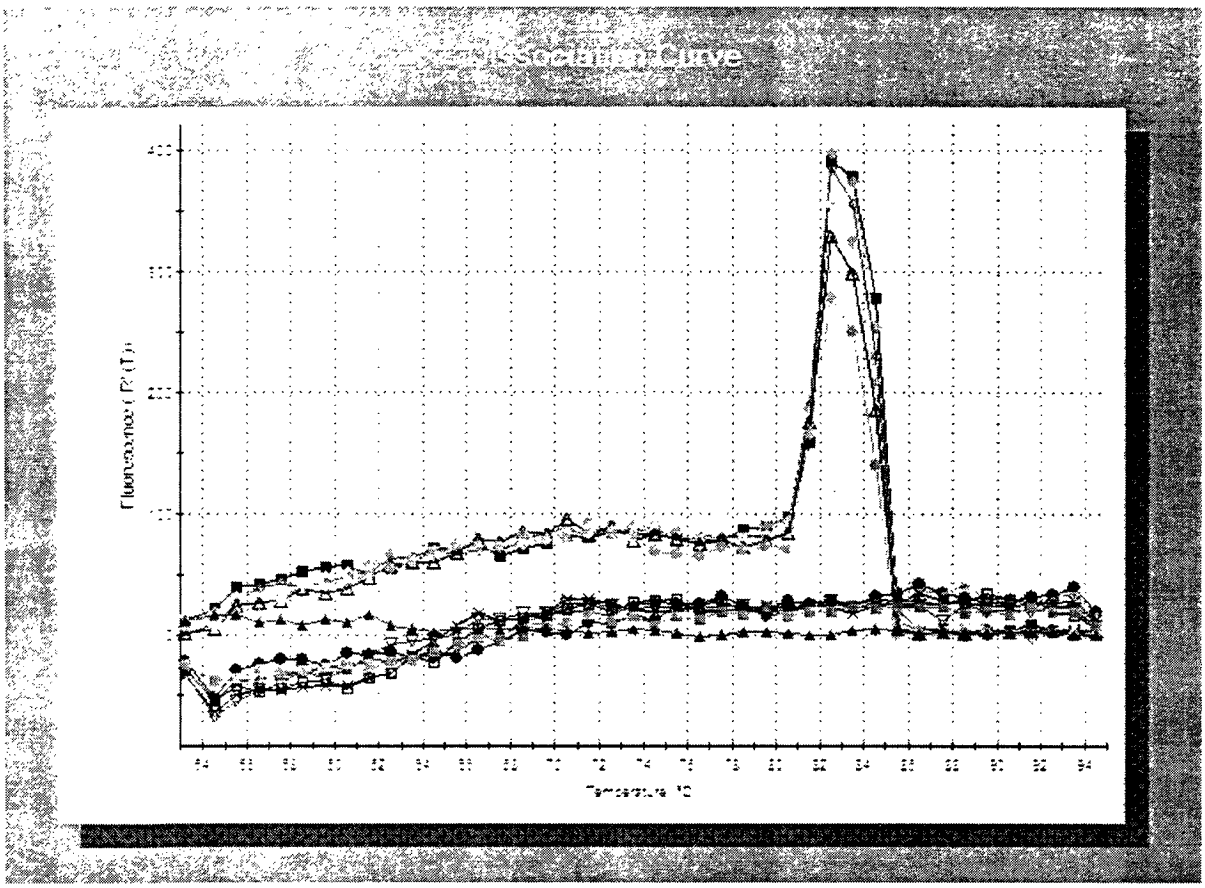

Figure 3: Dissociation curve analysis of *Rpl32* in RT-QPR showing no non-specific products.