Caenorhabditis elegans as a model for nematode metabolism of the anthelmintic drugs ivermectin and albendazole

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Abstract

Resistance to anthelmintics used to treat parasitic nematodes of veterinary importance represents a serious welfare and economic problem for the livestock production industry. Research into the mechanisms by which parasites develop resistance is necessary to prolong the life of the available drugs and to minimise development of resistance to new classes. Metabolism of anthelmintic compounds by parasites is a possible mechanism of resistance that has received little research, despite there being precedence in the case of insecticide resistance. Due to the more advanced molecular tools available and comparative ease of manipulation; we have used the model nematode *Caenorhabditis elegans* to investigate the metabolism of two important anthelmintic drugs, ivermectin and albendazole.

Whole genome microarrays and RT-QPCR were used to identify clusters of genes, which are significantly up-regulated upon exposure of *C. elegans* to anthelmintic. The transcriptomic response to albendazole is characterised by genes potentially involved in xenobiotic metabolism. These include members of the cytochrome P450 family and the UDP-glucuronosyl/ glucosyl transferase family. In contrast, the response to ivermectin appears to represent a fasting response caused by the phenotype of drug exposed nematodes. Recombinant worms carrying GFP reporter constructs of several genes of interest demonstrated their expression in the intestine, which is thought to be the main site of xenobiotic detoxification in nematodes. HPLC-MS techniques have definitively shown that *C. elegans* is able to metabolise albendazole to two glucose conjugates. These metabolites are compatible with the transcriptomic response to the drug and are similar to albendazole metabolites produced by the parasitic nematode *Haemonchus contortus*. No ivermectin metabolites were identified in the current study.

The data presented confirms the ability of the nematode *C. elegans* to respond to and metabolise anthelmintic compounds. In addition, the study validates the use of *C. elegans* as a model organism for parasitic nematodes and provides a platform upon which to investigate nematode metabolism further.

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CD containing:

- Full microarray data-
  - Normalised expression data for for each gene on each gene chip
  - Lists of significantly up-regulated and down-regulated genes in each experiment
- List of primers used for real-time quantitative PCR
- List of primers used for fusion-PCR
- List of primers used for sequencing

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Finally, a big thanks to all of the students and staff I now call friends and with whom I have spent the last three years drinking tea and having laughs.

Declaration

The work presented in this thesis was performed entirely by the author except where indicated. This thesis contains unique work and will not be submitted for any other degree, diploma or qualification at any other university.

Steven Laing BVMS (Hons) MRCVS, April 2010.

Definitions/ Abbreviations

ABZ	Albendazole
ABZ-SO	Albendazole sulphoxide
ABZ-SO_2	Albendazole sulphone
BH	Benjamini Hochberg
BSA	Bovine Serum Albumin
CAR	Constitutive androstane receptor
CYP	Cytochrome P450
DTT	Dithiothreitol
EDTA	Ethlenediaminetetraacetic acid
EHT	Egg hatch test
FA	Formic acid
FAD	Flavin adenine dinucleotide
FDR	False discovery rate
FECRT	Faecal egg count reduction test
FMN	Flavin mononucleotide
GluCl	Glutamate-gated chloride channel
GST	Glutathione-s-transferase
HLM	Human liver microsomes

IVM	Ivermectin
KOG	KOGs are a eukaryote-specific version of the Conserved Orthologous Groups (COGs)
MeCN	Acetonitrile
MRM	Multiple Reaction Monitoring
m/z	mass/ charge ratio
nAChR	nicotinic acetylcholine receptor
NGM	Nematode growth medium
PGE	Parasitic gastroenteritis
PMSF	Phenylmethylsulphonylfluoride
PPAR	Peroxisome proliferator- activated receptor
PXR	Pregnane X receptor
RP	Rank products
RT-QPCR	Real-time quantitative polymerase chain reaction
TOF	Time of flight (mass spectrometry)
UGT	UDP-glucuronosyl transferase
XME	Xenobiotic metabolising enzyme

Chapter 1: Introduction

1.1 Introduction

Resistance to commonly used anthelmintic drugs is a major problem in veterinary medicine (Getachew *et al.*, 2007; Pomroy, 2006; Gilleard, 2006; Kaplan, 2004; Wolstenholme *et al.*, 2004) and is becoming recognised in helminth parasites of humans (Osei-Atweneboana *et al.*, 2007; Awadzi *et al.*, 2004a; Albonico *et al.*, 2002; De *et al.*, 1997; Eberhard *et al.*, 1991). Parasitic gastroenteritis is though to cost the UK sheep production industry alone in the region of £84 million per year (Nieuwhof and Bishop, 2005). Additionally, it is thought that up to one billion people in sub-Saharan Africa, Asia and the Americas are affected by helminthoses, the most common being GI nematodes (Hotez *et al.*, 2008). In order to maintain the efficacy of the currently available anthelmintics, and to aid in the development of novel synergists and therapeutics, the molecular mechanisms resulting in resistance must be elucidated. However, with the possible exception of the benzimidazoles, where genotyping of ß-tubulin isotype-1 genes may be diagnostic, convincing evidence of conserved population-wide mutations resulting in resistance to the other drug classes is lacking (von Samson-Himmelstjerna *et al.*, 2009).

Metabolism of chemotherapeutics is a common mechanism of resistance in many classes of organism. Notably, insecticide resistance has been associated with overexpression of many classes of metabolising enzymes and in several cases a causative relationship has been proven (Li *et al.*, 2007; Daborn *et al.*, 2002). Studies investigating the genetics of anthelmintic resistance have largely focussed on mutations in the target gene of the drugs and recently the role of ABC transporters such as the PGPs, reviewed by Gilleard (Gilleard, 2006). The role of xenobiotic metabolising enzymes (XME) in anthelmintic resistance has been largely overlooked in the genomic era, but several recent studies have suggested that these pathways could be involved (Cvilink *et al.*, 2009a; Kotze *et al.*, 2006a).

This study has made use of whole genome microarrays and high performance liquid chromatography with tandem mass spectrometry (HPLC-MS) to begin to assess XME pathways in nematodes more fully.

1.2 The emergence of anthelmintic resistance

Anthelmintic therapy remains the mainstay of control of parasitic disease in both human and veterinary medicine. However, resistance to anthelmintic drugs has arisen quickly following their clinical application. Resistance to thiabendazole, a benzimidazole drug introduced in 1961 as the first widely used anthelmintic in veterinary species, was reported in the barber pole nematode of sheep, *Haemonchus contortus,* within a few years of its use (Conway, 1964; Drudge *et al.*, 1964). Resistance to all three major drug classes: the benzimidazoles, the imidazothiazole- tetrahydropyrimidines and the avermectin- milbemycins (or macrocyclic lactones), is now commonplace (Sargison *et al.*, 2007; Gilleard, 2006; Pomroy, 2006).

Alleles of genes which confer a resistant phenotype are hypothesised to be present within drug susceptible parasite populations at a low frequency (Sargison *et al.*, 2007; Le Jambre, 1978). Selection by anthelmintic therapy results in an increase in frequency of these alleles until the population becomes sufficiently resistant to lead to treatment failure. Although poorly understood, the method and frequency of anthelmintic administration is considered to affect the rate at which resistance emerges in a parasite population. A recent study investigated anthelmintic practice in four sheep flocks in the South-East of Scotland where multi-resistant populations of *Teladorsagia circumcincta* have arisen (Sargison *et al.*, 2007). Under-dosing of larger animals and over-frequent dosing were found to be a problem on several of the farms. Inadequate treatment of animals newly arrived on a farm, which may be harbouring resistant parasites, was also found to be a problem. In addition, many of the farms adopted a “dose and move” strategy, meaning that the sheep are moved to clean (parasite-free) pasture after having been treated with anthelmintic. The major drawback with this practice is that the largely anthelmintic susceptible population of eggs and larvae left in the original field will die due to the lack of the presence of the host. Consequently the *in refugia* population of parasites, i.e. those on the

pasture and not affected by anthelmintic dosing of the host, will consist entirely of the progeny of any resistant worms that the sheep were harbouring. Thus the selection pressure on the effective population is increased (Sargison *et al.*, 2007; van Wyk, 2001). It should be noted that whilst resistance of human parasites is not currently recognised as a common clinical problem, the mass dosing approach used to treat and prevent diseases such as human onchocerciasis (river blindness), applies similar pressures on the parasite population. There are now several reports of reduced efficacy of anthelmintics against nematodes of humans (Osei-Atweneboana *et al.*, 2007; Awadzi *et al.*, 2004b; Albonico *et al.*, 2002; De *et al.*, 1997; Eberhard *et al.*, 1988).

Resistant populations of veterinary parasitic nematodes are widespread. **Table 1-1**, modified and updated from Kaplan (2004), summarises the main problems with reference to cyathostomes in horses and trichostrongyloid nematodes of ruminants (unless otherwise specified):

Drug	Hosts with high resistance	Hosts with emerging resistance	Major livestock- producing areas where drug is still highly effective in sheep, goats and horses
Benzimidazoles	Sheep, goats, horses	Cattle	None
Levamisole (ruminants)	Sheep, goats	Cattle	None
Pyrantel (horses)	Horses (USA only)	Horses	Unknown- few recent studies outside USA
Ivermectin	Sheep, goats, cattle	Cattle, horses	Horses- worldwide Sheep, Goats- Europe, Canada
Moxidectin	Goats	Sheep, goats, cattle, horses	Horses- worldwide Sheep- most regions

Table 1-1: Current prevalence of anthelmintic resistance in veterinary species Adapted from Kaplan (2004).

More recently, resistance to pyrantel has been reported in both cyathostomins and *Parascaris equorum*, including in the UK (Lyons *et al.*, 2008b; Comer *et al.*, 2006). In addition, *P. equorum* resistance to macrocyclic lactones is now widespread (Reinemeyer, 2009; Lyons *et al.*, 2008a; Stoneham *et al.*, 2006). At the time of compiling the original table, Kaplan reported that no resistance against pyrantel or the macrocyclic lactones was seen in cyathostomin parasites of horses. However, there are now several reports of cyathostomin resistance to most of the available anthelmintics other than moxidectin (Traversa *et al.*,

2009; Edward *et al.*, 2008; Lyons *et al.*, 2008b; von Samson-Himmelstjerna *et al.*, 2007).

1.3 Diagnosis of resistance in field populations

The accurate diagnosis and quantification of resistance within a parasite population is vital so that appropriate treatment can be given on a farm to farm basis. Currently, anthelmintic efficacy is assessed using the undifferentiated faecal egg count reduction test. This is a crude test using the percentage decrease in egg counts taken before and after treatment as an assay of the level of resistance in a parasite population (McKenna, 2006). It is not specific to a particular parasite species and is insensitive when resistance is emerging. Other tests of anthelmintic resistance rely on *in vitro* exposure of the free living stages of parasites to drug. Several parameters can then be assessed such as egg hatching (EHT), larval feeding inhibition (LFIA) and larval migration and development (Coles *et al.*, 2006; Kotze *et al.*, 2006b; Alvarez-Sanchez *et al.*, 2005). However, in all cases there is marked variation in the sensitivity of the assays between different nematode species. In addition the EHT and larval development tests can provide very variable results depending on the operator (Coles *et al.*, 2006). Finally, these assays provide no information regarding the mechanism of resistance, which may be pertinent in deciding on a therapeutic programme.

A molecular diagnostic tool, testing for the presence of resistance-conferring alleles in a population before resistance is clinically apparent, would allow more educated treatment protocols to be implemented. Recent work by von Samson-Himmelstjerna *et al.* (2009), has suggested that pyrosequencing of ß-tubulin isotype 1 codon 200 may be used as a diagnostic test of benzimidazole resistance in *H. contortus*, discussed in **Section 1.7.2**. In order for this to be achieved for other anthelmintics, a thorough understanding of both the mechanism of action and the molecular mechanism(s) of resistance will be necessary.

1.4 Novel chemotherapeutics

Since the introduction of the macrocyclic lactones in the early 1980s there have been no new classes of anthelmintic licensed for use in small ruminants. In recent years, the growing problem of anthelmintic resistance has led to increased research and interest in the area by several of the major pharmaceutical companies. In the coming months two new products are to be released. The first to be commercialised will be monepantel, marketed as Zolvix by Novartis. Monepantel is an amino-acetonitrile derivative, and is thought to be an agonist of a novel nematode–specific nicotinic acetylcholine receptor (nAChR) (Rufener *et al.*, 2009b; Kaminsky *et al.*, 2008a). Members of this class have a broad spectrum of action and have been shown to be effective against parasite isolates resistant to the currently available anthelmintics. The mechanism of action was first investigated and mapped to the DEG-3 class of nAChR in *Caenorhabditis elegans*. *In vitro* exposure of *H. contortus* larvae to increasing doses of monepantel resulted in resistant strains within eight generations. Mutations in three nAChR genes within the DEG-3 subfamily were found in the resistant strains (Rufener *et al.*, 2009b; Kaminsky *et al.*, 2008b).

Derquantel (2-deoxyparaherquamide) is a paraherquamide derivative that is to be licensed as a drench in combination with abamectin. This class of drug is an antagonist of nAChR (Zinser *et al.*, 2002). In *Ascaris suum* muscle strips derquantel is thought to exert its affect through the *B*-subtype of nAChR, distinct from the *L*-subtype through which levamisole exerts its effect (Qian *et al.*, 2006). Interestingly, it is difficult to detect the effects of paraherquamide derivatives in *C. elegans* even at doses of up to 50μM (*pers. comm.*, Dr. Tim Geary & Dr. Eileen Coscarelli). The spectrum of activity of the paraherquamide derivatives is not as broad as monepantel alone, but in combination with abamectin the spectrum is increased and resistant isolates are also effectively treated. Derquantel and abamectin will be released in the UK as Startect by Pfizer (WAAVP conference 2009).

In addition to the amino-acetonitrile derivatives and paraherquamide derivatives, the cyclooctadepsipeptides have been shown to be active against resistant isolates of small ruminant parasites (Harder *et al.*, 2003). Emodepside,

a member of this class, has been licensed for use as a wormer in cats and dogs. However, due to the expense of production it has not yet been licensed for use in ruminants. Emodepside inhibits development, paralyses the pharynx and body and stops egg production in *C. elegans*. It mediates these effects via the latrophilin-like receptors, LAT-1 and LAT-2, and the calcium activated potassium channel SLO-1 (Guest *et al.*, 2007; Harder *et al.*, 2003).

The advent of these novel classes of anthelmintic is a welcome relief to the small ruminant industry. However, investigation of the mechanism of action of monepantel has already shown how readily *H. contortus* populations could become resistant to the drug. It is likely that this will be the case for derquantel and emodepside too. In the face of these possibilities it is imperative that research continues into the mechanisms by which parasites become resistant to all anthelmintics. Only with this level of understanding can appropriate diagnostic tests be developed to allow the educated use of new drugs and minimise the development of resistance.

1.5 Alternatives to anthelmintic control

It has been suggested that parasite control that relies entirely on anthelmintic dosing is not sustainable (van Wyk, 2002). Several alternatives or adjuncts to chemotherapeutics have been proposed to minimise the impact of parasitic gastroenteritis, reviewed by Sayers *et al.*(2005). Novel grazing management strategies such as rotational grazing between cattle and sheep; no dosing before moving to clean pasture in order to keep a susceptible *in refugia* population and alternative pasture species have been shown to reduce parasite burden and improve weight gain (Niezen *et al.*, 2002; Githigia *et al.*, 2001).

The use of predacious microfungi such as *Duddingtonia flagrans*, which traps nematode larvae, has had mixed success in improving production parameters. Some authors report increase in weight gain and decrease in anaemia in parasitized sheep following introduction of the fungi, but others saw no statistical improvement (Silva *et al.*, 2009; Epe *et al.*, 2009; Chandrawathani *et al.*, 2004; Fontenot *et al.*, 2003). Certain plant extracts have also been shown to reduce nematode burden. Recent studies have investigated the use of *Zizphus*

nummularia bark, *Acacia nilotica* fruit, *Maesa lanceolata* leaves and fruit, aerial parts of *Plectranthus punctatus* leaves and *Artemisia absinthium* (Bachaya *et al.*, 2009; Tadesse *et al.*, 2009; Tariq *et al.*, 2009). All of these plants were found to have varying degrees of anthelmintic potency. However, the active compounds in these plants are unknown and further research would be required before such plants could be used commercially in this country. In addition, resistance to these naturally derived anthelmintics is just as likely to arise as for synthesised drugs.

Breeding sheep for resistance to gastrointestinal parasites is a continued aim of many groups and has had some success. Quantitative trait loci for resistance to PGE are currently being mapped and assessed (Marshall *et al.*, 2009; Crawford *et al.*, 2006; Kahn *et al.*, 2003). In addition, the nutritional status of sheep greatly affects susceptibility to parasitic nematodes (Valderrabano *et al.*, 2006). Protein supplementation has been shown to improve immunity to several gastrointestinal parasites (Sykes *et al.*, 2001; Stear *et al.*, 2000).

Many of these strategies have been shown to have a positive effect on productivity and reduce worm burdens in affected animals. However, whilst they may reduce the need for anthelmintic dosing they do not preclude it entirely. Therefore, these strategies may only serve to delay the emergence of a resistant population and where multi-anthelmintic resistant parasite populations are already present, they offer little respite.

There has been a great deal of research into viable vaccine candidates for gastrointestinal nematodes. Bethony *et al.* (2006) reviewed the available vaccine candidates for the blood feeding nematodes of both humans and livestock, such as whole irradiated worms and proteins involved in penetration (Hookworm species) and blood meal digestion. Several protective antigens for *H. contortus* have been discovered. The most effective single protein to date has been the H11 antigen (Andrews *et al.*, 1997; Andrews *et al.*, 1995). This represents a gut expressed aminopeptidase and vaccination with the native protein results in up to 90% decrease in worm burden. However, trials of recombinant protein vaccines have not provided an equivalent protection. The only vaccine against any nematode infection currently in use is an irradiated larvae vaccine of

Dictyocaulus viviparous, the cause of parasitic bronchitis in cattle. This is licensed as Dictol or Huskavac from Intervet (McKeand, 2000).

The main drawback with any vaccine strategy thus far proposed for the prophylaxis of parasitic gastroenteritis (PGE) is the lack of a broad spectrum of action. PGE is rarely caused by a single species and anthelmintic drugs are useful in their ability to treat many co-infecting parasites simultaneously. In order for a single vaccine to have this effect it would need to induce a response against a shared antigen or contain antigens from many different species. Therefore, it is likely that for the foreseeable future anthelmintic drugs will remain the mainstay of control for parasitic helminthoses.

1.6 *C. elegans* as a model organism

Most of the nematodes of veterinary importance are obligatory parasites, making them very difficult to work with directly. For example, studies carried out using *H. contortus* are labour intensive due to the necessity of infecting sheep to maintain the reproductive stages of the parasite (Le Jambre *et al.*, 2000). It is mainly for these reasons that the use of model organisms, which are more easily manipulated, has become more common.

C. elegans is a free-living nematode that was first used in 1965 to study animal development and behaviour by Sydney Brenner (Riddle *et al.*, 1997). The nematode can be grown on agar plates with a bacterial food source and as such is easily manipulated for a variety of experiments. *C. elegans* was originally used to investigate neural anatomy and development. However, with the complete sequencing of the *C. elegans* genome in 1998 and the production of many advanced genetic tools, the organism is now used as a model for many different processes. These range from the investigation of muscle development in zero-gravity to the pathogenesis of Alzheimer's disease in humans (Higashibata *et al.*, 2006; Link *et al.*, 2003).

The use of *C. elegans* as a model for parasitic nematodes has slowly increased since it was first used to screen potential anthelmintic compounds in 1981 (Simpkin *et al.*, 1981). However, there are several areas of parasite specific biology, including feeding and host immune system evasion, for which it is not a

suitable model (Gilleard *et al.*, 2005). The appropriateness of *C. elegans* as a model for parasites can be expected to vary depending on the parasite species being investigated. For example, the trichostrongylid parasites have free-living larval stages and the adults are non-invasive, meaning they remain in the gut lumen of the host and do not migrate through other tissues. These may be expected to have more similar biology to the free-living nematode than a filarial nematode, such as *Dirofilaria immitus*, which has no free-living stages would (Geary *et al.*, 2001). Phylogenetic analysis of the phylum Nematoda, would also suggest that trichostrongylids, including *H. contortus*, are more closely related to *C. elegans,* see **Fig. 1-1** (Dorris *et al.*, 1999). *C. elegans'* use as a model is likely to be more appropriate for these species. However, transcriptomic analysis of *C. elegans* and 28 parasitic nematodes revealed that even closely related nematodes such as *H. contortus* shared only approximately 60% genome similarity to *C. elegans* (Parkinson *et al.*, 2004). On average 23% of genes were unique to the species they were derived from. Therefore, *C. elegans* will be of most use as a model to investigate core biology and conserved pathways. Cytochrome P450 genes are ubiquitous, having been found in vertebrates, invertebrates, fungi and plants as well as in prokaryotes (Nelson *et al.*, 1996). Many of these enzymes have important roles in core biological processes. For example *C. elegans daf-9* (*cyp-22A1*) is involved in regulating larval development and adult lifespan, possibly through the production of a steroidogenic ligand for DAF-12 (Jia *et al.*, 2002). Therefore conservation of function between *C. elegans* and parasitic nematodes may be expected.

C. elegans has been validated as a model for the core biology of closely related nematodes through many different experiments. Transgenic *C. elegans* have successfully been used to drive the expression of an *H. contortus* pepsinogen, under the control of the promoter region of *C. elegans cpr-5* (Redmond *et al.*, 1999). The *H. contortus* homologue of *elt-2*, a *C. elegans* GATA transcription, was shown to have conservation of function in the free-living nematode (Couthier *et al.*, 2004). However, there are also several examples where function is not completely conserved. Transgenes containing LacZ reporters under the control of promoter regions of genes from the parasitic nematodes *H. contortus* and *T. circumcincta* drove expression in a tissue specific manner (Britton *et al.*, 1999). However, the timing of expression was not as expected. In

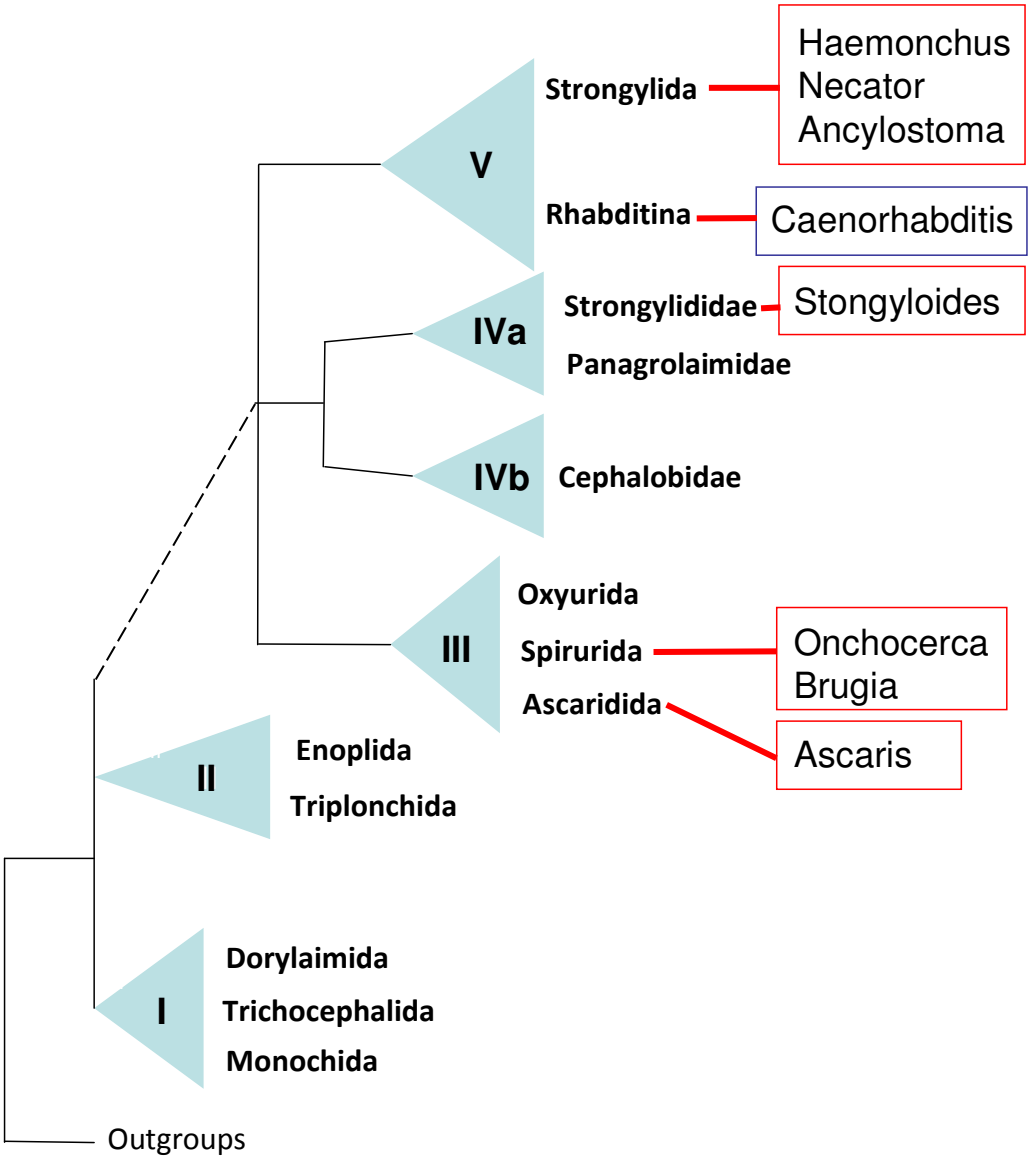


Figure 1-1: Phylogenetic relationship between the major phylogenetic clades (I-V) of the phylum Nematoda based on SSU RNA sequence
Adapted from Dorris *et al.* (1999). The red boxes contain examples of parasitic members of the associated order or family. *Caenorhabditis elegans* belongs to the suborder Rhabditina and is clustered in the same phylogenetic clade as *Haemonchus contortus* and other parasites of veterinary and human importance.

addition, a recent paper investigating HSP-90, revealed that neither *H. contortus* or *Brugia pahangi hsp-90* homologues were able to completely rescue a *C. elegans daf-21 (hsp-90)* null mutant (Gillan *et al.*, 2009).

Anthelmintic mode of action is an area in which *C. elegans* has already been very useful as a model organism. Experiments with the organism have been fundamental in discovering the mechanism of action of all three main groups of anthelmintic, as well as many of the novel compounds discussed in **Section 1.4** (Rufener *et al.*, 2009b; Brown *et al.*, 2006; Gilleard, 2006; Dent *et al.*, 2000; Cully *et al.*, 1996; Fleming *et al.*, 1996; Driscoll *et al.*, 1989; Brenner *et al.*, 1974). Importantly, the conclusions drawn from work with *C. elegans* have consistently been validated in parasitic nematode species. *C. elegans* has also been successfully used to elucidate the mechanism of resistance to the benzimidazole class of anthelmintics, discussed in **Section 1.8** (Kwa *et al.*, 1993a; Kwa *et al.*, 1993b). However, there has been limited success for the avermectins and levamisole. The major problem has been that genes identified as sufficient to confer resistance in the model organism have not been found to be universally present in resistant parasite populations.

Clearly any conclusions derived from work with *C. elegans* must be verified in the species of interest. However, the ability to undertake forward genetic approaches in the model organism is a powerful tool for the identification of genes that confer resistance to anthelmintics. Many parasitic nematode species have on-going genome projects in varying states of completion (see www.nematode.net; www.sanger.ac.uk/Projects/Helminths/). However, thus far none of the gastrointestinal nematodes of veterinary importance have completely sequenced genomes and as such the same genetic tools are not available. The use of high throughput techniques such as microarrays and SAGE analysis allows the entire genome to be investigated, decreasing the chance that a novel route of resistance will be missed. It also allows better investigation of resistance which is not caused by simple SNP (single nucleotide polymorphism) mutation of a gene. Furthermore, it has been noted by many authors that genetic techniques such as RNA inhibition, which is now commonly used in *C. elegans* research, may not be so easily applied to parasitic species (Lendner *et al.*, 2008; Geldhof *et al.*, 2006).

In summary, *C. elegans* has shown itself to be extremely useful as a model organism for many nematode processes. Whilst several aspects of parasite biology can be expected to be divergent, the free-living nematode currently offers the best available platform to carry out high-throughput genetic experiments. Providing that these studies are carried out in parallel with experiments in the parasitic species of interest, it is likely that *C. elegans* will continue to be fundamental in the investigation of anthelmintic resistance.

1.7 Ivermectin

1.7.1 Mechanism of action

It is generally accepted that the main mode of action of the drug is brought about by irreversibly binding to and activating ligand-gated ion channels, particularly glutamate-gated chloride channels (Holden-Dye *et al.*, 2006; Yates *et al.*, 2003; Brownlee *et al.*, 1997). Activation results in hyperpolarisation of the affected cell and inhibition of neuromuscular stimuli. This process can explain most of the effects seen in the whole nematode under experimental conditions and *in vivo*: decreased motility and feeding and a lower reproductive rate (Gilleard, 2006; Yates *et al.*, 2003). A direct link between decreased fecundity and glutamate-gated chloride channels has yet to be established.

Glutamate-gated chloride channels (GluCl) are thought to be heteropentomeric transmembrane structures. There have been six genes encoding GluCl subunits noted in the *C. elegans* genome: *avr-14, avr-15, glc-1, glc-2, glc-3* and *glc-4.* Both *avr-14* and *avr-15* are thought to encode two subunits each by alternative splicing (Dent *et al.*, 2000; Dent *et al.*, 1997). The *H. contortus* genome contains three genes encoding four GluCl subunits. Two of the genes are clear homologues of those found in *C. elegans, Hc-glc-2* and *Hc-avr-14* (Jagannathan *et al.*, 1999; Delany *et al.*, 1998). Interestingly, the *Hc-avr-14* gene is also thought to be alternatively spliced, a feature that is conserved in all nematodes in which homologues have been studied (Yates *et al.*, 2003; Jagannathan *et al.*, 1999). Recent studies have also shown that an *Hc-avr-14* transgene is able to rescue *avr-14* mutations in *C. elegans* (McCavera *et al.*, 2009).

A particular GluCl channel may contain a different combination of subunits depending on the species investigated and the anatomical location of the channel within a species. This is likely to affect where ivermectin has the greatest effect, as binding to different subunits, or combination of subunits, differentially activates a channel. For example, *C. elegans* GluClß homomeric channels, cloned in *Xenopus* oocytes, are insensitive to ivermectin whereas GluClα1 homomeric channels are highly sensitive to ivermectin (Etter *et al.*, 1996). The pharyngeal muscles of *C. elegans* are particularly sensitive to the effects of ivermectin; this is thought to be dependant on the presence a GluClα2 subunit encoded by *avr-15* (Pemberton *et al.*, 2001; Dent *et al.*, 1997). Differences in subunit expression between different species of nematode, results in ivermectin having slightly different effects on different parasites (Holden-Dye *et al.*, 2006).

Other proposed targets for ivermectin include GABA receptors, which may play a role in the pharyngeal phenotype of ivermectin-exposed *Ascaris suum* (Brownlee *et al.*, 1997). Chick or human α7 nicotinic acetylcholine receptors expressed in *Xenopus* oocytes exhibited sensitivity to ivermectin exposure as did human $P2X_4$ receptors (Khakh *et al.*, 1999; Krause *et al.*, 1998). A histamine-gated chloride channel (HisCl) has been implicated in avermectin sensitivity in *Drosophila melanogaster* (Gisselmann et al., 2002). However, HisCl channels are not present in the *C. elegans* genome. Whilst the GluCl channels are still accepted to be the main target of ivermectin in nematodes, it is clear that the mechanism of action of the drug is very complex. Therefore, multiple mechanisms of resistance may be employed by resistant isolates (Gilleard, 2006; Yates *et al.*, 2003).

1.7.2 The molecular basis of avermectin resistance in nematodes

Early theories on the mechanism of ivermectin resistance have focussed on mutations of the receptors to which the drug binds. Selection for specific alleles of genes encoding several ligand-gated ion channel subunits, including glutamate-gated channel subunits, has been noted in ivermectin-resistant strains of *H. contortus* (Gilleard, 2006). Blackhall *et al.* (1998b) examined the frequency of different glutamate-gated chloride channel alpha subunit alleles in unexposed and avermectin exposed isolates of *H. c*ontortus. They found that one allele was

consistently more frequent in drug selected (resistant) strains compared to unselected isolates, whilst another was reduced in frequency. This suggests that IVM exposure exerts selective pressure on GluCl channels. Njue *et al.* (2004) showed selection for GluCl3α subunit amino acid changes in ivermectin resistant *Cooperia oncophora* and demonstrated that one of these changes, L256F, resulted in decreased ivermectin sensitivity in channels expressed in *Xenopus* oocytes. More recently, the same L256F mutation in *H. contortus* GluClalpha3B subunit has been shown to affect ivermectin binding to the channels (McCavera *et al.*, 2009). However, in both cases the change in sensitivity of the channels was small and a direct relationship between this and the degree of resistance in field strains remains to be ascertained.

P-glycoproteins, members of the ABC transporter family, have also been proposed to be under selection pressure in ivermectin exposed strains of *H. contortus* (Sangster *et al.*, 1999; Blackhall *et al.*, 1998a). This was also found to be the case in ivermectin-exposed strains of the human parasite *O. volvulus* (Ardelli *et al.*, 2006). In addition, resistant isolates of *H. contortus* have been associated with mutations in ß- tubulin alleles; down regulation of dopamine-gated ion channels and up regulation of thioredoxin genes (Rao *et al.*, 2009; Sotirchos *et al.*, 2008; Eng *et al.*, 2006). Whilst all of these studies propose plausible mechanisms of resistance, they are, for the most part, based entirely on associations with ivermectin exposure or resistance. There has been a dearth of work into the functional importance of these polymorphisms and their frequency throughout parasitic nematode populations.

Gill *et al.* (1998) carried out a relatively simple study comparing differences in larval motility and development, as well as response to paraherquamide in three different laboratory-induced ivermectin-resistant strains of *H. contortus*. One of the isolates responded as per field-resistant *H. contortus* isolates, showing reduced sensitivity to ivermectin induced inhibition of development and motility but increased sensitivity to paraherquamide. The other two strains did not show a decrease in sensitivity to avermectin inhibition of development or motility, despite requiring a 10- fold greater concentration of ivermectin to kill 95% of the adults compared to parent strains. This study clearly shows that multiple mechanisms of resistance may be present and that experiments using ivermectin-resistant strains created in the laboratory must be interpreted with

care as the mechanisms used may be completely different to those used in field isolates.

Several ivermectin-resistant strains of *C. elegans* have been produced *in vitro*. Mutation of three important glutamate-gated chloride channel subunits (GLUClα3, GLUClα2, and GLUClα1) confers a very high level of resistance, EC_{37} 4264ng/ml (4.86μM) IVM. However, it is interesting to note that mutation of just one or two of these subunits results in much lower resistance to ivermectin, EC_{37} 13.8ng/ml (15.73nM) IVM or less (Dent *et al.*, 2000). Mutations to several other genes, not encoding known drug targets, have also been shown to confer ivermectin resistance to the nematode. These include innexins, components of nematode gap junctions, and *Dyf* mutants, which are thought to take up less ivermectin resulting in decreased sensitivity (Gilleard, 2006). More recently, selection of ivermectin-resistant strains of *C. elegans* produced by ivermectin exposure, rather than EMS mutagenesis, has shown that up-regulation of *pgps* and glutathione synthesis activities are associated with ivermectin resistance. However, no functional studies were undertaken and the ABC transporter family were the only genes to be analysed using real-time QPCR (James *et al.*, 2009).

In summary, it has been shown in parasitic species that mutations or overexpression of many genes may be associated with ivermectin resistance. *Caenorhabditis elegans* has been extremely useful in the initial identification and characterisation of many of these mutations. However, no single mutation has consistently been found in all ivermectin-resistant parasite populations. The functionality of the associated changes has not been assessed within parasite species. It seems increasingly likely that multiple mechanisms of resistance to ivermectin may be employed by parasites and that these mechanisms may differ between and within species. Therefore, further investigation of this complex problem will greatly benefit from the use of forward genetic techniques that allow an unbiased evaluation of the whole genome of nematodes under selective pressure from anthelmintics.

1.8 Albendazole

1.8.1 Mechanism of action

Albendazole belongs to the benzimidazole (BZ) class of anthelmintics. The major drug target of this group, ß-tubulins, have been well characterised in many species including *C. elegans* and parasitic nematodes (Driscoll *et al.*, 1989; Lacey *et al.*, 1986; Laclette *et al.*, 1980; Ireland *et al.*, 1979). Driscoll *et al.* (1989) first mapped BZ resistance to the *ben-1*, ß-tubulin, gene in *C. elegans* by creating resistant mutants with deletions in that gene. Several years later ß-tubulin was shown to be the target of the BZ drug group in *H. contortus* by showing that tubulin genes from the parasite could restore sensitivity when expressed in *ben-1* mutants of *C. elegans* (Kwa *et al.*, 1995). By binding to tubulins the BZs are postulated to inhibit polymerisation and the formation of microtubules, primarily in the gut. The downstream effects of this process have been studied in *H. contortus* and result in inhibition of egg hatching, slowed development and flaccid paralysis of the nematodes (Jasmer *et al.*, 2000).

1.8.2 The molecular basis of benzimidazole resistance in nematodes

The mechanism of action of the benzimidazole drugs appears to be far less complex than that of the avermectins. Mutations in the drug target, ß-tubulin, have generally been accepted as the major mechanism of resistance. Driscoll *et al.* (1989) used EMS mutagenesis to create several BZ-resistant strains of *C. elegans*. The resistance conferring mutations in all of these strains was mapped to the ß-tubulin gene, *ben-1*. Following that it was discovered that a phenylalanine to tyrosine substitution at position 200 of the isotype-1 ß-tubulin gene was consistently present in BZ-resistant strains of *H. contortus* (Kwa *et al.*, 1993a; Kwa *et al.*, 1993b). The functional importance of these mutations was confirmed by heterologous expression of *H. contortus* ß-tubulin alleles in transgenic *C. elegans* (Kwa *et al.*, 1995). Mutations of homologous tubulin genes have been associated with BZ resistance in many other parasitic nematode species including *Cooperia oncophora, Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (Winterrowd *et al.*, 2003; Silvestre *et al.*, 2002;

Grant *et al.*, 1996). Importantly, a recent study of a BZ resistant population of *Trichostrongylus axei*, carrying the F200Y mutation, has revealed that there was no reversion to wild-type genotype following a period of 7 years with no exposure to the drug (Palcy *et al.*, 2008). This suggests that this mutation can occur with no fitness cost to the nematode and that once BZ-resistant populations of nematodes are present on a farm they are likely to remain so.

Recent research has proposed that other mutations in the ß-tubulin protein may also be able to confer resistance to the BZs. These include glutamic acid to alanine substitutions at codon 198 in *H. contortus* and *Teladorsagia circumcincta* and phenylalanine to tyrosine substitutions at codon 167 in *H. contortus, T. circumcincta* and cyathostomin species (*Pers. comm.*, Dr. E. Redman; Rufener *et al.*, 2009a; Hodgkinson *et al.*, 2008; Silvestre *et al.*, 2002). Interestingly, benzimidazole resistant strains of *Ancylostoma caninum, Ancylostoma duodenale* and *Necator americanus* do not appear to be associated with mutations of tubulin genes at the usual codons (167 and 200) (Schwenkenbecher *et al.*, 2007). In addition, BZ-resistant strains of the liver fluke *Fasciola hepatica* do not appear to be consistently associated with any mutations of ß-tubulin genes (Ryan *et al.*, 2008).

Further evidence that multiple mechanisms of resistance to benzimidazoles may be employed came from von Samson- Himmelstjerna *et al.* (2009) who compared SNP frequency to thiabendazole resistance in different populations of *Haemonchus contortus,* see **Fig. 1-2.** This study showed that populations in which the susceptible TTC allele at codon 200 was not present, were all resistant to thiabendazole. However, the level of resistance varied greatly. In several circumstances, the variation between isolates classed as resistant was greater than that between some resistant and susceptible isolates. It is possible that these differences in resistance result from combinations of mutations in the ß-tubulin gene. However, there are increasing reports of BZ resistance being associated with other mechanisms such as metabolism of the drugs and changes in p-glycoprotein allele frequency. Certainly, in the case of triclabendazole resistance in *Fasciola hepatica* recent studies suggest that metabolism of the drug to an inactive form by the fluke is a mechanism of resistance (Devine *et al.*, 2009; Blackhall *et al.*, 2008; Mottier *et al.*, 2006).

In summary, whilst the mechanism of resistance to the benzimidazoles has been considered to be "solved", recent research suggests that the situation may be more complex. von Samson- Himmelstjerna *et al.*(2009) report that ß-tubulin codon 200 SNPs may be sufficient to diagnose an *H. contortus* population as resistant or susceptible. However, this classification may be rather crude, as it is not fully informative of the level of resistance. By examining other mechanisms of resistance involved it may be possible to propose protocols that can revert populations classified as resistant back to susceptibility.

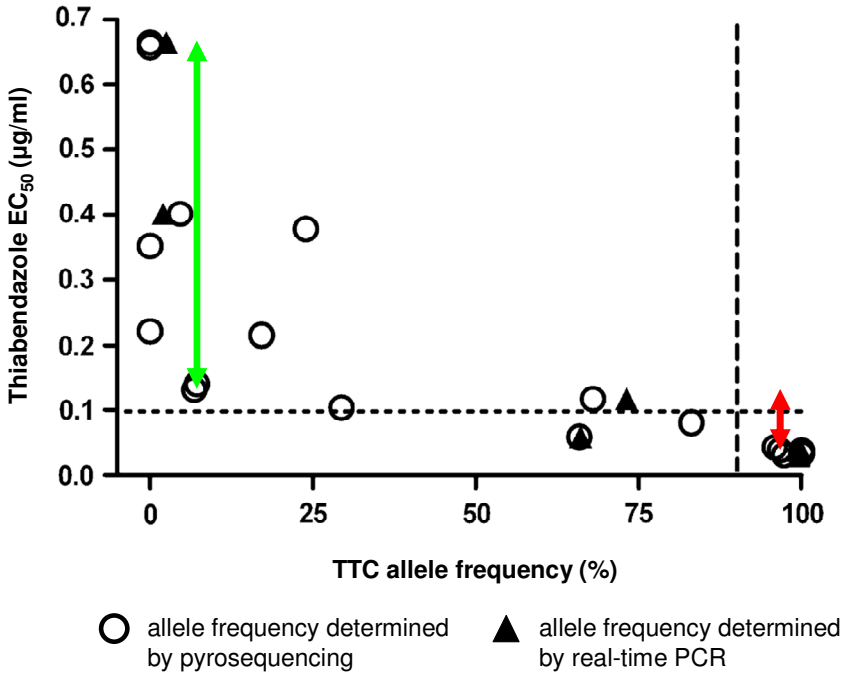


Figure 1-2: Codon 200 TTC frequency in *H. contortus* ß-tubulin isotype 1 gene related to thiabendazole (TBZ) sensitivity
Adapted from von Samson-Himmelstjerna *et al.* 2009. Populations with 100% TTC allele at codon 200 are always susceptible (plotted below the horizontal dashed line). However, the difference in TBZ EC_{50} between susceptible and resistant isolates (red arrow) is much smaller than between certain resistant isolates (green arrow).

1.9 Drug metabolism

1.9.1 Overview

Drug metabolism has been widely researched in humans due to the great effect this has on the therapeutic efficacy and toxicity of drugs (de Groot, 2006; Guengerich, 2006; Wells *et al.*, 2004). Enzymes involved in metabolism of toxins or drugs have historically been divided into two classes: the phase I enzymes, which serve to "functionalise" their substrate (i.e. add an active group such as a hydroxyl group to the substrate); and the phase II enzymes, which make use of the functional groups to conjugate the substrate, thus making it more polar and more easily excreted (Lindblom *et al.*, 2006; Rang *et al.*, 1999). ABC transporters, such as the p-glycoproteins, which aid in transporting the conjugated drug out of the cell, are sometimes referred to as phase III metabolism.

Many components of drug metabolism pathways are inducible upon exposure to their substrates, see **Fig. 1-3**. In this way the production of drug metabolising enzymes can be increased when they are needed. Although enzymes such as the cytochrome P450s may have a broad spectrum of activity, it is generally the case that a substrate will induce the up-regulation of enzymes specifically involved in the breakdown of the substrate. In *C. elegans* and other species, transcription of drug metabolising enzymes is regulated by members of the nuclear hormone receptor superfamily (Lindblom *et al.*, 2006). This is not only biologically important, but provides an interesting route to examine the possible mechanisms of metabolism of specific drugs. Using a whole genome microarray or RT-QPCR approach, it should be possible to identify enzymes potentially involved in xenobiotic metabolism due to their up-regulation following exposure to the xenobiotic (Rodriguez-Antona *et al.*, 2000). There have been several studies using *C. elegans* to investigate the response to environmental xenobiotics or toxins using these techniques (Lewis *et al.*, 2009; Hasegawa *et al.*, 2008; Lindblom *et al.*, 2006; Reichert *et al.*, 2005). Reichert and Menzel (2005) assessed changes in transcription in response to exposure to atrazine (an herbicide), clofibrate (active ingredient in certain antidiuretic and antihyperlipidaemic drugs), fluoranthene and DES. The results showed that over

203 genes were over-expressed in response to the various xenobiotics, including nine cytochrome P450 genes: *cyp-35A1, 35A2, 35C1, 14A5, 37B1, 35B2, 35B1, 35A5* and *22A1*. In addition, several other drug metabolising enzymes were induced along with genes of the collagen family and c-type lectins (involved in immune defence). However, only 26 of these genes could be induced by more than one of the tested compounds, showing the specificity of the response to a specific xenobiotic.

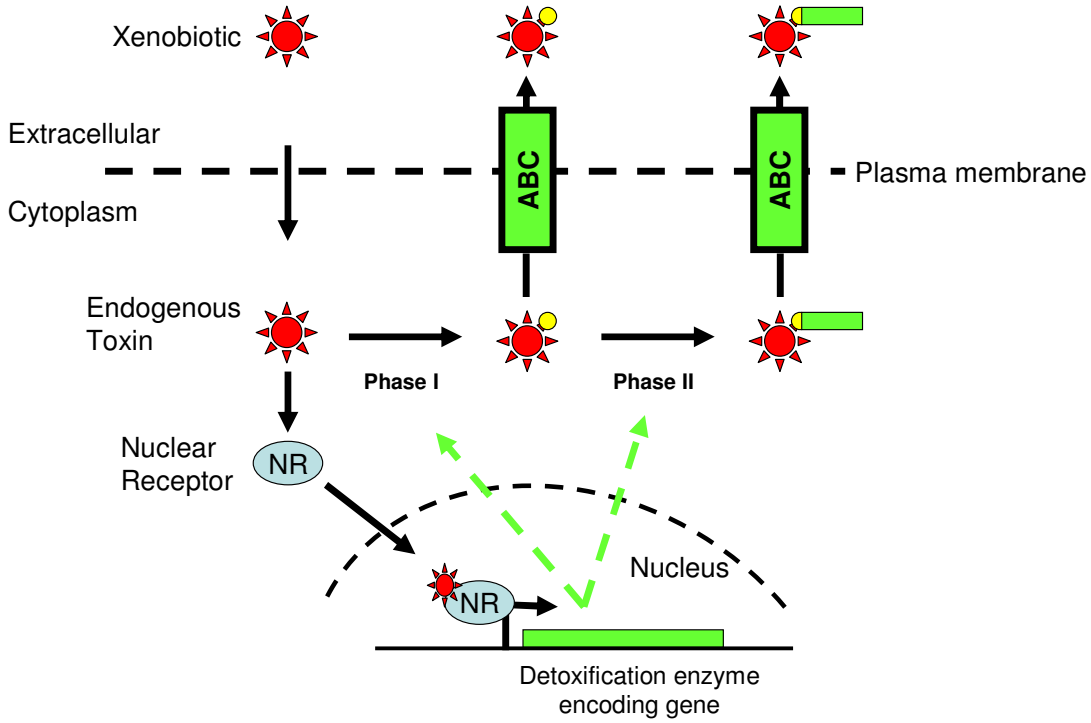


Figure 1-3: Schematic of xenobiotic metabolising enzyme induction
Adapted from Lindblom and Dodd (2006). Xenobiotics or endogenous toxins are proposed to bind to nuclear receptors thus allowing them to cross the nuclear membrane and up-regulate transcription of XME. Phase I enzymes, such as cytochrome P450s and flavin monooxygenases, and/ or phase II enzymes, such as glutathione-s-transferases and UDP-glucuronosyl transferases, metabolise the toxin to inactive forms or to allow efflux through ABC transporters such as the p-glycoproteins.

1.9.2 Nematode genomes encode enzymes potentially involved in drug metabolism

The cytochrome P450s (CYPs) are an example of phase 1 enzymes involved in oxidation/reduction reactions. They are a large, ubiquitous family of haem containing enzymes, which are separated into families and subfamilies based on

amino acid sequence identity. The CYPs have a wide substrate range and are important in many constitutive metabolic pathways as well as in the metabolism of xenobiotics. In fact, members of the cytochrome P450 family are drug targets themselves in several infectious agents including many fungi and in *Mycobacterium tuberculosis,* the bacterial cause of tuberculosis (McLean *et al.*, 2007; Mellado *et al.*, 2007).

The human genome contains 57 P450s, but 90% of CYP drug metabolism can be accounted for by just five of these (Guengerich, 2006). Despite this, the cytochrome P450s metabolise more drugs than any other enzyme system in humans (de Groot, 2006). Until recently nematodes were thought to lack the cytochrome P450 family (Barrett, 1997; Precious *et al.*, 1989a). However, the genome of *C. elegans* contains 75 full length cytochrome P450 genes, most of which belong to the CYP2, CYP3 and CYP4 families, which are involved in xenobiotic metabolism in humans (Gotoh, 1998). The function of most of these genes is unknown, but several are associated with the dauer pathway and others are involved in fatty acid metabolism and eggshell development (Benenati *et al.*, 2009; Kulas *et al.*, 2008; Motola *et al.*, 2006). Cytochrome P450 enzymes are found in the smooth endoplasmic reticulum of cells and as such are associated with the microsomal fraction. Identification of these proteins in microsome preparations relies on the characteristic peak in absorbance (soret peak) at 450nm of the carbon monoxide-complexed, reduced protein. Interestingly, Kulas *et al.* (2008) recently reported the first convincing 450nm soret peak in *C. elegans* derived microsomal protein. Spectral evidence of P450 proteins in nematode derived microsomes have proved difficult to demonstrate due to a large peak at approximately 420nm that appears to be present in most nematode microsomes tested. This peak has been proposed to represent a “nemo-protein”, which has unknown activity and function (Rocha-e-Silva TA *et al.*, 2001).

Certain members of the cytochrome P450 family of *C. elegans* have been shown to be inducible following exposure to xenobiotics (Menzel *et al.*, 2005; Reichert *et al.*, 2005). Recent studies by Schafer *et al.* (2009) used RNAi of *cyp* genes to show that members of the CYP-14A family and CYP-34A6 were directly involved in *C. elegans* metabolism of PCB52, an example of the environmental pollutants polychlorinated biphenyls. Typical CYP activity, assessed by enzymatic assays, was found to be present in homogenates of *Heligmosomoides polygyrus,* a

parasite of the rodent small intestine, *H. contortus* and *A. suum* (Solana *et al.*, 2001; Kotze, 1999; Kotze, 1997; Kerboeuf *et al.*, 1995). Extracts of *A. suum* have been found to be able to oxidise albendazole to albendazole sulphoxide (Solana *et al.*, 2001). However, no work has been presented comparing the relative rates of ABZ metabolism between resistant and susceptible isolates. Additionally, recent work on the *H. contortus* genome has uncovered the presence of a large family of cytochrome P450 genes to be present in this nematode (*pers. comm.*, R. Laing and Dr. J. S. Gilleard*)*.

The peroxidases represent another group of xenobiotic metabolising enzymes that are potentially involved in xenobiotic metabolism. The presence of peroxidases in parasitic nematodes is accepted and their activity is thought to help protect the nematode from both endogenous reactive oxygen species and those produced by the host immune system (Kotze *et al.*, 2001). Flavin containing monooxygenases and several reductase and hydrolase enzymes could also contribute to xenobiotic metabolism. The *C. elegans* genome contains genes thought to encode homologues of each of these enzymes. Enzyme activity has been noted in several parasitic nematodes: carbonyl group reduction activities, such as those catalysed by short chain dehydrogenases, have been noted in *H. contortus*, against several model substrates (Cvilink *et al.*, 2008). In addition, *Ascaris lumbricoides*, a parasitic roundworm of man, has been found to have reductive activity against azo and nitro compounds as well as hydrolytic activity against several substrates (Precious *et al.*, 1989b). However, the specific identity of the enzymes and their function within parasitic nematodes is largely unknown (Cvilink *et al.*, 2009a).

Phase 2 enzymes include uridine dinucleotide phosphate- glucuronosyl transferases (UGT), glutathione-s-transferases (GST), N-acetyl transferases, methyltransferases and sulphotransferases. After the cytochrome P450s, the UGTs are the enzyme family most commonly involved in xenobiotic metabolism in humans (Guengerich, 2006; Wells *et al.*, 2004). Little is known about the function of these enzymes in nematodes. However, the *C. elegans* genome contains 65 genes of this family, several of which have been shown to be induced upon exposure to xenobiotics (Lewis *et al.*, 2009; Reichert *et al.*, 2005).

The GST enzymes have been more fully characterised in both *C. elegans* and parasitic nematodes due to their role in oxidative stress adaptation and in surviving the host immune response. The family is organised into several sub-types based upon amino acid sequence and homologues are present in all nematode species (Cvilink *et al.*, 2009a). The *C. elegans* genome contains 48 putative *gst* genes. Several of these may belong to a nematode specific class, of which *H. contortus* is known to have two representatives, *Hc-GST1* and *Hc-GSTE* (Lindblom *et al.*, 2006; Campbell *et al.*, 2001).

GST enzymes do not function solely by conjugating glutathione to substrates. They may also act as peroxidases or bind substrates without biotransformation (Salinas *et al.*, 1999). They are involved in many constitutive biological processes and their function may not be conserved between species. For example the *H. contortus* and *Ancylostoma caninum* GSTs, *Hc*-GST1 and *Ac*-GST1, have been shown to bind haematin and are thought to be involved in blood meal digestion (Zhan *et al.*, 2005; van Rossum *et al.*, 2004). As such they have attracted a great deal of attention as potential vaccine candidates. *C. elegans* is not a blood feeder and the homologous GST protein does not bind haematin. However, other *C. elegans* GSTs have been shown to be able to bind haematin and GST-19 production is increased at high concentrations of haem (Perally *et al.*, 2008). Nematodes are unable to synthesise haem and it is thought that these GSTs may be important in trafficking of the potentially toxic haem molecule. GST sequence and activity analysis have been undertaken in *Ascaris suum* (parasitic nematode of pigs), where activity of the enzyme has been localised to the intestine, suggesting a role in xenobiotic metabolism (Liebau *et al.*, 1997). Both *Onchocerca volvulus* and *Ascaridia galli* (parasitic nematode of poultry) have GSTs involved in prostaglandin synthesis. One of the three *O. volvulus* GST genes, *OvGST1,* has been shown to be protective against oxidative stress in transgenic *C. elegans* (Sommer *et al.*, 2003; Kampkotter *et al.*, 2003; Meyer *et al.*, 1996). GST activity has been shown to be inducible in *Setaria cervi* (parasite of ruminants) in response to exposure to phenobarbital, diethyl carbamazine and butylated hydroxyanisole (Gupta *et al.*, 2005). Phenobarbital was also able to induce GST production in cestodes In addition, the presence of GSTs has been reported in *Heligmosomoides polygyrus bakeri* (parasitic nematode of rodents); *Wuchereria bancrofti* and *Brugia malayi*

(filarial parasites of man) and *Necator americanus* (human hookworm); reviewed by Cvilink *et al.* (Cvilink *et al.*, 2009a). Analysis of GST activity in most cases was carried out using standardised enzyme assays, but due to the wide substrate specificities of this class, the specific function of many of these enzymes within the organisms is not known. Many GSTs have been shown to have potent antioxidant activities. Those with characterised prostaglandin synthesis activity, isolated from *O. volvulus* and *A. galli*, may be involved in direct modulation of the host immune response (Sommer *et al.*, 2003; Meyer *et al.*, 1996; Brophy *et al.*, 1995; Brophy *et al.*, 1994). Exposure to xenobiotics has been shown to induce *gst* gene expression in *C. elegans* as well as several of the parasites described above (Lewis *et al.*, 2009; Hasegawa *et al.*, 2008; Custodia *et al.*, 2001). The functional importance of GST induction has not been elucidated. However, there is substantial evidence that these proteins can bind to anthelmintics, even if they do not conjugate glutathione to the drugs (Brophy and Barrett, 1990). This could explain the inhibition of activity of recombinant GST, from *A. suum* and *O. volvulus,* in the presence of several anthelmintic compounds (Fakae *et al.*, 2000; Liebau *et al.*, 1997). Albendazole had limited inhibitory affect on recombinant *A. suum* GST1, with an IC_{50} of 520 μM.

There are limited reports in the literature of other conjugation systems in parasitic nematodes, though all of the enzyme systems listed above are putatively present in the *C. elegans* genome. N-acetyl transferase activity has been detected in *Brugia pahangi*, *A. galli*, *A. suum* and *O. volvulus*. However, this activity has only been detected against naturally occurring diamines, not against exogenous compounds. Sulphotransferase activity has been noted in *C. elegans*, again involving endogenous structural proteins (Cvilink *et al.*, 2009a). It is clear that these enzyme systems have been under researched in the nematode family. The presence of these enzymes in the *C. elegans* genome suggests that they must at least perform constitutive biological functions. The similarity of many therapeutic compounds to naturally occurring compounds would suggest that these could also be substrates for the same enzymes.

1.9.3 Xenobiotic metabolising enzymes associated with drug resistance

1.9.3.1 Phase I enzymes

Daborn *et al.* (2002) reported that overexpression of a single P450 allele, *Cyp6g1*, is sufficient to result in a DDT resistant phenotype in *Drosophila melanogaster*. The study made use of microarrays carrying only cytochrome P450 probesets, which were hybridised with whole organism cDNA from DDT resistant and susceptible strains of *D. melanogaster*. Results from these experiments were then quantified using real-time QPCR. In the resistant strain *Cyp6g1* alone was over-expressed 10 to 100-fold compared to two susceptible strains. Sequencing of the DDT-resistant allele, in two different resistant strains, suggested that overexpression of the *Cyp6g1* gene was due to insertion of a 5′-*Accord* transposable element. In addition, the same *Accord* element was found in all DDT-resistant field strains tested (a total of 20) and exhibited local linkage disequilibrium. This suggests that the resistant mutation originated from a single event that has since spread globally. Overexpression of *cyp6g1* in a susceptible *D. melanogaster* background was proved to be sufficient to confer DDT resistance (Daborn *et al.*, 2002). The catalytic activity of CYP6G1 against DDT and imidacloprid has recently been defined using heterologous expression (Joussen *et al.*, 2008). DDT was converted to the inactive compound DDD by dechlorination and imidacloprid was hydroxylated to at least two metabolites.

Overexpression of cytochrome P450 genes has been associated with insecticide resistance in many other insect species. These include *Anopheles funestus* and *Anopheles gambiae*, both important vectors of malaria; *Aedes aegypti*, the mosquito vector of yellow fever and dengue fever; *Bemisia tabaci*, a whitefly that is an important causes of crop destruction; and house flies (Marcombe *et al.*, 2009; Amenya *et al.*, 2008; Djouaka *et al.*, 2008; Karunker *et al.*, 2008; Zhu *et al.*, 2008a). In addition, resistance of the ticks *Boophilus microplus* and *Rhipicephalus bursa* to various acaracides has also been associated with cytochrome P450 activity (Rosario-Cruz *et al.*, 2009; Villarino *et al.*, 2002). In each case the actual cytochrome genes involved are varied. Recent work has shown that while insecticide resistance in *D. melanogaster* in field populations is

associated with *cyp6g1*, overexpression of several other *cyp* genes could also result in resistance (Daborn *et al.*, 2007). Ffrench-Constant *et al.* (2004) have suggested cytochrome P450s with broad substrate specificities may be preferentially up-regulated in field strains as they allow for resistance to multiple drugs. Interestingly, Schlenke *et al.* (2004) reported that a *Doc* transposable element was found 200bp upstream of the *cyp6g1* gene in eight Californian isolates of *Drosophila simulans*. These isolates were shown to be more resistant to DDT than isolates without the insertion. This suggests that a very similar mechanism of resistance may have evolved in completely separate populations, which were under similar selective pressure. It is important to note that most of the reports described above are just associations. Other than CYP6G1, CYP6A2 and CYP12D1 the activity of very few of these cytochrome P450s against insecticides has been defined (Giraudo *et al.*, 2009). Intriguingly, the mechanism of overexpression of CYPs associated with insecticide resistance has thus far only been found to be due to up-regulation of specific genes, due to mutations in the cis- or trans- regulatory regions, rather than gene amplification events (Li *et al.*, 2007).

The avermectins are used to treat both endoparasites and ectoparasites and resistance of several strains of insect to this drug class has been reported. In some cases this is thought to be associated with monooxygenation of the drugs. Piperonyl butoxide, a potent inhibitor of CYPs, was shown to be highly synergistic with abamectin in both a mutagenised resistant strain and a resistant strain created by abamectin selection of the Colorado potato beetle, *Leptinotarsa decemlineata* (Clark *et al.*, 1995). In the same strains, cytochrome P450 content was found to be between 1.6 fold and 1.9 fold higher than that of susceptible strains. Significantly higher levels of the abamectin metabolites 24-desmethyl abamectin, 24-hydroxyabamectin and an unknown metabolite were found in the excrement of resistant strains compared to susceptible strains. However, general oxidase substrate assays did not find any increase in activity, suggesting that a specific cytochrome P450 was overexpressed. The same experiments revealed a strong association between increased carboxylesterase activity and abamectin resistance. Similar associations have also been noted in abamectin resistant house flies (*M. domestica*), but no PBO synergism suggestive

of monooxygenase derived resistance was noted in abamectin resistant two-spotted spider mites (*Tetranychus urticae*) (Clark *et al.*, 1995).

Mutations in the catalytic site and overexpression of esterase genes have both been shown to be involved in insecticide resistance. In the Australian blow-fly, *Lucilia caprine,* and the mosquito, *Culex pipiens,* single amino acid replacements from glycine to aspartic acid in the active sites of enzyme E3 and the acetylcholine esterase-1 protein respectively result in resistance to organophosphates (Weill *et al.*, 2003; Newcomb *et al.*, 1997). This is thought to occur via increased hydrolytic activity against the OP drugs. Insecticide resistance in *C. pipiens* and the aphid *Myzus persicae* may also be caused by overexpression of esterase genes (Field *et al.*, 1998; Raymond *et al.*, 1998). In addition, recent studies in lab selected organophosphate resistant strains of *A. aegypti* suggested that overexpression of several esterases and GSTs may be involved (Melo-Santos *et al.*, 2009).

The malarial parasites *Plasmodium falciparum* and *Plasmodium berghei* have become resistant to several of the drugs used to treat them. For the most part resistance to quinolone drugs has been associated with mutations in or increased expression of transport proteins such as MDR1 and PFCRT (Roepe, 2009). However, this is not sufficient to explain all examples of resistance developed in laboratory and field strains. Resistance to chloroquine, a 4-aminoquinolone drug used to treat malaria has been associated with increased CYP concentration, activities to standard substrates and *cyp* mRNA (Surolia *et al.*, 1993; Ndifor *et al.*, 1990). Increased chloroquine sensitivity of *P. falciparum* has been noted following exposure in combination with several P450 inhibitors *in vitro*. In addition, i*n vivo* sensitivity of *P. berghei* to chloroquine was increased in combination with the P450 inhibitor cimetidine (Ndifor *et al.*, 1993). However, other studies have found that was not always the case. Paciorkowski *et al.* (1997) reported that whilst cimetidine had clear synergistic effects in combination with both chloroquine and pyrimethamine, another P450 inhibitor, proadifen, showed no synergism, or caused antagonism of the drugs. This may be due to inhibition of specific CYP isoforms, but could also be due to cimetidine exerting its synergistic effect via another mechanism than P450 inhibition. *P. falciparum* strains are able to metabolise mefloquine, another antimalarial drug,

but no difference was found in the P450 content or metabolic rate between resistant and susceptible strains (Na-Bangchang *et al.*, 2007).

Resistance of certain trypanosome species has been related to enhanced metabolic processes. Portal *et al.* (2008) have reported that transgenic overexpression of a cytochrome P450 reductase (CPR) enzyme may increase resistance to benznidazole and to a lesser extent nifurtinox. CPR enzymes represent the rate limiting step in many CYP reactions. They serve to reduce the CYP enzyme back to its functional state following interaction with its substrate. By using transgenic trypanosome CPR enzymes in combination with rat microsome derived CYPS, Portal *et al.* (2008) were able to prove that these enzymes supported CYP mediated reactions. This represents an important discovery as CPR enzymes may interact with many different CYP isoforms. Thus by over-expressing a single CPR the activities of several CYP reactions may be enhanced.

The azoles represent a group of antifungal drugs which target the ergosterol synthesis pathway by inhibiting the action of 14-α-sterol demethylase, a cytochrome P450 enzyme. Azole resistant strains of *Aspergillus fumigatus* have been associated with overexpression of the *cyp51A* gene. Studies suggest that a mutation in the coding part of the gene and the promoter region are required to convey high level resistance (Mellado *et al.*, 2007). Overexpression of cytochrome P450s homologous to the *cyp51* gene in *Candida spp.* has also been associated with azole resistance. However, this represents a different mechanism of resistance to those discussed thus far as the CYP is the target of the drug class. Overexpression of *cyp51A* presumably allows the normal function of the enzyme, as no evidence of CYP51 mediated metabolism of azole drugs has been presented.

Antimicrobials represent the largest group of drugs specifically used to treat infectious agents. There are many different classes of drug available, but unfortunately microbe resistance to these drugs is widespread. Metabolism of antimicrobial agents to inactive forms is by far the most common mechanism of bacterial resistance (Harbottle *et al.*, 2006). There are several incidences of redox mechanisms being involved. The best described is the presence of the *tetx* gene which encodes an oxygen-requiring flavoprotein active against tetracycline

(Wright, 2005). Interestingly, this gene was discovered in a transposon of an obligate anaerobe bacterium, *Bacteroides fragilis*. Redox enzymes have also been discovered in *Rhodococcus equi*, active against rifampicin, and *Streptomyces virginae*, which protects the bacterium from virginiamycin M1, an antibiotic produced by the bacterium itself. There are also several examples of hydrolase enzymes. The most well-known being the ß-lactamases that cleave the lactam ring of penicillin antibiotics. Enzymatic mechanisms of resistance to antimicrobials are reviewed by Wright, 2005. Conjugation reactions represent the most common mechanism of resistance to antibiotics and are described in **Section 1.9.3.2**.

There is growing evidence to suggest that flavin monooxygenases (FMOs) may be involved in triclabendazole resistance in the liver fluke *Fasciola hepatica*. Unlike benzimidazole resistance in most nematodes, resistant fluke isolates have none of the expected mutations in ß-tubulin genes (Brennan *et al.*, 2007). *F. hepatica* has been shown to be able to carry out sulphoxidation of triclabendazole to the active metabolite triclabendazole sulphoxide and to produce low levels of the inactive metabolite triclabendazole sulphone. Robinson *et al.* (2004) demonstrated that production of the inactive TBZ-SO_2 was on average 20.29% greater in resistant Sligo isolates compared to susceptible Cullompton isolates. Studies using P450 inhibitors such as piperonyl butoxide and FMO inhibitors such as methimazole, suggest that whilst both enzyme systems may be involved in this process, the FMOs are more important (Alvarez *et al.*, 2005). A recent study by Devine *et al.* (2009) demonstrated an increase in disruption of the tegument of the resistant Oberon isolate of *F. hepatica* following exposure to both TBZ and TBZ-sulphoxide when coincubated with methimazole (Devine *et al.*, 2009). Interestingly, coincubation of the susceptible Cullompton isolate with methimazole and the drugs appeared to decrease disruption compared to the incubations with TBZ and TBZ-SO alone. *F. hepatica* does not have an annotated genome, thus the individual genes that may be involved have not been identified and further studies will be necessary to define molecular nature of triclabendazole resistance.

Oxidase activities have been investigated in anthelmintic resistant and susceptible strains of *H. contortus* (Kotze, 2000; Kotze, 1997). No differences in activity were noted, but the assays used examined only aldrin epoxide (AE)

activity and 7-ethoxycoumarin-O-deethylase (ECOD). These activities are thought to be associated with CYP2B/ CYP3A (AE) and CYP1A1/ 2B1 (ECOD) activities. *H. contortus* microsomes were found to be inactive against several other cytochrome substrates standardised with human microsomes. Given the large family of cytochrome P450s present in the *H. contortus* genome it is likely that the enzymes are present but simply have different substrate specificities. Alvinerie *et al.* (2001) have shown that *H. contortus* is capable of producing a P450 derived metabolite of moxidectin. In contrast, earlier studies investigating *H. contortus* metabolism of closantel, using reverse-phase HPLC and C^{14} labelled closantel, revealed no metabolites in either resistant or susceptible isolates (Rothwell *et al.*, 1997). However, in resistant isolates, 40-95% of radioactivity was associated with the closantel peak. This technique may not have detected very small concentrations of metabolite, which may still be physiologically important.

Differences in esterase content or activity between resistant and susceptible nematodes have been noted by several groups (Gimenez-Pardo *et al.*, 2004; Gimenez-Pardo *et al.*, 2003; Sutherland *et al.*, 1993; Echevarria *et al.*, 1992). Gimenez-Pardo *et al.* (2003) used substrate assays to show that cholinesterase activities were six times higher in a resistant *H. contortus* isolate compared to a susceptible isolate. However, these experiments were only carried out on one resistant and one susceptible isolate and the functional importance of these differences remains to be assessed. Similar increases in acetylcholine esterase activity were found in benzimidazole resistant isolates of *H. contortus, T. circumcincta* and *T. colubriformis* (Sutherland *et al.*, 1993).

Compared to the plethora of data concerning the monooxygenase enzymes of insects and bacteria, there has been a dearth of research investigating these pathways in parasites. As can be seen, several studies have suggested strong associations between resistance and overexpression of the enzyme systems, but complete characterisation of the activity of the enzymes is lacking. In addition, the mechanisms underlying overexpression of metabolising genes have not been investigated in the nematode phylum. However, transposable elements are very common in *C. elegans* (Witherspoon *et al.*, 2003; Le *et al.*, 2001) and the genomes of parasitic nematodes are likely to contain many transposable elements as suggested by analysis of the genome of *Brugia malayi* and *H.*

contortus (*pers. comm.*, R. Laing; Underwood *et al.*, 1999). It is therefore possible that a disruption of the normal promoter region of a CYP or other XME could be involved in up-regulation of gene expression resulting in anthelmintic resistance in nematodes.

1.9.3.2 Phase II (conjugation) enzymes

As was previously discussed, the glutathione-s-transferases are known to be extremely important in protection from reactive oxygen species. Many chemotherapeutics rely on creation of these radicals to kill infectious agents. Therefore, it is not surprising that up-regulation or enhanced activity of these enzymes has been associated with drug resistance.

Overexpression of GST enzymes has been associated with insect resistance to organophosphates, DDT and pyrethroids. In this role GSTs do not always act to conjugate the drugs. DDT resistance in *Anopheles gambiae, Aedes aegypti* and *D. melanogaster* may be mediated by a dehydrochlorination reaction catalysed by GST enzymes which use glutathione as a co-factor. Pyrethroid resistance has also been associated with GST overexpression in both *Nilaparvata lugens*, the brown planthopper, and in *A. aegypti*. In addition, recent studies have shown pyrethroid resistant strains of the plant bollworm, *Helicoverpa armigera,* to be associated with increased oxidase activity and GST activity (Omer *et al.*, 2009). GSTs do not directly metabolise pyrethroids, but instead may be involved in binding and sequestering the drugs or in detoxification of lipid peroxidation products produced by the action of the drug (Li *et al.*, 2007). In contrast to the situation with cytochrome P450s, overexpression of GST enzymes associated with insecticide resistance may be caused by gene amplification events, as occurs with *Md-GSTD3* in *M. domestica*, or by up-regulation of specific genes, as with *Aa-GSTD1* and *Aa-GSTE2* in *A. aegypti* and *Ag-GSTE2* in *A. gambiae* (Ranson *et al.*, 2001; Zhou *et al.*, 1997; Grant *et al.*, 1992).

Diethyl maleate, which binds glutathione, has also been shown to have synergistic effects in combination with diazinon in the cattle tick *Boophilus microplus* (Li *et al.*, 2003a). This may suggest that GST activity is involved in resistance to diazinon, but further functional assays were not undertaken.

Direct interaction of UGTs, another family of conjugating enzymes, with insecticide drugs has not thus far been investigated. However, a microarray experiment carried out by Vontas *et al.* (2005) found several conjugating enzymes to be up-regulated following permethrin exposure of resistant strains of *A. gambiae*. These included several members of the UDP-glucuronosyl transferase family. Similarly, three UGT genes were found to be up-regulated in DDT resistant *D. melanogaster* compared to susceptible strains (Pedra *et al.*, 2004).

The mechanisms behind the resistance of *Leishmania* spp, the protozoal agents which cause cutaneous and visceral leishmaniasis, to many of the compounds used to treat it have not been greatly researched (Croft *et al.*, 2006). The most commonly used drugs are the antimonials, including meglumine antimonate and sodium stibogluconate, which may act by interfering with glycolysis and fatty acid ß-oxidation. Additionally, these drugs may decrease thiol content (of which glutathione is an example) in the amastigote thus reducing the resistance of the parasite to oxidative stress (Wyllie *et al.*, 2004; Berman *et al.*, 1987). Resistant laboratory and field strains of *Leishmania spp.* have been associated with increased levels of thiols; especially trypanothione, a glutathione spermidine conjugate; via up-regulation of thiol synthesising enzymes (Haimeur *et al.*, 1999; Grondin *et al.*, 1997; Mukhopadhyay *et al.*, 1996). Work carried out in *L. tarentolae,* a parasite of geckos commonly used as a model *Leishmania* organism, showed that overexpression of ornithine decarboxylase, a key enzyme in spermidine synthesis, could confer resistance to arsenite in combination with overexpression of the efflux protein PGPA. Overexpression of PGPA alone did not confer a similar level of resistance (Haimeur *et al.*, 1999). Glutathione-s-transferase activities are known to be increased in mammalian cells selected for arsenite resistance, but GSTs are not present in *Leishmania spp.* (Lo *et al.*, 1992). However, a GST-like trypanothione-s-transferase (TST) activity has been noted in several *Leishmania spp.* (Vickers *et al.*, 2004). In addition to s-transferase activity, TST is thought to be a functional peroxidase. Wyllie *et al.* (2008) demonstrated that peroxidase activity is significantly increased, between 4 and 8.5-fold, in resistant isolates of *L. tarentolae.*

Resistant isolates of *Trypansoma cruzi* have been shown to have higher glutathione levels than susceptible isolates (Faundez *et al.*, 2005; Moncada *et*

al., 1989). Buthiomine sulphoxomine was shown to decrease the glutathione content in *T. cruzi* and to have synergistic effects in combination with nifurtinox and benznidazole. However, no functional assays were undertaken, so the role by which increased GST may result in resistance to trypanocidal drugs remains uncharacterised.

Chloroquine resistance in malaria parasites is thought to arise mainly through the action of transport proteins and P450s. However, a recent study has shown that chloroquine sensitivity can be increased using chemicals that affect intracellular glutathione concentrations (He *et al.*, 2009). Resistant *P. falciparum* isolates were shown to have increased intracellular glutathione, glutathione-s-transferase activity and glutathione peroxidise activity compared to sensitive isolates. Glutathione reductase activity was lower in resistant isolates. Enzyme activities were similarly affected in *P. chabaudi*, except that there were no differences in glutathione peroxidise activity between resistant and susceptible isolates. Ritonair, a potent protease inhibitor, increased the sensitivity to chloroquine and simultaneously reduced GST activity in the resistant isolates. This strongly suggests that GST-like activity may be involved in the resistance of *P. falciparum* and *P. chabaudi* to chloroquine. Interestingly, *P. falciparum* appears to encode only one GST gene, *pcGST,* making this a potential drug target (Deponte *et al.*, 2005).

Several bacterial species make use of conjugating enzymes to resist the action of antibiotic drugs. These include acetyl-transferases, phosphoryl-transferases, thiol-transferases, nucleotidyl-transferases, ADP-ribosyl-transferases and glycosyl transferases. These enzymes may be constitutively up-regulated or may be induced upon exposure to the antibiotics (Harbottle *et al.*, 2006). Many different classes of antibiotic may be affected by these mechanisms, reviewed by Wright (2005). For example, the macrolide antibiotics may be inactivated by the addition of a glucose group at position 2′ of the desosamine sugar, using a UDP-glucosyl transferase enzyme encoded by the *mtg* gene of *Streptomyces lividans* (Jenkins *et al.*, 1991). In all cases, the addition of the conjugate interferes with the action of the drug, usually by reducing the binding efficiency to the target. However, efflux pumps may also be involved in antibiotic resistance and in some cases drug conjugation may be used to aid in effective efflux of the drug (Harbottle *et al.*, 2006).

Praziquantel is the only drug currently used against the blood fluke *Schistosoma japonicum*. Praziquantel has been shown to bind to *S. japonicum* GST and therefore up-regulation of GST may be a mechanism of resistance (McTigue *et al.*, 1995). In the related species *Schistosoma mansoni,* non-conjugating GST activity was noted. Intact parasites or cytosol preparations incubated with dichlorvos produced *O*-demethylated dichlorvos and S-methyl glutathione (O'Leary *et al.*, 1991). Des-methylated dichlorvos is pharmacologically inactive and this route of metabolism may explain why *S. mansoni* is resistant to dichlorvos unlike the related fluke *Schistosoma haematobium*. However, this association has not been seen in all trematodes. *F. hepatica* isolates resistant to salicylanilide anthelmintics have been shown to have lower GST activities than comparable susceptible isolates (Miller *et al.*, 1994). It is hypothesised that GST binding may in fact increase the uptake of certain drugs. Therefore, the role of GST activity in drug resistance in trematodes is unclear.

IVM selected strains of *C. elegans* have also been associated with changes in intracellular glutathione content. James *et al.* (2009) showed that as well as overexpression of drug transport proteins the concentration of glutathione was reduced and expression of the glutathione-s-transferase gene *gstp-1* was increased in low-level resistant strains (6ng/ml [6.84nM] IVM) of the nematode. The low concentration of glutathione was hypothesised to be due to increased binding to ivermectin, as strains allowed to grow on standard NGM plates were able to return their glutathione levels to wild-type levels, but confirmation of this will require further work. In worms resistant to concentrations of up to 10ng/ml (11.4nM) IVM, the glutathione level was consistently wild-type level. However, expression of *gcs-1*, a γ-gluatamyl-cysteine synthetase homologue which is thought to be the rate limiting step in glutathione synthesis, was significantly increased. Interestingly, *C. elegans gcs-1* has previously been shown to confer resistance to arsenic and may explain the role of this gene in antimonial resistance in *Leishmania spp.* (Liao *et al.*, 2005).

There are few reports of the investigation of the role of phase II conjugation enzymes in anthelmintic resistance. Resistance of *H. contortus* to cambendazole has been associated with increased GST-like activities and recombinant *A. suum* GSTs can bind anthelmintic compounds (Liebau *et al.*, 1997; Kawalek *et al.*, 1984). Sangster *et al.* (1986) demonstrated that *T. colubriformis* was capable of

producing a sulphate conjugate of hydroxythiabendazole. However, hydroxyTBZ is an inactive form of thiabendazole and no differences in the rate of conjugation were seen between resistant and susceptible isolates. In all of these reports and in the study in *C. elegans*, presented by James *et al.* (2009), no direct relationship between conjugating enzyme activity and resistance has been demonstrated. Therefore, further work will be required to investigate the role of these pathways in anthelmintic resistance.

1.9.4 Anthelmintics as substrates for xenobiotic metabolising enzymes

As summarised above, metabolism of chemotherapeutic compounds is a common mechanism of resistance utilised by many infectious agents. Whilst these pathways have been neglected in current research into anthelmintic resistance, examination of the *C.* elegans genome and work by several authors would suggest that these pathways are present in nematode species. Analysis of the structures of the anthelmintic compounds currently in use reveals them to have chemical bonds or functional groups that could potentially be acted upon by xenobiotic metabolising enzymes. In addition, the avermectins, benzimidazoles, levamisole and monepantel, are known to undergo metabolism in mammalian hosts to varying degrees (Gonzalez *et al.*, 2009; Karadzovska *et al.*, 2009; Li *et al.*, 2003b; Zeng *et al.*, 1998; Paulson *et al.*, 1996; Fargetton *et al.*, 1986).

Both ivermectin and albendazole are known substrates for cytochrome P450 mediated metabolism in mammals. Albendazole is also thought to be metabolised by mammalian flavin monooxygenases (Rawden *et al.*, 2000; Fargetton *et al.*, 1986). The main metabolites of albendazole are the active metabolite albendazole sulphoxide and the inactive albendazole sulphone. In contrast, ivermectin is metabolised to at least ten different metabolites by human microsomes (Zeng *et al.*, 1998). Both drugs may be conjugated to glucuronate and ivermectin is known to undergo extensive enterohepatic recycling via this pathway (Gonzalez *et al.*, 2009).

In order for metabolism of anthelmintic to be a plausible mechanism of resistance, the metabolites must be inactive or be better substrates for efflux

pumps than the parent compounds. However, in many cases metabolism can lead to bioactivation of a compound. The sulphoxidation of albendazole to the active compound albendazole sulphoxide is just one example of this. However, in many cases, including that of ivermectin, whilst some of the metabolites have been defined their relative activity compared to the parent compound has not been assessed. Therefore, elucidating the mechanisms by which nematodes may metabolise anthelmintics is only the first step in discovering whether or not this is a likely mechanism of resistance. Further studies investigating the chemotherapeutic efficacy of any metabolites discovered will be necessary.

1.10 Specific aims of this study

a) To use whole genome microarrays to compare the transcriptome of *C. elegans* following exposure to anthelmintic to that of an unexposed control. Specifically, the anthelmintic drugs to be investigated were ivermectin, an example of a macrocyclic lactone drug, and albendazole, an example of a benzimidazole drug.

b) To characterise the response of genes identified as differentially expressed in microarray experiments using real-time quantitative PCR.

c) To characterise genes identified as differentially expressed in microarray experiments using GFP reporter constructs.

d) To assess the metabolism of ivermectin and albendazole by *C. elegans* and *H. contortus* using HPLC-MS.

e) To provide a framework upon which to investigate transcriptomic and metabolomic responses to anthelmintics in *Haemonchus contortus* and other parasitic nematodes.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Standard reagents and Media

Ampicillin:	100mg/ml ampicillin (Sigma, A9393) in sterile distilled H_2O. Filter sterilised and stored at -20^oC.
Chloramphenicol:	12.5mg/ml chloramphenicol (Sigma, C0378) in 100% ethanol. Stored at -20^oC.
EDTA:	ethylenediaminetetra-acetic acid in sterile distilled H_2O. Stock solution of 0.5M, pH 8.0. Autoclaved and stored at room temperature.
Ethidium Bromide:	10mg/ml in sterile distilled H_2O. Stored at room temperature.
L-broth:	1% tryptone (Oxoid, LP0042)), 0.5% yeast extract (Oxoid, LP0021), 1% NaCl in sterile distilled H_2O. Autoclaved and stored at room temperature.
LB-agar:	L-broth + 1.5% agar (Oxoid, LP0011). Autoclaved and stored at room temperature.
Loading buffer (5X):	100mM EDTA pH 7.5, 22% Ficoll (Sigma, F2637), 0.05% Bromophenol Blue (Sigma, B0126).
M9 Buffer (10X):	3% KH_2PO_4, 6% Na_2HPO_4, 5% NaCl, 10mM $MgSO_4$. Autoclaved and stored at room temperature.
MF4 HPLC mobile phase:	Methanol (H411) 10%; H_2O (H949) 90%; formic acid (H353) 0.027%; ammonium acetate (HR079) 2mM. Prepared for Pfizer by Romil.

MF5 HPLC mobile phase:	Methanol (H411) 90%; H_2O (H949) 10%; formic acid (H353) 0.027%; ammonium acetate (HR079) 2mM. Prepared for Pfizer by Romil.
NGM-agar:	0.3% NaCl, 1.7% agar (Oxoid, LP0011), 0.25% peptone (Oxoid, L37), 0.0003% cholesterol (1ml/L of 5mg/ml stock in ethanol), in sterile distilled H_2O. Autoclaved then supplemented with 1ml/L 1M $CaCl_2$, 1ml/L 1M $MgSO_4$ and 25ml/L KPO_4 buffer pH 6.0.
Proteinase K:	10 mg/ml proteinase K (Roche, 03115836001) in sterile distilled H_2O. Stored at -20^oC.
S-basal	0.1M NaCl, 0.05M $KHPO_4$ buffer pH 6.0, 12.5mg/L PEG water soluble cholesterol (Sigma, C1145).
S-buffer:	129 ml/L 0.05M K_2HPO_4, 871 ml/L 0.05M KH_2PO_4, 0.1M NaCl, pH 6.0.
Superbroth:	Per 1L: 12g tryptone (Oxoid, LP0042); 24g yeast extract (Oxoid, LP0021), 8ml of 50% glycerol stock. Autoclaved then supplemented with 100ml of 0.17M KH_2PO_4/0.72M K_2HPO_4.
TAE (50X):	2M Tris-base, 100ml/L 0.5M EDTA, 57.1ml/L glacial acetic acid. Autoclaved and stored at room temperature.
TBE (5X):	0.45M Tris-base, 0.45M Boric acid, 100ml/L 0.5M EDTA. Autoclaved and stored at room temperature.
TE buffer:	10mM Tris, 1mM EDTA pH 8.0

2.1.2 Caenorhabditis elegans strains and culture conditions

Bristol N2: *C. elegans* wild type, DR subclone of CB original (Tc1 pattern I). Gift from the CGC.

CB3474 : *ben-1(e1880)III* (Driscoll *et al.*, 1989) Mutation ß-tubulin gene resulting in high level resistance to benzimidazoles. Dominant at 25°C, recessive at 15°C. Gift from CGC.

DA1316: *avr-14(ad1302)*; *avr-15(ad1051); glc-1(pk54)*. Mutations of three major subunits of glutamate-gated chloride channels resulting in high level resistance to ivermectin (Dent *et al.*, 2000).

Culture of *Caenorhabditis elegans* was carried out as per standard protocols (Brenner, 1974). Worms were maintained at 15-20°C on NGM plates with an OP50 bacterial lawn unless otherwise specified.

2.1.3 E. coli strains

OP50: A variant of the uracil requiring OP50 strain (Brenner, 1974) with a streptomycin selectable marker. Strain received from CGC.

2.2 Standard methods

2.2.1 Freezing and storage of nematode strains

Strains to be frozen were grown on 5-6 5cm diameter NGM plates with OP50 bacterial lawns until just starved and many L1-L2 larvae were present. Nematodes were washed from plates with 2-3ml S-buffer. The nematodes were suspended in approximately 1ml S-basal which was split equally between two 1.8ml cryotubes. An equal volume of S-buffer plus 30% glycerol was added to each tube. The tubes were placed into a polystyrene rack and placed at -80^{o}C overnight. The polystyrene ensures that the worms do not freeze too rapidly and die. One tube was thawed the following day to ensure successful recovery and the other stored in a permanent freezer location.

To recover strains, tubes were thawed completely at room temperature. Approximately 500µl of the supernatant was removed and discarded. The remaining buffer and worm pellet was transferred to a fresh, dry NGM plate with an OP50 lawn. The plates were left at 20^{o}C overnight then assessed for live worms. These were then picked to fresh NGM plates.

2.2.2 Synchronisation of L1 larvae

Nematodes were grown on 10-15 standard NGM plates with OP50 bacterial lawns for approximately three days until many gravid hermaphrodites were present. Adults and eggs were washed off each plate in M9 buffer and transferred to a 50ml falcon tube. The tube was filled to 50ml with M9 and allowed to chill on ice for 15-30min. The falcon tube was centrifuged at 2500rpm, 4^{o}C for 3min, in a table top centrifuge. The supernatant was removed to 2ml with a 10ml pipette then completely using a 1ml pipette without disturbing the pellet. 10ml of bleach solution (625µl 4M NaOH, 1500µl concentrated bleach [Sigma, 425044] and 7875µl distilled water) was added to the worm pellet and the tube agitated. After approx 3min, and every minute thereafter, a 10µl sample was removed and examined on a microscope slide under a dissecting microscope. Once the adult worms began to lyse and release their eggs the falcon tube was filled to the top with ice cold M9 buffer. The tube was immediately centrifuged at 2000rpm, 4^{o}C

for 2min. The pellet of eggs was washed in this manner a further two times. Finally, the supernatant was completely removed and the pellet resuspended in approx 5-7 ml of S-buffer and transferred to a 5cm diameter petri dish. The eggs were incubated at 20°C overnight to allow the eggs to hatch.

The following day 10µl of the L1 suspension was removed and the number of L1 larvae counted. This was repeated three times and the mean number of worms per 10µl calculated.

2.2.3 Preparation of worm lysates

Worm lysates were used as template for PCR reactions unless otherwise stated.

Lysis buffer: 10mM Tris (pH 8.0); 50mM KCl; 2.5mM $MgCl_2$; 0.05% gelatin. Autoclaved and supplemented with 0.45% Tween-20 and 0.5µg/ml Proteinase K.

Young adult stage *C. elegans* were picked into a total volume of 20µl lysis buffer. Using a GeneAmp PCR system 9700 (Applied Biosystems) the samples were heated to 65°C for 90min, followed by 95°C for 15min to denature the proteinase K. Samples were immediately stored at -80°C until use.

2.2.4 Standard Polymerase Chain Reaction (PCR)

PCR reactions were performed using a GeneAmp PCR system 9700 (Applied Biosystems) in a 20µl volume unless otherwise stated. Routine PCR conditions used were 95°C for 30sec, primer annealing at 55-59°C for 30sec and extension at 72°C for 1-2min per 1kb of target sequence. A total of 35-40 cycles were used. Final concentrations of 250-500nM of forward and reverse primers and 250µM of each dNTP were used. Oligonucleotide primers were purchased from Eurofins MWG Operon. The sequences of all primers used are presented in the Appendices and on the accompanying CD. Amplitaq DNA polymerase (5U/µl) and GeneAmp 10X PCR buffer (Applied Biosystems- N808-0160) were used at a final concentration of 1 unit of enzyme per reaction. Where appropriate a combination of Amplitaq DNA polymerase (5U/µl; Applied Biosystems, N808-0160) and cloned Pfu polymerase (2.5Uµl; Stratagene, 600153-81), 5:1 by

volume, was used at a final concentration of 0.8 units Amplitaq DNA polymerase and 0.1 units *Pfu* polymerase per reaction. *Pfu* is a proof-reading polymerase that contains 3′-5′-exonuclease activity that enables it to proof-read for nucleotide mis-incorporations. This was used for all fragments amplified for sequencing.

GeneAmp 10X PCR Buffer: 100mM Tris-HCl pH 8.3 (at 25°C); 500mM KCl; 15mM $MgCl_2$; 0.01% w/v gelatine; autoclaved

2.2.5 PCR for GFP fusion constructs

GFP fusion constructs were all in the region of 3kb long and a slightly modified PCR protocol was used. PCR conditions consisted of 10 cycles of 94°C for 10sec, primer annealing at 55°C for 30sec, and extension at 68°C for 4min; followed by 25 cycles of 94°C for 15sec, primer annealing at 55°C for 30sec, and extension at 68°C for 4min plus an increment of 20sec each cycle. A combination of Amplitaq DNA polymerase (5U/µl; Applied Biosystems, N808-0160) and cloned Pfu polymerase (2.5Uµl; Stratagene, 600153-81), 5:1 by volume, was used at a final concentration of 0.8 units Amplitaq DNA polymerase and 0.1 units *Pfu* polymerase per reaction.

2.2.6 Agarose gel electrophoresis

Nucleic acids were separated on 1-2% (w/v) agarose gels. Agarose (Invitrogen, 15510-027) was melted in 1X TAE, or 1X TBE for RNA separation, by heating until in solution. Ethidium bromide was then added to a final concentration of 0.1µg/ml and gels cast. Gels were electrophoresed in 1X TAE or 1X TBE as appropriate using electrophoresis equipment from Amersham Pharmacia Biotech. Gels were imaged using a Fluorchem 5500 UV transilluminator and image capture system (Alpha Inotech).

2.2.7 Preparation of drug plates

Nematode growth medium (NGM) was prepared to standard specifications other than the addition of PEG water soluble cholesterol 25mg/ml in H_2O (Sigma, C1145) in place of cholesterol 5mg/ml in ethanol (Stiernagle, 1999). The use of

water soluble cholesterol increased the solubility of the compounds in NGM whilst allowing normal growth of the nematodes.

Molten NGM was allowed to cool to 55°C in a water bath before being split between drug and control aliquots. Stock drug (ivermectin [Sigma, I8898] or albendazole [Sigma, A4673]) dissolved in DMSO (Sigma, D8418) was added to the required concentration for the drug aliquot and an equal volume of DMSO alone was added to the control aliquot. NGM was then poured into standard triple vent petri dishes, approximately 8-10 ml for 5cm diameter plates and 20-25 ml for 9 cm diameter plates.

2.2.8 Liquid culture conditions

Drug exposure to high dose albendazole (300µg/ml; 1.13mM) and preparation of *C. elegans* for microsome extraction was carried out in liquid culture. *C. elegans* was cultured in S-basal with the following supplements added prior to use (per 500ml S-basal): 1.5ml 1M $MgSO_4$; 3ml 0.5M $CaCl_2$; 5ml 100X trace metal solution (0.346g $FeSO_4.7H_2O$, 0.930g NA_2EDTA, 0.098g $MnCL_2.4H_2O$, 0.144g $ZnSO_4.7H_2O$, 0.012g $CuSO_4.5H_2O$ in 500ml dsH_2O); 5ml 1M KCitrate, pH 6.0.

Concentrated OP50 was used as a food source and was prepared by inoculating 1L superbroth with 1ml OP50 in L-broth and incubating overnight at 37°C with shaking at 200rpm. The bacteria were pelleted in a Beckman Coulter Avanti J-E centrifuge at 4000rpm, 4°C for 20min. The bacterial pellet was resuspended in 10ml S-basal. The pellets were either stored at -20°C or kept refrigerated and used within two weeks.

Cultures were initiated either with synchronised L1 larvae or 5-8 9cm diameter NGM plates containing many mixed stage *C. elegans*. They were maintained at 20°C with shaking at 240rpm for a maximum of 5 days. Nematodes were then harvested by sucrose floatation as follows. The nematodes were pelleted by centrifugation at 3000rpm, 4°C for 3min. The pellet was resuspended in ice cold 0.1M NaCl and pelleted by centrifugation at 2000rpm, 4°C for 3min. The pellet was then resuspended in approximately 20ml of ice cold 0.1M NaCl (in a 50ml falcon tube) and left on ice for 5min to ensure it was thoroughly chilled. An equal volume of ice cold 60% sucrose solution was added to each of the tubes.

These were immediately inverted several times and centrifuged at 3500rpm, 4°C for 5min.The top 20ml from each tube was removed and split between 2 fresh 50ml falcon tubes. These tubes were immediately filled with ice cold 0.1M NaCl and centrifuged at 3100rpm, 4°C for 3min. The supernatant was removed from each of the tubes and the pellets resuspended and transferred to 2ml eppendorf tubes. Finally the samples were centrifuged at 2000rpm for 1min in a tabletop centrifuge, the supernatant removed and the pellets snap frozen and stored in liquid nitrogen until RNA extraction.

2.2.9 RNA extraction

RNA was extracted using a Trizol procedure as per the manufacturer's guidelines. Briefly, four volumes of TRIzol reagent (Invitrogen, 15596-026) was added per *C. elegans* pellet (100-1000µl). The sample was homogenised and vortexed and left at room temperature for at least 5min. Insoluble debris was removed by centrifuging at full speed at 4°C for 10min in an Eppendorf Centrifuge 5810 R tabletop centrifuge. The supernatant was removed to a fresh tube and 20% volume of chloroform added. The mixture was vortexed for 15sec and left at room temperature for 3min. Following centrifuging at full speed, 4°C for 15min the aqueous layer was removed and the chloroform wash repeated. Finally, 500µl isopropanol was added and the RNA precipitated at -80°C. The RNA was pelleted by centrifugation at full speed, 4°C for 10min. The RNA pellet was resuspended in RNase free water and treated with DNase I (Qiagen, 79254) in solution for 10min, before purification and concentrating using RNeasy columns (Qiagen, 74104).

Individual RNA samples were initially quantified by 260/280 absorption on a Gene Quant *pro* spectrophotometer (Amersham Biosciences) and were analysed by gel electrophoresis (1.2% agarose TBE gel, 100V, 1hr). Samples were then appropriately diluted for analysis on an Agilent Bioanalyser 2100. This is a microfluidics-based platform, which separates RNA fragments based on size and detects them via laser-induced fluorescence. Data is compared to that of a standard ladder to produce accurate quantification of RNA concentration. RNA integrity is assessed based on the whole electrophoretic trace including ribosomal RNA ratios, the "inter region" between the 18S and 28S ribosomal RNA

fragments and background fluorescence. An RNA integrity number (RIN) between 1 and 10 is then assigned, 1= degraded and 10= intact. In this study, RIN of greater than 8 out of 10 were accepted for further analysis by microarray. Samples for microarray analysis were re-precipitated in ethanol for storage and delivery to the Wellcome Trust Sanger Institute on dry ice.

2.2.10 Microarray hybridisation and analysis

2.2.10.1 Pre-processing

Sample labelling and hybridisation to Affymetrix *C. elegans* GeneChip arrays were performed at the Wellcome Trust Sanger Institute, using standard Affymetrix protocols (performed in Dr. Al Ivens' laboratory). The DNA microarray contained 22625 gene probes corresponding to 22150 *C. elegans* genes (http://www.affymetrix.com/index.affx). Scanned array images (CEL files) were quality control assessed using the arrayQualityMetrics Bioconductor package (www.bioconductor.org) in the R environment (www.r-project.org). Arrays identified as possible outliers were removed from subsequent analyses.

2.2.10.2 Annotation

An updated annotation dataset was assembled for the *C. elegans* probesets (genes) present on the Affymetrix GeneChip. Data were sourced from WormBase (Sept. 2008).

2.2.10.3 Processing

Linear model fitting of the array data was undertaken, taking into account bioreplicates using the limma (Linear Models for Microarray Data) Bioconductor package (www.bioconductor.org/packages/bioc/html/limma.html). A series of pairwise comparisons (test relative to control) was subsequently performed to identify differentially expressed genes. Significance of the differential expression values was assessed using two approaches. Firstly, an empirical Bayesian approach, with a multiple testing correction (Benjamini & Hochberg) was undertaken at the Sanger Institute (Benjamini *et al.*, 1995). Secondary analysis was carried out using a Rank Products methodology, which has been

proposed to be less discriminative against microarray experiments with lower numbers of biological replicates (Breitling *et al.*, 2004). In both cases initial analysis of significance was carried out using a False Discovery Rate cut-off of 5%.

2.2.10.4 Ontology analysis

Further analysis was carried out, using the freely available DAVID software (the Database for Annotation, Visualisation and Integrated Discovery) from the National Institutes of Health (Huang *et al.*, 2009; Dennis, Jr. *et al.*, 2003), to assess the functional annotation and clustering of the genes noted to be differentially expressed between samples. Input into the program consisted of genes shown to be significantly altered in expression using the Rank Products algorithm, with a False Discovery Rate cut off of less than 10%. The gene lists were compared to a whole genome background to provide information regarding enrichment of particular families or biological functions. Initially gene functional classification clustering was carried out using medium stringency.

2.2.11 Real-time quantitative PCR

Microarray experiments can be insensitive leading to false negative, or alternatively, false positive results. Consequently, we have used RT-QPCR to confirm the results for those genes represented on the array by probes showing the greatest differential expression between drug-exposed and non-exposed worms. SYBR green I is a fluorescent dye that can be used to quantitate DNA. When bound to double-stranded DNA the dye absorbs light of wavelength 488nm and emits light of wavelength 522nm with intensity proportional to the amount of bound dye.

SYBR green I will bind to any double-stranded DNA. Therefore, several steps must be taken to ensure accurate results. All RNA samples were subject to DNase I treatment prior to cDNA synthesis using a cloned AMV first strand synthesis kit (Invitrogen, 12328-032). No template controls and no reverse transcriptase controls, only differing from the experimental samples by the absence of reverse transcriptase, were included for all samples. Finally, dissociation curves were carried out for all samples in all analyses to ensure that

a single product was amplified in each reaction. In the case of primers designed to span an intron, this will help to identify gDNA contamination as gDNA would be expected to be a larger product and produce a melting curve at a higher temperature.

2.2.11.1 Primer design and analysis

Where possible RT-QPCR primers were designed to the following criteria: primers were all between 20 and 25-bp long; the product of the PCR was between 160-200bp in length; the product spanned an intron to give differentially sized genomic and cDNA products; the melting temperatures of the primer pairs were matched and were between 55 and 60^{o}C. *ama-1*, encoding a subunit of RNA polymerase II, was used as a normalising gene. This constitutively expressed gene showed no significant changes on microarray analysis and has been extensively used as a normalising gene in differential expression studies in *C. elegans* (Johnstone *et al.*, 1996). All primer sequences were compared to the current *C. elegans* genome using a BLASTn search to ensure that they amplified a unique DNA fragment (www.wormbase.org/db/searches/blast_blat). In addition, all primers were used with standard PCR methods to amplify fragments from both *C. elegans* genomic and cDNA and analysed by gel electrophoresis. Only primer sets showing single bands of the expected size amplified from both gDNA and cDNA were used for RT-QPCR analysis. Primer sequences may be found in **Appendix 7.1** and on the accompanying CD.

2.2.11.2 RT-QPCR reaction parameters

All reactions were carried out using Brilliant SYBR Green QPCR mastermix (Stratagene, 600548). The final concentration of primers was between 300 and 400nM in a total reaction volume of 25µl. A Stratagene Mx3000P QPCR system was used with the following parameters: 7.5min at 95^{o}C; 40 cycles of 0.5min 95^{o}C, 0.5 min 59^{o}C, 0.5min 72^{o}C; and finally 1min 95^{o}C followed by 0.5min 59^{o}C and a gradient to 95^{o}C. Fluorescence was measured at the end of the elongation phase (72^{o}C) during each cycle, for quantitation, and continuously during the final gradient from 59-95^{o}C, to assess dissociation curves. Data was captured and analysed using Stratagene MxPro software.

Standard curves for all primer sets were run over 5-fold dilutions of sample cDNA from 1:25- 1:625. Where possible, primer sets used for analysis of experimental samples had a standard curve with an efficiency of 90-105% and an Rsq of 0.99 or above, over the range of experimental sample concentrations. Rsq is a measure of the fit of all data to the standard curve plot, where 1.00 equals perfect alignment. In some cases despite attempts to optimise the PCR this was not possible. However, standard curves were assessed alongside all experiments and their efficiencies applied to the quantitation algorithm for that experiment. All samples were analysed in duplicate or triplicate on every plate and no template controls and no reverse transcriptase controls for all experimental samples were included. 10µl of a 1:50 dilution of the sample cDNA was used to compare the relative quantity of each gene within each biological replicate, using the ΔΔCt method outlined below:

$$\text{Normalised } \frac{\text{Unknown}}{\text{Control}} = \frac{(1+E\ \text{target})^{-\Delta Ct\ \text{target}}}{(1+E\ \text{norm})^{-\Delta Ct\ \text{norm}}}$$

Where, E = efficiency of PCR amplification; maximum 1 (or 100%)

ΔCt = difference in threshold cycles between samples (unknown-control)

target = gene of interest

norm = normalising gene (*ama-1*)

2.2.11.3 Statistical analysis

Where stated, normalised threshold cycle values from real-time QPCR studies were subject to statistical analysis using a paired student's *t*-test.

2.2.12 Determination of expression patterns using Green Fluorescent Protein (GFP)

2.2.12.1 Preparation of GFP constructs

GFP reporter constructs were created using a PCR fusion protocol as described by Hobert *et al.* (2002). The promoter region of the gene of interest was amplified with a forward primer approximately 3Kb upstream from the ATG start site of the gene of interest (primer A), and a reverse primer immediately upstream of the ATG (primer B). Primer B was designed with a 5′ 24bp tag that was complimentary to primer C. Primer C and D are the forward and reverse primers used to amplify the GFP gene, including synthetic introns and *unc-54* 3′ UTR, from Fire vector pPD95.67 (Fire *et al.*, 1990). Primer sequences are available in **Appendix 7.2** and on the accompanying CD.

The products of these two reactions were assessed by gel electrophoresis to ensure that bands of the expected size were present. 1µl of each of the PCR reactions was then used in a final PCR using the nested primers A* and D* which amplified a single linear fragment consisting of the promoter region of the gene of interest fused to the GFP gene.

2.2.12.2 Microinjection of the GFP fusion constructs

Constructs were injected into the syncitial gonad of young adult hermaphrodites, of the ivermectin resistant strain DA1316, along with the marker construct pRF-4 and p-Bluescript KS+ added to a total DNA concentration of 160-200ng/µl (Mello *et al.*, 1991). pRF-4 is a plasmid used as a cotransformation marker to identify transgenic worms. It contains the mutant allele *rol-6(su1006)*, which encodes a cuticle collagen gene that produces a dominant roller phenotype. Progeny carrying the transgene exhibit an inability to move in a normal sinusoidal pattern, instead rotating around their longitudinal axis and rolling in circles. F2 worms showing the roller phenotype were selected and maintained as a transmitting line.

2.2.12.3 Imaging of GFP expressing *C. elegans*

Nematodes were picked on to microscope slides with 2% agarose/ 0.065% sodium azide pads. 5-10µl of M9 buffer was applied to the pad to prevent desiccation of the nematodes and a cover slip was placed on top and sealed with Vaseline. Expression patterns were visualised using a Zeiss, Axioscop 2 plus microscope. Images were collected and processed using Improvision Openlab software (www.improvision.com).

Chapter 3: *C. elegans transcriptomic response to ivermectin*

3.1 Introduction

Ivermectin is an avermectin drug and has been used by the veterinary profession as an endectoside, treating both endoparasites and ectoparasites, since the early 1980s. The drug is also used in human medicine to treat a variety of parasitic diseases, most importantly the filarial helminthoses caused by *Onchocerca volvulus* and *Brugia malayi* infection (Boatin *et al.*, 2006; Horton *et al.*, 2000).

The pharmacokinetics of ivermectin has been examined in many mammalian species including humans and veterinary species such as cattle, sheep, pigs, horses and dogs, reviewed by Gonzalez *et al.* (2009 and 2008). Ivermectin is a highly lipophilic drug which is readily absorbed following ingestion, subcutaneous or intramuscular injection and topical application. The drug has a long plasma half life in all species; in humans this has been estimated at approximately 1 day, but may be up to a week depending on species and formulation of ivermectin (Gonzalez *et al.*, 2009; Bousquet-Melou *et al.*, 2004). This is thought to be partially due to the large volume of distribution and extensive enterohepatic recycling of the drug (Gonzalez *et al.*, 2008). There are few studies investigating the routes of metabolism of ivermectin in humans. However, Zeng *et al.* (1998) have shown that ivermectin is metabolised to at least ten metabolites by human liver microsome preparations. Using a combination of microsomes containing specific CYP isoforms and CYP3A4 antibodies, their work suggested that the predominant enzyme involved in this biotransformation is CYP3A4.

In animals, most of the dose of ivermectin is excreted unchanged with minimal metabolism (Gonzalez *et al.*, 2009). 24-hydroxy-methyl metabolites predominate in sheep, cattle and rats and as a result fat esters are also found. 3′-*O*-desmethyl metabolites are more common in pigs and goats (Gonzalez *et al.*, 2009; Chiu *et al.*, 1986). The enzymes responsible for biotransformation and the

pharmacological activity of metabolites have not been assessed. However, altering the composition of the side groups of macrocyclic lactone drugs, where biotransformation may occur, is known to dramatically affect their potency (Michael *et al.*, 2001).

There have been few studies investigating the induction of cytochrome P450s and other potentially xenobiotic metabolising enzymes by ivermectin. Skalova *et al.* (2001) have reported that CYP activities are induced in rats and mouflon but not in fallow deer following exposure to ivermectin. These studies made use of substrate assays which are thought to distinguish specific CYP isoforms. A single therapeutic dose of ivermectin resulted in induction of CYP1A1/2, CYP2B and CYP3A activities in mouflon. However, in rats, CYP1A1 and CYP1A2 activities were only significantly induced after exposure to high doses of ivermectin (20 to 30-fold the therapeutic dose), no induction in CYP2B/ CYP3A4 activities were noted. Bapiro *et al.* (2002) found that ivermectin caused no induction in CYP1A1 and CYP1A2 activities in human HepG2 cells. However, enzyme activities specific to other CYP isoforms were not assessed.

Induction of cytochrome P450 gene expression is thought to occur via binding to and activation of nuclear hormone receptors such as the constitutive androstane receptor (CAR), pregnane X receptor (PXR) and peroxisome proliferator activated receptor (PPAR), as well as several others (Wei *et al.*, 2000; Kliewer *et al.*, 1999). Specific studies investigating the pathways by which ivermectin may induce CYP activity have not been carried out.

In order to investigate the potential for ivermectin to induce nematode xenobiotic metabolising enzymes (XMEs), the transcriptomes of *Caenorhabditis elegans* exposed to ivermectin and an unexposed control group were compared. Ivermectin is an extremely potent drug, effective plasma concentrations in cattle are between 0.5-1ng/ml (0.57-1.14nM; Lifschitz, 1999). DA1316 is a laboratory created strain of *C. elegans* with mutations in three glutamate-gated chloride channel subunits, which are reported to confer extremely high resistance to ivermectin. In order to minimise transcriptomic changes resulting from the phenotype of drug intoxicated worms and generalised stress responses, DA1316 was used for intial microarray experiments.

3.2 Methods

3.2.1 Preparation of nematodes for microarray analysis- chronic exposure

Initially a chronic exposure to low dose ivermectin was used. Approximately 10000 synchronised DA1316 L1 larvae per experimental condition were added to OP50-seeded NGM plates containing 0.5 or 5ng/ml (0.57-1.14nM) ivermectin (Sigma, I8898) or control plates at approximately 500 nematodes per 5cm diameter plate. The nematodes were grown in standard conditions (20^{o}C) for approximately 60hrs until greater than 90% of worms had reached the L4 stage. The nematodes were then washed into 15ml falcon tubes with M9 buffer and centrifuged at 2500rpm, 4^{o}C for 3min. The pellet of worms was washed twice by removing the supernatant, refilling the tube with fresh M9 buffer and repeating the centrifugation step. Finally the pellet was snap frozen and stored in liquid nitrogen until RNA extraction.

3.2.2 Preparation of nematodes for microarray analysis- acute exposure

Synchronised DA1316 L1 larvae (approximately 10000 per experimental condition) were grown for 53hrs at 20^{o}C on standard NGM plates with OP50 bacterial lawns. The nematodes were washed from the plates with M9 buffer into a 50ml falcon tube and washed twice in M9 buffer as per **Section 3.2.1**. The supernatant was again removed and the worms resuspended in 2-3ml of fresh M9. The suspension of worms was split equally between control plates and plates containing 100ng or 1µg/ml (114nM or 1.14µM) IVM (Sigma, I8898) at a density of 500- 600 worms per 5cm diameter plate. After 4hrs exposure the nematodes were harvested and stored in the same manner as described in **Section 3.2.1**.

Similar experiments were carried out using the Bristol N2 strain, with exposure to 100ng/ml (114nM) IVM. These were chronologically identical to previous experiments, but due to the N2 strain growing slightly faster than the DA1316 strain the nematodes were at the young adult stage at the time of harvesting.

RNA extraction and microarray hybridisation were carried out as described in **Chapter 2**.

3.2.3 Preparation of nematodes for Real-time quantitative PCR

Three separate biological replicates from those sent for microarray analysis were used for RT-QPCR assays. The protocol used to prepare these replicates was identical to that described for the microarray experiments except that a commercial pour-on preparation of ivermectin (Virbamec 5mg/ml, Virbac Animal Heath) was used as the source of drug. RNA was extracted and cDNA synthesised from 5µg total RNA for each sample using a cloned AMV first strand synthesis kit (Invitrogen, 12328-032) with random hexamer primers. For each sample an identical reaction lacking reverse transcriptase enzyme was carried out. cDNA was then purified using PCR purification columns (Qiagen, 28106) according to the manufacturer's protocol.

Investigation of gene up-regulation following exposure to a gradient of ivermectin concentrations was also undertaken. The method was the same as the microarray experiments but five matched cultures of *C. elegans* were prepared. Cultures were exposed for 4hrs to 1, 10, 100 and 1000ng/ml (1.14, 11.4, 114 and 1140nM) IVM or to no IVM as a control. Ivermectin (Sigma, I8898) dissolved in DMSO (Sigma, D8418), stock 10mg/ml (11.4mM), was used and all cultures contained an identical volume of DMSO.

3.2.4 Pharyngeal pumping assay

Ivermectin (Sigma, I8988) plates were prepared to final concentrations of 1, 10, 100 and 1000ng/ml (1.14, 11.4, 114 and 1140nM) IVM and a matched no drug control. Synchronised N2 and DA1316 L1 larvae were allowed to grow on standard NGM plates at 20^{o}C for 53hrs. The L4/ young adults were then picked on to drug plates and allowed to remain at 20^{o}C for a further 4hrs. The pharyngeal pumping rate of ten worms of each strain at each concentration of drug was then assessed over a period of 1min.

3.2.5 Genotyping of strain DA1316

DA1316 has mutations in three glutamate-gated chloride channel subunit genes: *avr-14, avr-15* and *glc-1*. *avr-14(ad1302)* and *avr-15(1051)* represent single nucleotide substitutions. Analysis of these mutations was assessed by amplifying an approximately 300bp region around the proposed mutation site and sequencing by direct PCR sequencing using both a forward and reverse primer. A combination of Taq: Pfu (10:1) DNA polymerase was used to increase the fidelity of the PCR reaction. *glc-1(pk54::Tc1)* represents a Tc1 transposon insertion at amino acid 255 of GLC-1. Analysis of this mutation was carried out using a primer within the *glc-1* gene and one within the Tc1 transposon, which would be expected to give a 666bp product in the mutant strain and no product in wild-type worms. All primers used for amplification and sequencing are available in **Appendix 7.3** and on the accompanying CD.

3.3 Results

3.3.1 Microarray analysis

3.3.1.1 Exposure to 0.5ng/ml and 5ng/ml IVM result in no significant changes to gene expression

Initial experiments used an extremely conservative dose of ivermectin. After 60hrs exposure to 0.5ng/ml (0.57nM) IVM there were no stage differences between the drug and control plates of strain DA1316. However, N2 worms grown on this concentration of drug had severely retarded development compared to control plates. In total three biological replicates (three 0.5ng/ml IVM and three controls) were sent for microarray hybridisation and analysis and none were dropped following quality control. Only two genes were found to be significantly up-regulated using an empirical Bayes t-test with Benjamini-Hochberg FDR correction to 5%. The top 10 up-regulated genes, based on $\log_2$ fold-change, were initially thought to be encouraging. **Table 3-1**, shows that two cytochrome P450 genes, one UGT and one GST-like gene were up-regulated. Interestingly, RNAi of several of these genes (*cyp-13A6*, T16G1.6, *cdr-1*, F15E11.2) results in cadmium hypersensitivity, suggesting a shared regulatory pathway (Cui *et al.*, 2007). However, QPCR analysis of the *cyp-13* family using the same biological replicates as were sent for microarray analysis revealed that only two of the three replicates showed up-regulation of *cyp-13A6*. The third replicate showed a significant down-regulation of the same gene **(Fig. 3-1).** Further biological replicates did not show any change in the expression level of *cyp-13A6* using real-time QPCR.

Range finding experiments were carried out and 5ng/ml (5.7nM) IVM was determined as the highest concentration of IVM that could be used over 60hrs without causing stage differences between DA1316 exposed to drug and control populations. Four biological replicates were sent for microarray analysis (four exposed to 5ng/ml IVM and four controls). However, two chips were dropped following quality control, both of which represented the transcriptome of nematodes exposed to IVM. Analysis of the remaining chips revealed no statistically significant changes in the expression of any genes.

Probeset	Gene ID	Log_2 FC	p-value	Adjusted p-value*	Ontology
189575_at	*cyp-13A6*	4.57	1.62E-13	3.66E-09	cytochrome P450 (CYP3/5/6/9 subfamily)
190651_at	*ugt-61*	2.36	2.27E-10	2.56E-06	UDP-glucuronosyl/ glucosyl transferase KOG
172020_x_at	*lin-36*	0.89	1.90E-03	4.07E-01	involved in vulval development
189457_at	*cyp-34A9*	0.86	2.49E-03	4.62E-01	cytochrome P450 (CYP2 family)
178563_at	T16G1.6	0.81	5.38E-04	2.40E-01	predicted small molecule kinase
191611_at	*cdr-1*	0.70	9.26E-05	1.59E-01	glutathione-s-transferase-like protein (microsomal).
177676_s_at	C53B4.3	0.63	7.16E-04	2.52E-01	uncharacterised
174112_at	*cogc-2*	0.63	6.21E-03	5.98E-01	orthologue of mammalian conserved oligomeric golgi complex subunit.
186519_at	F15E11.12| F15E11.15	0.63	2.90E-01	1.00E+00	uncharacterised
187628_s_at	C30G12.6	0.56	6.66E-03	6.09E-01	uncharacterised

Table 3-1: Top 10 up-regulated probesets based on fold change following 60hrs exposure of DA1316 to 0.5ng/ml (0.57nM) IVM
***cyp-13A6, cyp-34A9, ugt-61* and *cdr-1* represent genes that are potentially involved in xenobiotic metabolising pathways.**
***Benjamini Hochberg False Discovery Rate correction**

Only three genes were up-regulated more than 2-fold following 60hrs exposure to 5ng/ml (0.57nM) IVM: *cgh-1* represents a dead-box RNA helicase which is extremely important in oocyte and spermatocyte development; *rpn-2* represents a non-ATPase subunit of 26S proteasomes 19S regulatory particle and is required for embryonic, larval and germline development; *prp-17* is uncharacterised but encodes an mRNA splicing factor KOG. None of the top 10 were genes potentially involved in xenobiotic metabolism.

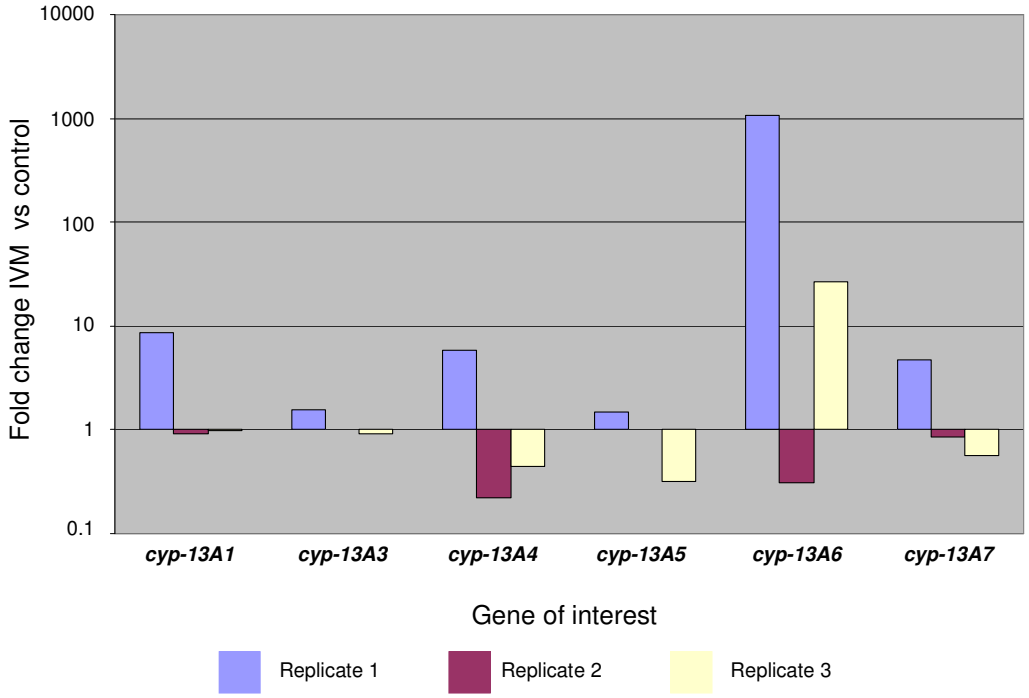


Figure 3-1: Real-time QPCR of individual bioreplicates sent for microarray analysis; 0.5ng/ml (0.57nM) IVM vs. control
A logarithmic scale is used due to the highly variable up-regulation. *cyp-13A6* is over 1000 fold up-regulated in one biological replicate, but significantly down-regulated in another.

It was decided that increasing the number of biological replicates at this dose of drug was unlikely to improve the results and that a different approach was needed. Despite strain DA1316 being reported to be unaffected by doses of ivermectin up to 4µg/ml (4.56µM), we found that significant stage differences occurred between drug exposed and control *C. elegans* over 60hrs. Therefore, we decided to use a shorter exposure of 4hrs and increase the dose of drug significantly.

3.3.1.2 Acute exposure to 100ng and 1µg/ml IVM results in differential expression of a distinct set of genes

Experiments were carried out as per **Section 3.2.2.2**. Exposure to 100ng/ml (114nM) IVM was assessed first. In total, six drug exposed and six matched control RNA samples were sent for analysis. Two chips were dropped following quality control, one drug exposed and one control. Analysis of the remaining chips revealed there to be no probesets with significantly altered expression using a Bayesian t-test with FDR correction to 5%. However, using the rank

products algorithm there were twelve probesets considered to be significantly up-regulated and three considered to be significantly down-regulated (FDR <5%). The top 10 up-regulated genes, based on $\log_2$ fold change, are listed in **Table 3-2**. Considering the number of biological replicates and high dose of drug this is still a surprisingly small list of genes whose expression levels were significantly changed.

Probeset	Gene ID	Log_2 FC	BH FDR*	RP FDR$^+$	Ontology
172744_at	*mtl-1*	1.59	5.86E-01	0	metallothionein
184913_s_at	T22F3.11	1.44	6.40E-01	0	permease of major facilitator family KOG
192737_at	*scl-2*	1.31	9.04E-01	0	sterol carrier-like protein
189221_at	*cyp-37B1*	1.27	7.31E-01	0	cytochrome P450 (CYP4/19/26 subfamilies)
186971_at	C23G10.11	1.23	8.59E-01	0	uncharacterised
173729_at	T22F3.11	1.21	8.84E-01	0	permease of major facilitator family KOG
183381_at	C50F7.5	1.12	8.65E-01	1.00E-02	uncharacterised
186521_at	F21C10.10	1.10	7.10E-01	1.11E-02	uncharacterised
173550_at	F45D3.4	1.08	9.99E-01	1.25E-02	uncharacterised
190978_at	*sodh-1*	1.07	8.65E-01	1.82E-02	alcohol dehydrogenase class V KOG

Table 3-2: Top 10 up-regulated probesets based on fold change following 4hrs exposure of DA1316 to 100ng/ml (114nM) IVM
***cyp-37B1* and *sodh*-1 represent the only genes in the top 10 that may potentially be involved in "classical" xenobiotic metabolising pathways. However, there are many uncharacterised genes that may have novel roles in the response to ivermectin.**
***BH Benjamini Hochberg correction of Bayesian t-test.**
$^+$RP Rank Products analysis

Experiments were carried out using 1µg/ml (1.14µM) IVM in a similar manner. Again six drug exposed and six matched controls were sent for analysis. Only one control chip was dropped. At this concentration of ivermectin there were 1352 genes with significantly altered expression following analysis with the empirical Bayesian t-test and a FDR cut off of 5% (786 up-regulated and 565 down-regulated). Analysis of the five complete biological replicates using the rank products algorithm suggested that only 369 probesets were significantly altered in expression with the same FDR correction (216 up-regulated and 153 down-regulated). All genes considered significant in the rank products analysis are also considered significant in the t-test analysis. **Fig. 3-2** summarises the microarray

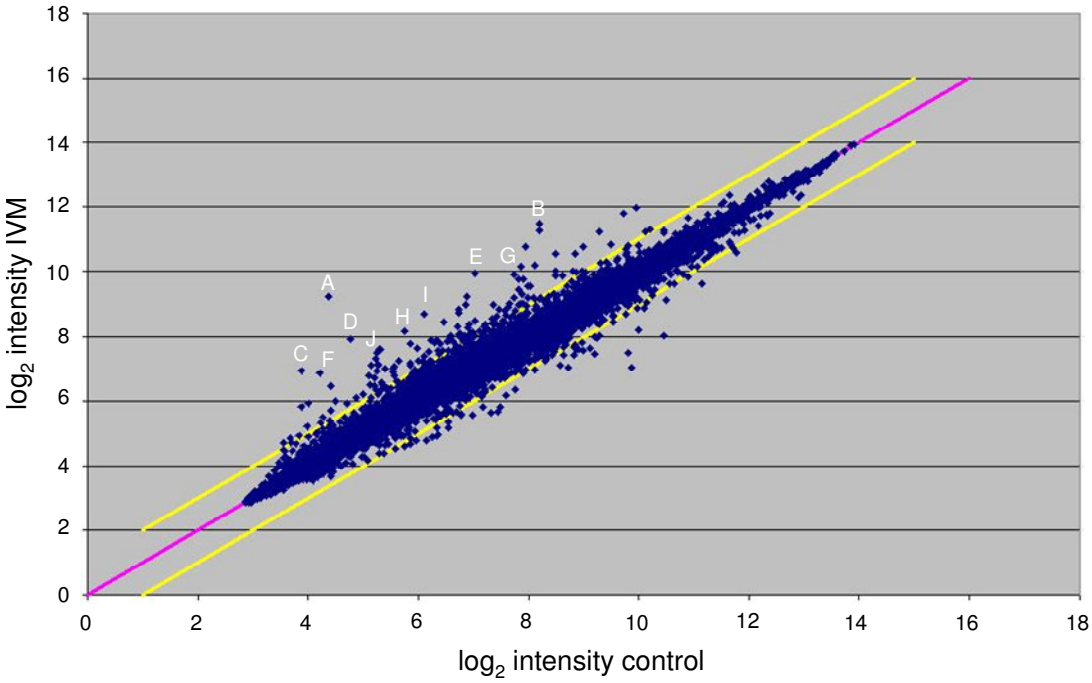


Figure 3-2: Model fitted log_2 control chip intensity vs. log_2 IVM (1µg/ml, 1.14µM) chip intensity
The scatter plot represents the entire 22625 probesets represented on the Affymetrix chips. The upper and lower yellow lines represent up-regulation greater than 2-fold and down-regulation greater than 2-fold respectively. The plots marked A-H represent the top 10 up-regulated genes in Table 3-3.

data and **Tables 3-3** and **3-4** list the top 10 up-regulated and down- regulated probesets based on log_2 fold change. Full microarray data can be found on the accompanying CD.

The top 10 up-regulated genes are not immediately striking as those potentially involved in xenobiotic metabolism pathways in either the 100ng/ml (114nM) or 1µg/ml (1.14µM) IVM experiments. However, there are several similarities between the lists, including the presence of *mtl*-1, *scl*-2 and *cyp*-37B1, which suggests there is a consistent response at the two doses of drug. *cyp*-37B1 represents a cytochrome P450 and therefore could potentially be involved in oxidoreductive metabolism. This gene has previously been shown to be up-regulated in microarray experiments investigating the response to other xenobiotics including PCB52, fluoranthene, progesterone and oestrogen (Menzel *et al.*, 2007; Reichert *et al.*, 2005; Custodia *et al.*, 2001). *sodh-1* is represented in the top 10 up-regulated in response to 100ng/ml (114nM) IVM and is also significantly up-regulated in the 1µg/ml (1.14µM) IVM experiment. This gene

Probeset	Gene ID	Log_2 FC	BH FDR	RP FDR	Ontology
172744_at	*mtl-1*	4.99	8.48E-09	0	metallothionein
192737_at	*scl-2*	3.27	6.90E-04	0	sterol carrier-like protein
186971_at	C23G10.11	3.20	1.58E-03	0	uncharacterised
189221_at	*cyp-37B1*	3.09	1.07E-05	0	cytochrome P450 (CYP4/19/26 subfamilies)
177613_at	F57G8.7	3.01	2.26E-08	0	uncharacterised
177671_at	K03D3.2	2.83	8.41E-09	0	uncharacterised
178900_s_at	F45D3.4	2.77	2.11E-03	0	uncharacterised
187964_at	F54F3.3	2.51	1.31E-04	0	triglyceride lipase-cholesterol esterase KOG
180946_at	*ilys-3*	2.51	4.47E-07	0	invertebrate lysozyme
173335_s_at	*dod-3*	2.33	2.26E-08	0	down stream of daf-16

Table 3-3: Top 10 up-regulated genes based on fold change following 4hrs exposure of DA1316 to 1µg/ml (1.14µM) IVM
***cyp-37B1* represents the only gene potentially involved in xenobiotic metabolism pathways. However, there is good correlation with the 100ng/ml (114nM) IVM experiment. Five genes represented in this table were also present in the top 10 up-regulated genes in the 100ng/ml IVM experiment.**

Probeset	Gene ID	Log_2 FC	BH FDR	RP FDR	Ontology
176939_at	*spp-23*	-2.79	1.10E-05	0	saposin-like protein family
190404_s_at	*folt-2*	-2.55	3.23E-04	0	putative folate transporter
179187_s_at	F46F2.3	-2.36	1.10E-02	0	uncharacterised
189345_at	*pho-13*	-1.88	2.70E-04	0	predicted intestinal acid phosphatase
192528_at	C35A5.3	-1.83	1.84E-05	0	uncharacterised
187085_s_at	*gst-10*	-1.77	5.60E-05	0	glutathione-s-transferase
190744_at	*ugt-63*	-1.77	2.74E-03	0	UDP-glucuronosyl/ glucosyl transferase KOG
175489_at	F18E3.11	-1.72	1.18E-04	0	uncharacterised
177747_at	F58G6.9| *srm-3*	-1.72	9.95E-04	0	uncharacterised
188441_at	F21F8.4	-1.70	1.59E-03	0	KOG- aspartyl protease

Table 3-4: Top 10 down-regulated genes based on fold change following 4hrs exposure to 1µg/ml (1.14µM) IVM
***ugt-63* and *gst*-10, both potentially involved in xenobiotic metabolism pathways, are significantly down-regulated.**

encodes a putative class V alcohol dehydrogenase, an important class of xenobiotic metabolising enzyme. There are no reports of *sodh*-1 being responsive to xenobiotics in the literature, but the related gene *sodh-2* has been reported to be ethanol responsive in *Caenorhabditis elegans* (Kwon *et al.*, 2004).

mtl-1 was the most highly up-regulated gene in the current study and represents a metallothionein gene, which is known to be highly inducible in response to oxidative stress and heavy metal intoxication (Cui *et al.*, 2007). However, this gene has also been shown to be induced in the presence of many xenobiotics including clofibrate, ß-naphthoflavone and steroid hormones (Reichert *et al.*, 2005; Custodia *et al.*, 2001). *scl-2* encodes a protein whose function is largely unknown. The gene contains a sterol carrier-like protein domain (www.wormbase.org).Therefore, SCL-2 may be involved in lipid metabolism, as may F54F3.3 which is a putative cholesterol esterase. Many of the other up-regulated genes are completely uncharacterised and so their potential role in the response to ivermectin exposure is unclear. Interestingly, many of these genes have been shown to be regulated together in the response to bacterial infection. Exposure to *Pseudomonas aeruginosa* was shown to result in up-regulation of *mtl-1, scl-2, cyp-37B1* and *sodh-1* as well as C23G10.11, F45D3.4, F54F3.3, C50F7.5 and *dod-3* (Troemel *et al.*, 2006). In addition *mtl-1, ilys-3, dod-3, sodh-1,* T22F3.11, C50F7.5, F21C10.10 and F45D3.4 are proposed downstream targets of the FOXO family transcription factor DAF-16 (Murphy *et al.*, 2003).

The top 10 down-regulated genes include a glutathione-s-transferase and an UDP-glucuronosyl/ glucosyl transferase. These both represent gene families that would be expected to be up-regulated if ivermectin were inducing xenobiotic metabolising genes. Experiments examining *gst-10(RNAi)* have proposed it to be integral to the response to heat, electrophilic stress and paraquat intoxication (Ayyadevara *et al.*, 2007). Therefore, if *C. elegans* was exhibiting a general stress response following exposure to ivermectin this gene may be expected to be up-regulated. *ugt-63* represents a putative UDP-glucuronosyl/ glucosyl transferase and has been proposed to be up-regulated in *C. elegans* exposed to

ethanol (Kwon *et al.*, 2004). Induction of expression of this gene was also seen in response to albendazole exposure, see **Chapter 4**.

The remainder of the top 10 down-regulated probesets represent a diverse group of genes, most of which are largely uncharacterised. However, KOG domains present in many of the genes would suggest that many are involved in general metabolism of lipids and proteins. *spp-23* represents a saposin-like protein, which is potentially involved in lipid binding and metabolism. However, proteins with a saposin-like domain may have numerous functions including antimicrobial action (Bruhn, 2005). Also down-regulated are a putative intestinal acid phosphatase (*pho-13)*, an aspartyl protease (F21F8.4) and two genes potentially encoding mineral transport proteins (*srm-3* and *folt-2*). Interestingly seven of the ten genes have also previously been shown to be down-regulated in response to *Pseudomonas aeruginosa* infection. Only *gst-10*, *srm-3* and F21F8.4 were not down-regulated in the microarray screen carried out by Troemel *et al*. (2006). However, none of these genes were proposed as targets of DAF-16 mediated suppression (Murphy *et al.*, 2003).

3.3.2 Real-time QPCR confirms up-regulation of genes in response to IVM exposure

QPCR primers were designed for several of the most interesting up-regulated genes following exposure to 1µg/ml (1.14µM) IVM. Analysis was carried out using three separate biological replicates independent to those sent for microarray analysis. The purity of ivermectin from Sigma, as was used for the microarray experiments, is stated to be ≥90% ivermectin B1a and ≤5% ivermectin B1b. Therefore it was possible that the changes seen in the microarray were as a result of impurities rather than a response to ivermectin itself. Virbamec is a commercial preparation of ivermectin licensed for use in cattle, and as such is presumed to be pure. However, the exact make up of the excipient was not detailed and experiments were carried out comparing nematodes exposed to Virbamec and those containing no additional supplements to the standard NGM. Real-time quantitative PCR results are summarised in **Fig. 3-3**.

All genes examined that were considered to be up-regulated in the microarray experiments were validated using RT-QPCR experiments. The fold-change of specific genes was higher using RT-QPCR than that suggested by microarray experiments. This was likely due to RT-QPCR being much more sensitive than microarrays which compare many genes simultaneously. In addition, random hexamer primers were used in the reverse transcriptase step, which may exaggerate differences in expression. The absolute fold-change is likely unimportant as the purpose of the real-time QPCR was to confirm the results of the microarray experiments and the biological significance of absolute up-regulation of a gene is unknown. The control genes were selected on the basis of them showing no significant changes on the microarray. *col-19* is an adult specific collagen gene. The lack of any change in the expression of this gene between the experimental groups also confirms the accurate staging of the

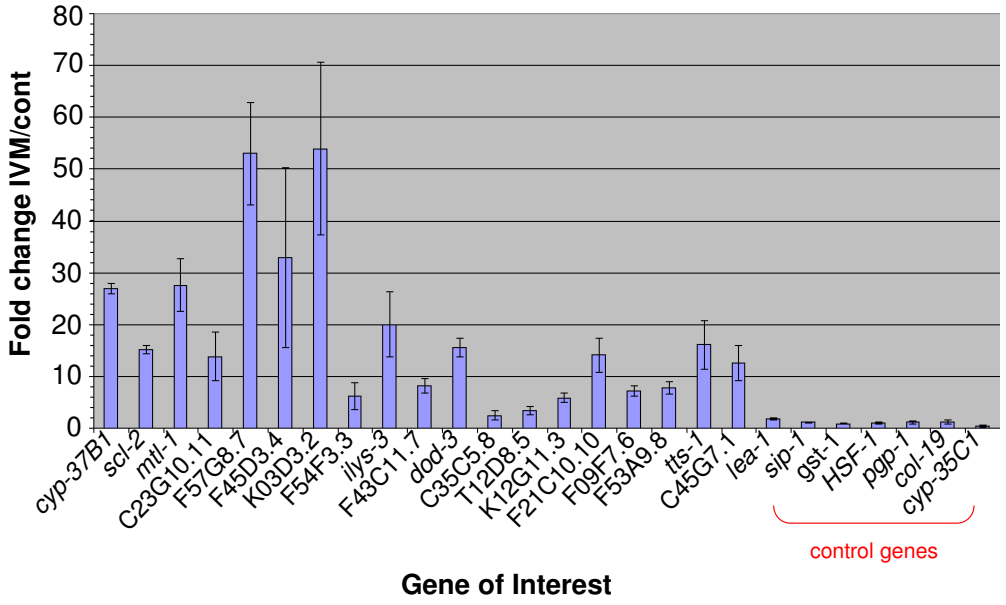


Figure 3-3: RT-QPCR results following 4 hrs exposure of DA1316 to Virbamec (1µg/ml [1.14 µM] IVM)
All genes proposed to be up-regulated by microarray were confirmed by RT-QPCR. The control genes showed no significant changes on microarray analysis and confirm that the response to ivermectin is not a general stress response. *cyp-35C1* is up-regulated in response to albendazole exposure (Chapter 4), but appears down-regulated in response to Virbamec exposure.

biological replicates. Several genes on the control panel were chosen because they are proposed to be involved in general stress responses: *sip-1, HSF-1* and *gst-1* (www.wormbase.org; Ayyadevara *et al.*, 2007; Cohen *et al.*, 2006;

Halaschek-Wiener *et al.*, 2005). In addition, an example of the p-glycoprotein family, *pgp-1,* was investigated. This gene has been proposed to be constitutively up-regulated in ivermectin selected lines of *C. elegans* and members of this family have also been proposed to be induced following IVM exposure of resistant isolates of *H. contortus* (James *et al.*, 2008; Prichard *et al.*, 2007). None of these genes showed any significant alteration of expression following exposure to Virbamec.

cyp-35C1 was chosen as a control since it is up-regulated in response to albendazole (see **Chapter 4**) as well as several other xenobiotics (Reichert *et al.*, 2005; Menzel *et al.*, 2001). This gene is not significantly down-regulated on microarray analysis and has a $\log_2$ fold change of -0.49. However, real-time QPCR demonstrated *cyp-35C1* to be consistently down-regulated following exposure to Virbamec (fold change 0.423).

3.3.3 DAVID analysis of genes with significant changes in expression following ivermectin exposure

3.3.3.1 Up-regulated genes

Global analysis of function was carried out using the freely available DAVID software from the National Institute of Allergy and Infectious Disease (NIAID), National Institutes of Health (NIH). The gene lists assessed consisted of up-regulated genes with a false discovery rate cut-off of less than 10%, as assessed by the rank products method. This up-regulated data set contained 292 probesets, which represented 254 genes in the *Caenorhabditis elegans* genome.

DAVID software aids in the interpretation of biological function of large gene lists by assigning annotation terms to each gene. DAVID makes use of gene ontology terms but also integrates information from several other gene identifier databases (Huang *et al.*, 2009). Prevalence of annotation terms within a gene list are compared to the prevalence in the background list, in this case the whole *C. elegans* genome. Fold enrichment is then calculated and a modified Fishers exact test (EASE score) used to assign significance. Finally, DAVID software can be used to cluster genes within a list based on similar functional annotation terms.

3.3.3.1.1 Gene ontology analysis

113 probesets represented genes encoding hypothetical proteins with no associated gene ontology terms. These probesets could not be included in the analysis, but may represent novel genes which are important in the response to ivermectin. **Fig. 3-4** and **3-5** represent ontology terms associated with a minimum of two genes and with an associated EASE score (p-value) of ≤0.1, a total of 99 genes. The terms are listed in order of the calculated significance of enrichment. The pie charts represent the actual number of genes associated with each of the ontology terms.

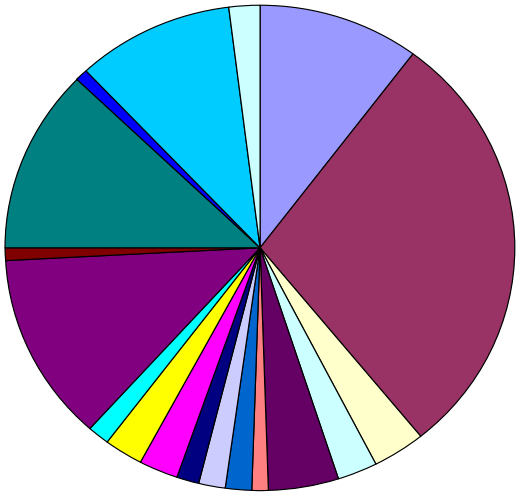
Fig. 3-4 represents the molecular function ontology terms. There is a significant enrichment of genes with *oxidoreductase activity*. This includes five cytochrome P450 genes, two flavin containing monooxygenases (FMO), two short chain dehydrogenase genes and an alcohol dehydrogenase, all of which could potentially be involved in xenobiotic metabolism. In addition, this term is also associated with three catalase genes, a fatty acid desaturase, a gamma butyrobetaine hydroxylase (potentially involved in carnitine biosynthesis) and a phytanol-CoA alpha-hydroxylase. These genes are all potentially involved in fatty acid breakdown and metabolism. The other molecular function ontology terms are essentially overlapping and represent the same genes.

Perhaps of more interest are the biological process ontology terms, **Fig. 3-5**. The most significantly enriched group are genes associated with the term *aging*, which includes *mtl-1, sodh-1, cyp-34A9* and *dod-3*. In addition, this group contains other down-stream targets of DAF-16 including catalase genes (*ctl-2, ctl-1*), a gut esterase (*ges-1)*, a fatty acid CoA synthetase gene (*acs-17*), a predicted isocitrate lyase/ malate synthase (*gei-7*) and an acylsphingosine amidohydrolase (*asah-1*), which may all be involved in fatty acid metabolism pathways. The terms *generation of precursor metabolites and energy*; *metabolic process*; *organic acid metabolic process*; *carboxylic acid metabolic process* and *catabolic process* all include genes potentially involved in lipid breakdown. Overall, there does not appear to be enrichment of terms that could be specifically associated with xenobiotic metabolism pathways. The analysis suggests that the nematodes are undergoing a stress response associated with an increase in lipid catabolism. Importantly, assessment of microarray data and

confirmatory real-time QPCR (**Fig. 3-3**), suggest that this is not a general stress response, as there is no significant up-regulation of heat shock proteins (*hsp-16.1, hsp-16.49, hsp-70*), stress associated glutathione-s-transferases (*gst-1, gst-4, gst-38*) or other stress associated genes (*sip-1, hsf-1*).

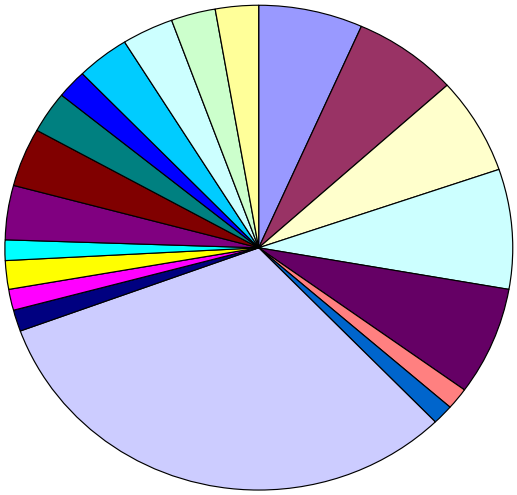
The only cellular component ontology terms associated with more than two genes and with an EASE score < 0.1 were: *Intrinsic to endoplasmic reticulum membrane* and *microsome and vesicular fraction*. These terms were associated with only two genes: *fmo-1* and *fmo-2*.

Increasing the number of annotation terms to include protein domains (INTERPRO, PIR_SUPERFAMILY, SMART), KEGG pathways and functional categories (COG_ONTOLOGY, SP_PIR_KEYWORDS, UP_SEQ_FEATURE), in addition to GOterms, did not significantly increase the number of genes annotated. The sole KEGG pathway term to be significantly enriched was f*atty acid metabolism*. This term was associated with five genes: F54F3.4, *acs-2*, *sodh-1*, *acs-17* and F58F9.7.



Molecular function Ontology Terms	p-val
oxidoreductase activity (26)	6.02E-08
catalytic activity (72)	1.12E-06
monooxygenase activity (9)	2.92E-05
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (6)	2.15E-04
iron ion binding (11)	4.87E-04
catalase activity (3)	8.12E-04
peroxidase activity (4)	4.65E-03
oxidoreductase activity, acting on peroxide as acceptor (4)	4.65E-03
antioxidant activity (4)	1.07E-02
heme binding (6)	2.47E-02
tetrapyrrole binding (6)	2.47E-02
hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds (4)	3.35E-02
metal ion binding (31)	4.75E-02
flavin-containing monooxygenase activity (2)	5.75E-02
ion binding (31)	6.39E-02
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NADH or NADPH as one donor, and incorporation of one atom of oxygen (2)	6.86E-02
transition metal ion binding (25)	7.89E-02
coenzyme binding (5)	9.52E-02

Figure 3-4: Molecular function ontology terms associated with genes up-regulated in response to exposure of DA1316 to 1µg/ml (1.14µM) ivermectin for 4hrs.
Terms are listed in order of significance as assessed by EASE score. The absolute number of genes associated with each term are shown in brackets and in the pie chart.



Biological Process Ontology Terms	p-val
Aging (14)	7.78E-08
multicellular organismal aging (14)	7.78E-08
determination of adult life span (14)	7.78E-08
generation of precursor metabolites and energy (17)	5.15E-06
electron transport (15)	8.28E-06
hydrogen peroxide catabolic process (3)	7.41E-04
hydrogen peroxide metabolic process (3)	7.41E-04
metabolic process (67)	1.21E-03
response to reactive oxygen species (3)	1.82E-03
response to hydrogen peroxide (3)	1.82E-03
response to oxidative stress (4)	3.69E-03
oxygen and reactive oxygen species metabolic process (3)	7.68E-03
organic acid metabolic process (8)	1.24E-02
carboxylic acid metabolic process (8)	1.24E-02
response to chemical stimulus (6)	2.29E-02
monocarboxylic acid metabolic process (4)	3.37E-02
response to stress (7)	4.25E-02
catabolic process (7)	6.14E-02
nitrogen compound metabolic process (6)	7.09E-02
cellular catabolic process (6)	7.71E-02

Figure 3-5: Biological Process ontology terms associated with genes up-regulated in response to exposure of DA1316 to 1µg/ml (1.14µM) ivermectin for 4hrs.
Terms are listed in order of significance as assessed by EASE score. The absolute number of genes associated with each term are shown in brackets and in the pie chart.

3.3.3.1.2 Gene functional classification clustering reveals CYPs and UGTs to be up-regulated in response to ivermectin exposure

Clustering up-regulated genes based on similar functional annotation aids in the elucidation of important pathways induced by ivermectin exposure. Enrichment scores for each group are the log converted geometric mean of the p-values associated with each of the annotation terms in the cluster. These provide a guide as to the significance of these clusters, a score over 1.3 represents a significant enrichment.

The genes up-regulated in response to ivermectin exposure clustered into six groups. However, these clusters contained only a total of 33 genes, 221 genes were not clustered. **Table 3-5**, shows the top four clusters all of which had enrichment scores of greater than 1. There are two groups that could potentially be involved in xenobiotic metabolism. Cluster 1, enrichment score 5.12, contains a group containing five cytochrome P450 genes and three catalase genes. These share annotation terms relating to *oxidoreductase activity*, *ion binding* and *multicellular organismal aging*. The up-regulated *cyp* genes belong to the CYP4 (*cyp-37B1* and *cyp-32B1*) and CYP2 families (*cyp-34A9, cyp-34A4* and *cyp-33C7*) (www.wormbase.org; Gotoh, 1998). Both *cyp-34A9* and *cyp-33C7* have been shown to be up-regulated in dauer constitutive TGF-beta mutants (Liu *et al.*, 2004) Interestingly, there are no members of the *cyp-35* group (also CYP2 family), which have been associated in the response to many xenobiotics (Menzel *et al.*, 2005).

Cluster 3, enrichment score 1.35, contains a group of four putative UDP-glucuronosyl/ glucosyl transferases. These are important enzymes in phase II metabolism, functioning by conjugating glucuronosyl/ glucosyl groups to endogenous and exogenous compounds to aid in their excretion from the organism.

The remaining two clusters contain a group of putative transcription factors (cluster 2; enrichment score 2.09) and a final group sharing terms associated with their location in the *cell membrane* (cluster 4, enrichment score 1.23).

Functional group 1: Enrichment score 5.12		
Probeset	**Gene ID**	**Ontology**
173480_s_at	*cyp-32B1*	cytochrome P450
AFFX-Ce_catalase_M_s_at, 188587_s_at, AFFX-Ce_catalase_5_s_at	*ctl-3*	catalase
189457_at	*cyp-34A9*	cytochrome P450
189309_at	*cyp-33C7*	cytochrome P450
188687_s_at	*ctl-2*	catalase
189343_at	*cyp-34A4*	cytochrome P450
AFFX-Ce_catalase_M_s_at, AFFX-Ce_catalase_5_s_at, 188587_s_at	*ctl-1*	catalase
189221_at	*cyp-37B1*	cytochrome P450
173490_s_at	F09F7.7	KOG- 2-oxoglutarate and iron dependent dioxygenase related proteins
Functional group 2: Enrichment score 2.09		
Probeset	**Gene ID**	**Ontology**
193927_s_at	Y48A6B.7	cytidine deaminase
192333_at	*pqm-1*	paraquat responsive (transcription factor)
171737_x_at, 190566_at	T12G3.1	KOG- ZZ type Zn finger
189967_at	C06G3.6	KOG- ZZ type Zn finger
176141_s_at	Y58A7A.4	Uncharacterised
Functional Group 3: Enrichment score 1.35		
Probeset	**Gene ID**	**Ontology**
176453_at	*ugt-31*	UDP-glucuronosyl/ glucosyl transferase
191053_at	*ugt-4*	UDP-glucuronosyl/ glucosyl transferase
191434_at	*ugt-54*	UDP-glucuronosyl/ glucosyl transferase
184602_at	*ugt-25*	UDP-glucuronosyl/ glucosyl transferase
Functional Group 4: Enrichment score 1.23		
Probeset	**Gene ID**	**Ontology**
173200_s_at	*inx-2*	innexin
186660_s_at	F46C5.1	uncharacterised
179396_at	C35A5.6	uncharacterised
188431_s_at	*dct-1*	DAF-16/FOXO controlled germline tumour affecting

Table 3-5: Gene functional classification of up-regulated genes following 4hrs exposure of DA1316 to 1µg/ml (1.14µM) IVM

3.3.3.2 DAVID analysis of down-regulated genes

217 probesets were down-regulated with a false discovery rate cut off of 10%, using rank products analysis. This represented a total of 192 genes that were analysed using DAVID software.

3.3.3.2.1 Gene ontology analysis

59 probesets represented genes with no annotation data. **Fig. 3-6** and **3-7** represent annotation terms associated with at least two genes in the list and an EASE score of ≤0.1, a total of 108 genes.

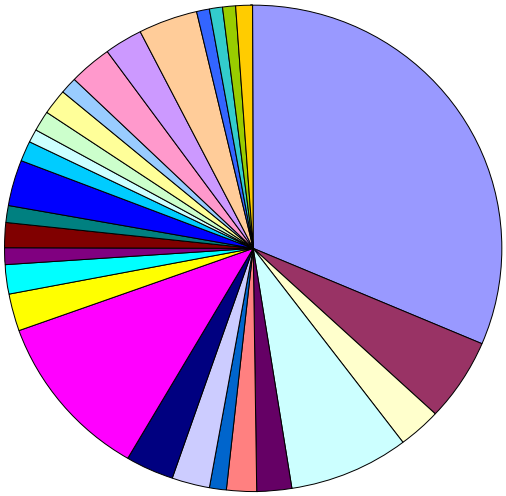
The most significantly down regulated molecular function annotation term is *catalytic activity*, **Fig. 3-6**. This term is associated with six UDP-glucuronosyl/glucosyl transferases, four glutathione-s-transferases, one cytochrome P450 and one short-chain dehydrogenase. More specific terms for each of these families, including *oxidoreductase* and *transferase activity*, are also significantly down-regulated. These represent gene families that would be expected to be up-regulated in a xenobiotic detoxification response.

In addition to XME gene families there is significant down-regulation of terms associated with lipid metabolism and biosynthetic processes. This is especially notable in the biological process ontology terms, **Fig. 3-7**. The most significantly enriched a term is *carboxylic acid metabolic process*, which is associated with the fatty acid desaturase genes *fat-5, fat-6* and *fat-7*; and several hypothetical proteins with acyl-CoA thioesterase, acyl-CoA dehydrogenase, acyl-CoA oxidase, glycine dehydrogenase KOGs. Several of these genes are also associated with *amino acid metabolic processes*. Genes involved in *lipid transport*, including vitellogenins, are down-regulated. *Carbohydrate metabolic processes*, exemplified by the UDP-glucuronosyl transferases *ugt-12, ugt-46*; the lysozyme genes *lys-5* and *lys-6; gale-1* (a putative UDP-galactose 4 epimerase) and *ger-1* (a putative GDP-keto-6-deoxymannose 3,5-epimerase/ 4-reductase) are also enriched in the down-regulated gene list.

Cellular component ontology terms associated with more than two genes in the down-regulated list were: *Cytoplasm and cytoplasmic part*, *endoplasmic*

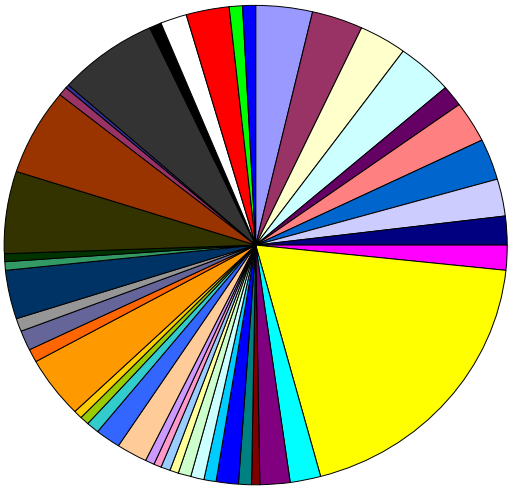
reticulum, *apical part of cell* and *apical plasma membrane*. These terms are associated with many of the genes involved in fatty acid metabolism listed above including the fatty acid desaturases *fat-5, fat-6* and *fat-7*. The terms *apical part of cell* and *apical plasma membrane* were both associated with the same two genes: *nhx-2*, a sodium/ proton exchanger, and *pep-2,* a peptide transporter. NHX-2 and PEP-2 are thought to be functionally coupled (Walker *et al.*, 2005). PEP-2 is thought to co-transport H^+ and peptides into the intestinal cells, whilst NHX-2 removes the H^+ to prevent excessive acidification of the cytoplasm. The expression of both of these genes was reduced in *daf-2* mutants (McElwee *et al.*, 2004). Decreased DAF-2 signalling is involved in formation of the long-lived, non-eating dauer stage.

As in **Section 3.3.1.1.1,** using protein domain, KEGG pathways and functional category annotation in addition to GOterms did not significantly increase the number of genes in the down-regulated list that were annotated. Three KEGG pathway terms were significantly enriched: Porphyrin and Chlorophyll Metabolism, 1- and 2- Methylnaphthalene degradation and Metabolism of Xenobiotics by Cytochrome P450s. These terms were associated with two UDP glucuronosyl transferases, two glutathione-s-transferases, a gene predicted to encode a short chain-type dehydrogenase and an alcohol dehydrogenase. The down-regulation of these pathways in response to ivermectin exposure is not consistent with a detoxification response.



Ontology term	p-val	Ontology term	p-val
catalytic activity (79)	2.69E-11	lipid transporter activity (3)	7.72E-03
cofactor binding (14)	5.90E-07	transferase activity, transferring glycosyl groups (8)	1.18E-02
oxidoreductase activity, acting on the CH-CH group of donors (7)	3.68E-05	glutathione transferase activity (3)	1.53E-02
oxidoreductase activity (20)	7.38E-05	lysozyme activity (2)	3.34E-02
acyl-CoA dehydrogenase activity (5)	2.20E-04	acyltransferase activity (4)	3.53E-02
acid phosphatase activity (5)	3.34E-04	exopeptidase activity (4)	3.68E-02
stearoyl-CoA 9-desaturase activity (3)	3.75E-04	carbon-oxygen lyase activity (3)	3.93E-02
FAD binding (6)	6.94E-04	iron ion binding (7)	5.21E-02
coenzyme binding (8)	1.33E-03	transferase activity, transferring hexosyl groups (6)	6.80E-02
transferase activity (28)	1.75E-03	peptidase activity (10)	6.94E-02
vitamin binding (6)	3.10E-03	copper ion TM transporter activity (2)	8.67E-02
pyridoxal phosphate binding (5)	3.13E-03	acyl-CoA oxidase activity (2)	8.67E-02
oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water (3)	4.30E-03	carbonate dehydratase activity (2)	9.70E-02
transferase activity, transferring alkyl or aryl (other than methyl) groups (4)	7.02E-03	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (3)	9.81E-02

Figure 3-6: Molecular function ontology terms associated with genes down-regulated following 4hrs exposure of DA1316 to 1µg/ml (1.14µM) IVM
Terms are listed in order of significance as assessed by EASE score. The absolute number of genes associated with each term are shown in brackets and in the pie chart.



Ontology term	p-val	Ontology term	p-val
carboxylic acid metabolic process (13)	1.34E-05	amine metabolic process (7)	2.70E-02
organic acid metabolic process (13)	1.34E-05	nitrogen compound metabolic process (7)	3.14E-02
cellular lipid metabolic process (11)	4.15E-05	lipid glycosylation (3)	3.34E-02
lipid metabolic process (13)	1.13E-04	purine base metabolic process (2)	3.57E-02
fatty acid metabolic process (6)	2.37E-04	peptidoglycan catabolic process (2)	3.57E-02
multicellular organismal aging (10)	2.70E-04	biosynthetic process (15)	3.63E-02
determination of adult life span (10)	2.70E-04	ion homeostasis (3)	3.87E-02
Aging (10)	2.70E-04	cofactor metabolic process (6)	4.08E-02
aromatic compound metabolic process (7)	3.37E-04	lipid modification (3)	5.33E-02
monocarboxylic acid metabolic process (6)	9.31E-04	cellular biosynthetic process (12)	5.36E-02
metabolic process (71)	1.29E-03	aromatic amino acid catabolic process (2)	5.88E-02
amino acid metabolic process (7)	6.31E-03	aromatic compound catabolic process (2)	5.88E-02
amino acid and derivative met. process (7)	1.26E-02	positive regulation of growth rate (21)	5.93E-02
transition metal ion transport (3)	1.74E-02	regulation of growth rate (21)	6.06E-02
lipid transport (3)	1.74E-02	nucleobase biosynthetic process (2)	7.01E-02
lipid biosynthetic process (5)	2.14E-02	peptidoglycan metabolic process (2)	7.01E-02
organic acid biosynthetic process (3)	2.37E-02	positive regulation of growth (23)	7.74E-02
fatty acid biosynthetic process (3)	2.37E-02	heterocycle metabolic process (3)	7.99E-02
carboxylic acid biosynthetic process (3)	2.37E-02	carbohydrate metabolic process (7)	8.09E-02
nutrient import (2)	2.39E-02	Proteolysis (10)	8.10E-02
long-chain fatty acid biosynthetic process (2)	2.39E-02	response to unfolded protein (3)	9.44E-02
purine base biosynthetic process (2)	2.39E-02	response to protein stimulus (3)	9.44E-02
long-chain fatty acid metabolic process (2)	2.39E-02		

Figure 3-7: Biological process ontology terms associated with down-regulated genes following 4 hours exposure of DA1316 to 1µg/ml (1.14µM) IVM
Terms are listed in order of significance as assessed by EASE score. The absolute number of genes associated with each term are shown in brackets and in the pie chart.

3.3.3.2.2 Gene functional classification reveals transferases and fatty acid elongases to be down-regulated following ivermectin exposure

Functional classification of the down-regulated genes confirms the decrease in transferase activities following ivermectin exposure, **Table 3-6**. The down-regulated genes contain a cluster of UDP-glucuronosyl transferases and a cluster of glutathione-s-transferases (cluster 2, enrichment 2.93; and cluster 3, enrichment 2.63, respectively). Members of the UGT family may perform constitutive functions, in addition to being involved in xenobiotic detoxification. The current data would suggest that certain pathways utilising glucuronidation/ glucosylation are up-regulated (*ugt-31, ugt-4, ugt-54* and *ugt-25*) and others down-regulated (*ugt-16, ugt-12, ugt-63* and *ugt-22*) in response to ivermectin exposure. It is possible that the UGTs within each group are involved in common pathways and further investigation of their promoter regions may provide a useful insight into their regulation.

Cluster 1, enrichment score 3.35, represents genes associated with fatty acid metabolism. Interestingly, all of these genes have been shown to be down-regulated during short-term fasting (Van Gilst *et al.*, 2005b). Reduction in the fatty acid elongase genes *elo-2, elo-5* and *elo-6* and the vitellogenin genes is consistent with a shift from fat storage to fat breakdown which would be expected in a fasting situation.

Functional group 1: Enrichment score 3.35		
Probeset	**Gene ID**	**Ontology**
190156_s_at	C48B4.1	KOG-Acyl-CoA oxidase
194059_at	F08A8.2	uncharacterised
188822_at	*acdh-1*	Acyl CoA dehydrogenase
190705_s_at	*acdh-2*	Acyl CoA dehydrogenase
187495_s_at	*acdh-9*	KOG- medium-chain acyl-CoA dehydrogenase
Functional group 2: Enrichment score 2.93		
Probeset	**Gene ID**	**Ontology**
191418_at	*ugt-16*	UDP-glucuronosyl/ glucosyl transferase
191778_s_at	*ugt-12*	UDP-glucuronosyl/ glucosyl transferase
190744_at	*ugt-63*	UDP-glucuronosyl/ glucosyl transferase
193604_at	*ugt-22*	UDP-glucuronosyl/ glucosyl transferase
Functional Group 3: Enrichment score 2.62		
Probeset	**Gene ID**	**Ontology**
192407_at	*gst-4*	putative glutathione requiring prostaglandin D synthase
187084_at, 187085_s_at	*gst-10*	glutathione-s-transferase
191393_s_at	*gst-27*	glutathione-s-transferase
191431_at	*gst-26*	glutathione-s-transferase
Functional Group 4: Enrichment score 2.39		
Probeset	**Gene ID**	**Ontology**
176872_at	*vit-3*	vitellogenin structural gene
187318_at	*lon-1*	PR-protein superfamily
171723_x_at, 172134_x_at	*vit-4*	vitellogenin structural gene
177065_at	*vit-1*	vitellogenin structural gene
Functional Group 5: Enrichment score 1.71		
Probeset	**Gene ID**	**Ontology**
189345_at	*pho-13*	intestinal acid phosphatase
177183_s_at	*acp-6*	acid phosphatase family
175238_s_at, 182487_s_at	*pho-1*	intestinal acid phosphatase
191091_at	*pho-7*	intestinal acid phosphatase
Functional Group 6: Enrichment score 1.25		
Probeset	**Gene ID**	**Ontology**
186362_s_at	*glf-1*	UDP-galactopyranose mutase
177190_at, 172177_x_at	Y71H2AL.1	KOG- Ca^{2+}/ calmodulin-dependent protein phosphatase
186757_s_at	F42A8.1	Uncharacterised
190067_at	F09F7.4	KOG- enoyl CoA hydratase
189019_at	C31E10.7	KOG- cytochrome B5
194203_x_at, 177650_at	*lpr-3*	Lipocalin- related protein
191276_s_at	*elo-5*	polyunsaturated fatty-acid elongase
184144_at	R193.2	uncharacterised
173725_s_at	*elo-6*	polyunsaturated fatty acid elongase
180055_at	ZC328.1	uncharacterised
189318_at	*elo-2*	palmitic acid elongase

Table 3-6: Gene functional classification of down-regulated genes following 4 hours exposure of DA1316 to 1µg/ml (1.14µM) IVM

3.3.3.3 Global analysis summary

Global analysis suggests that the response of *C. elegans* to 4hrs exposure to 1µg/ml (1.14µM) ivermectin is predominated by an up-regulation of genes involved in lipid catabolism and gluconeogenesis and a down-regulation of lipid biosynthesis and carbohydrate metabolism. This is consistent with a change in metabolic profile to use stored energy as would be expected in the fasting response. Van Gilst *et al.* (2005b) used real-time QPCR to investigate the response of 97 fat and glucose metabolism genes in response to fasting at all life stages over a period of 12hrs. 39 genes were found to have altered expression levels in one or more life stages and 18 were consistently altered in all stages. Changes in the level of expression of these genes were noted as soon as 30min after the withdrawal of food. The $\log_2$ FC of these 18 genes following exposure to ivermectin is presented in **Fig. 3-8**. There is excellent correlation in the response of these genes following fasting and upon exposure to ivermectin.

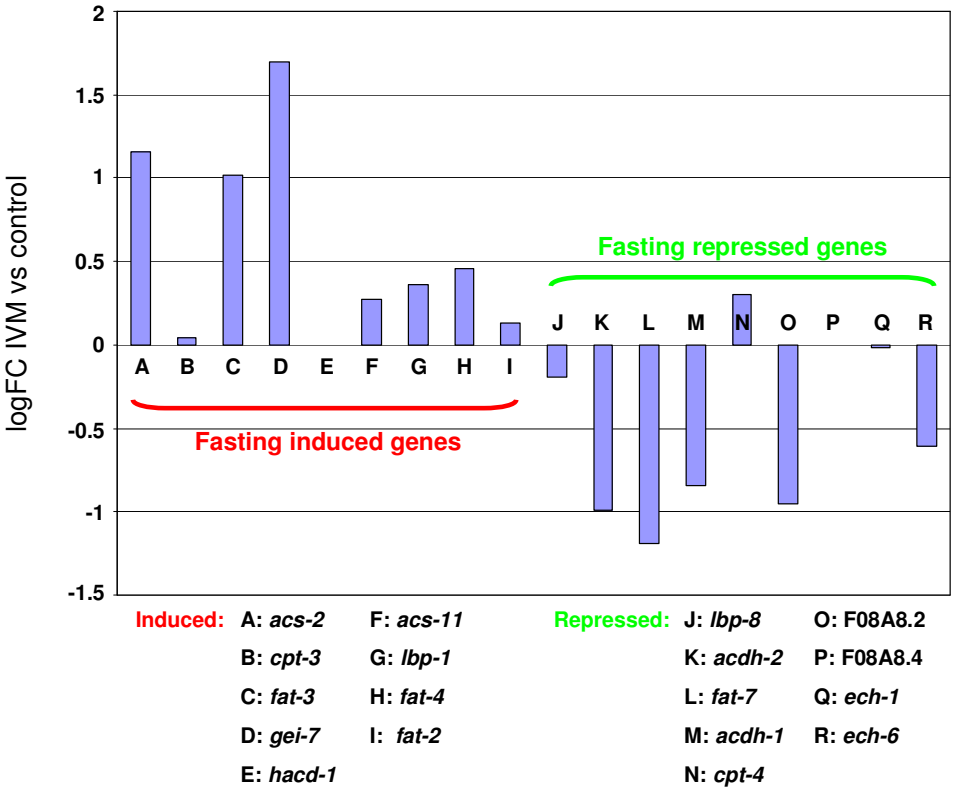


Figure 3-8: Fasting response genes change in expression following 4hrs exposure of DA1316 to1µg/ml (1.14µM) IVM.
The effect of ivermectin exposure on the expression level of 18 genes known to be responsive to fasting (van Gilst *et al.*, 2005) was assessed. In general genes that were shown to be induced by fasting were also induced following exposure to ivermectin and fasting repressed genes were also repressed by ivermectin exposure.

3.3.4 Pharyngeal pumping rate of strain DA1316 is reduced upon exposure to 1µg/ml IVM

Given the large number of genes whose alteration in expression intensity is consistent with a fasting response, it was important to more fully evaluate the phenotype of the DA1316 strain when exposed to 1µg/ml (1.14µM) IVM. Dent *et al*. (2000) report that the glutamate-gated chloride channel triple mutant rests in a slightly starved state, but that it is resistant to ivermectin doses of up to 5µM (4.5µg/ml) IVM. However, resistance was measured as the ability of synchronised eggs to reach adulthood, over a period of 2 weeks, on ivermectin-containing plates. After 4hrs exposure on NGM plates containing 1µg/ml (1.14µM) IVM, DA1316 was clearly phenotypically affected compared to controls, showing decreased movement. Of more interest, given the microarray results, was the response of the pharynx after 4hrs at this concentration of drug. **Fig. 3-9** demonstrates the pharyngeal pumping rate of both DA1316 and N2 worms after 4hrs exposure to a gradient of ivermectin concentrations. After 4hrs at 1µg/ml (1.14µM) IVM strain DA1316 has a significantly reduced pharyngeal pumping rate. This is contrary to the recent report by Ardelli *et al*. (2009) who saw no effect on pharyngeal pumping rate of an *avr-14*, *avr-15*, *glc*-1 triple mutant 2.5 hours after exposure to 5µM IVM.

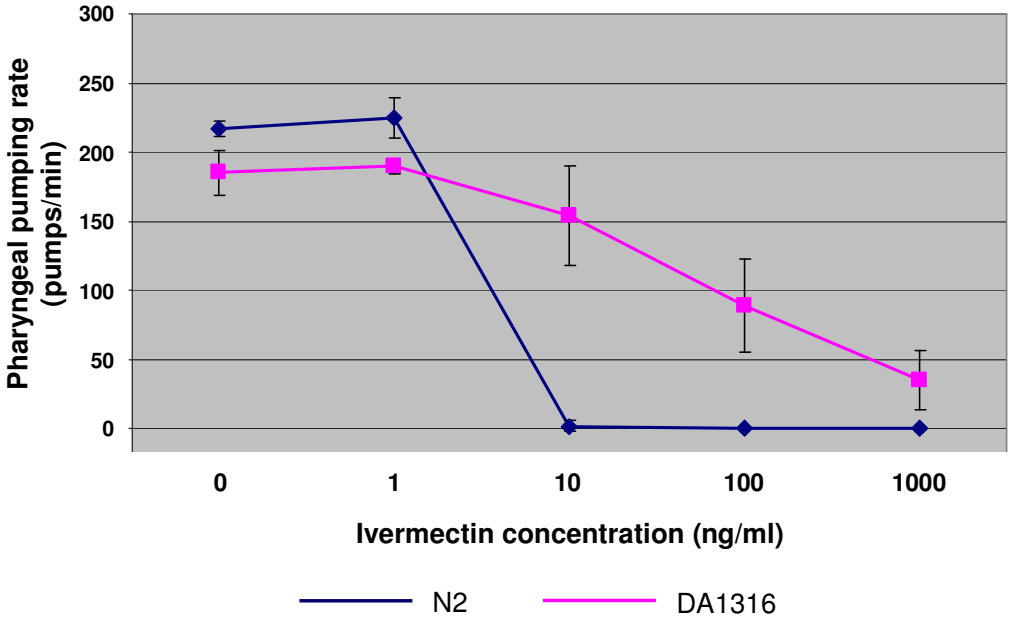
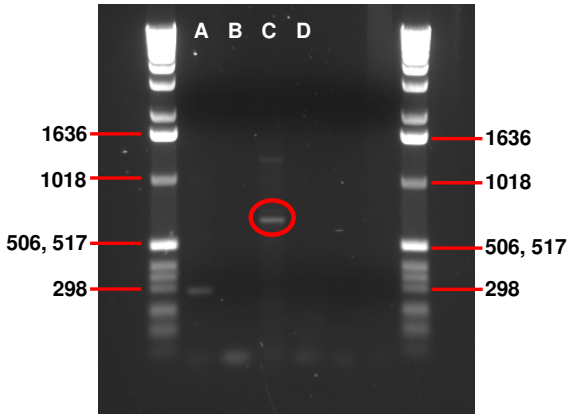


Figure 3-9: Pharyngeal pumping rate following 4hrs exposure of DA1316 and N2 to 1µg/ml (1.14µM) IVM.
Whilst strain DA1316 is more resistant to the effect of ivermectin on pharyngeal pumping, at concentrations greater than 100ng/ml (114nM) the pharyngeal pumping rate is significantly reduced.

3.3.5 avr-15 is wild-type in strain DA1316

Strain DA1316 did not appear to be responding to ivermectin in the manner reported by both Dent *et al*. (2000) and Ardelli *et al* (2009). Therefore, the three putative mutations resulting in the triple mutant phenotype were analysed using a combination of PCR diagnosis and PCR sequencing. The point mutation *avr-14*(*ad1302)* and the transposon insertion *glc-1(pk54)* were present as expected. However, the point mutation *avr-15(ad1051)* was not (**Fig. 3-10** to **3-12**). *avr-15* is thought to be a major subunit in post-synaptic glutamate-gated chloride channels at the neuromuscular junction of the pharynx (Dent *et al.*, 1997). The fact that this subunit appeared wild-type in the strain received from the CGC may explain why the pharynx of DA1316 is sensitive to ivermectin. It should be noted that wild-type *avr-15* was present in two separate batches of DA1316 received from the CGC.



1.2% agarose gel in TAE

100V, 90 min

A: 292bp fragment of *avr-14* (positive control).

B: No template control of A.

C: 666bp product of *glc-1* amplified from strain DA1316 confirming the presence of a Tc1 transposon insertion.

D: No template control for C.

Figure 3-10: PCR confirming the presence of glc-1(pk54::Tc1) in strain DA1316

```
                    351                                                 400
DA1316 seq      (1) ----------------------------------------CTGAA-CGG
    avr-14    (351) TTTGAAAACTGTGAAAATACGGTTCTTGAAAATTTACTTTGCTGAATCGG
consensus                                                   ***** ***

                    401                                                 450
DA1316 seq      (9) CAGGTTCAGGAGTTTTTTCTGTAAGAAATTGAGTTTTGCACTTGAAAAAC
    avr-14    (401) CAGGTTCAGGAGTTTTTTCTGTAAGAAATTGAGTTTTGCACTTGAAAAAC
consensus       ************************************************************

                    451                                                 500
DA1316 seq     (59) CACTTATTGCATAATCCCAAAAAAGTATTTCAAAAGATTTTTGACCCTTG
    avr-14    (451) CACTTATTGCATAATCCCAAAAAAGTATTTCAAAAGATTTTTGACCCTTG
consensus       ************************************************************

                    501                                                 550
DA1316 seq    (109) AAAATTTGCAAACCTTGGCAAAAATACAAATTTAAACTTCTATTATGACA
    avr-14    (501) AAAATTTGCAAACCTTGGCAAAAATACAAATTTAAACTTCTATTATGACA
consensus       ************************************************************

                    551                                                 600
DA1316 seq    (159) GAATTAATTCAAAAACTAAACTATCAGATACCGGTGGACCCGTTCTAGAG
    avr-14    (551) GAATTAATTCAAAAACTAAACTATCAGATACCGGTGGACCCGTTCTAGTG
consensus       ************************************************************

                    601                                                 650
DA1316 seq    (188) ACAGTAAACATTTATCTTCGATCAATATCAAAAATTGATGACGTAAATAT
    avr-14    (601) ACAGTAAACATTTATCTTCGATCAATATCAAAAATTGATGACGTAAATAT
consensus       ************************************************************

                    651                                                 700
DA1316 seq    (188) GGAATACAGTGCTCAATCCACATTCAGGAAGTC----------------
    avr-14    (651) GGAATACAGTGCTCAATTCACATTCAGAGAAGAATGGACAGATCAAAGAT
consensus       ****************************
```

Figure 3-11: Sequence of *avr*-14*(ad1302)* locus of strain DA1316
The red box highlights the T to A substitution expected in mutant strain DA1316

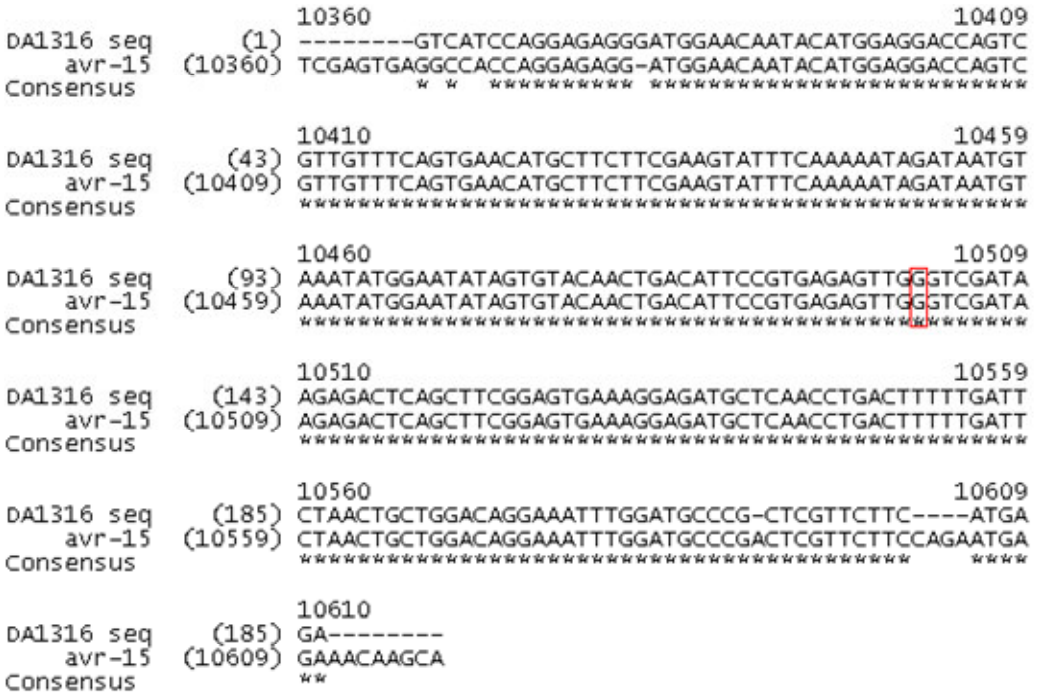


Figure 3-12: Sequence of *avr-15(ad1051)* locus of strain DA1316
The red box highlights where the G-A substitution should be in strain DA1316. However no mutation is present.

3.3.6 Comparison to dauer data and axenic culture

Axenic culture has been proposed to result in a change in lifestyle of *C. elegans*, resulting in a decrease in energy storage and slowing of growth rate (Castelein *et al.*, 2008; Szewczyk *et al.*, 2006). Ivermectin causes a decrease in pharyngeal pumping and the microarray analysis suggests that pathways involved in energy metabolism and storage are affected by ivermectin exposure. Therefore, significant overlap may be expected between the transcriptomes of nematodes grown in axenic culture and those exposed to ivermectin. Szewczyk *et al.* (2006) carried out microarray analysis of *Caenorhabditis elegans* grown in a defined axenic culture system and on *E. coli* seeded NGM plates. They defined a subset of 22 genes that were reliably up-regulated in nematodes grown in axenic culture. Most of these genes were uncharacterised, but the list included several genes involved in heavy metal response. Comparison of these data to the microarray data generated in the current study revealed that *mtl-1* was the only gene considered to be significantly up-regulated in axenic medium that was also significantly up-regulated in response to ivermectin.

The dauer larvae of *Caenorhabditis elegans* is a stress resistant, hypobiotic stage of the nematode. Dauers do not feed and it is possible that many of the pathways up-regulated in response to ivermectin induced pharyngeal paralysis in L4 worms may also be enriched in dauers compared to non-dauer L4 larvae. Wang *et al.* (2003) used microarray analysis to compare the transcriptomes of dauer worms to those that have been exposed to food for 12 hours and were exiting dauer stage. The analysis made use of 2 colour spotted arrays and each chip compared RNA derived from dauers at various stages of exit to a reference pool of RNA derived from mixed stage N2 worms. For this reason re-analysis of the data in a similar manner to the method used in the current study was not possible. Therefore, a comparison was drawn between the ivermectin responsive and dauer transcriptomes based on fold change alone (greater than 2-fold up or down-regulation) and is presented in **Fig. 3-13**. The number of genes differentially expressed between dauers and non-dauers is much larger than that between IVM exposed and unexposed. This is to be expected as the dauer stage represents a physiologically specialised life-stage that must resist long-term fasting, over a period of months, and associated metabolic stress (Riddle *et al.*, 1981). In the current study nematodes were exposed to ivermectin for 4hrs and other than a decrease in pharyngeal pumping rate were largely resistant to the effects of the drug. However, as can be seen, a significant number of the genes up-regulated in response to ivermectin exposure were also up-regulated in the dauer stage. An overlap of no more than ten genes would be expected by chance, but 64 genes were up-regulated in both experiments[1]. Therefore, many of these genes may represent a response to the fasting induced by ivermectin's effect on the pharynx.

[1] No. of genes expected to overlap by chance (Troemel *et al.*, 2006) =

$$\frac{\text{No. of genes up-regulated in current study}}{\text{No. of genes assessed by microarray (22150)}} \times \text{No. genes up-regulated in Wang (2003)}$$

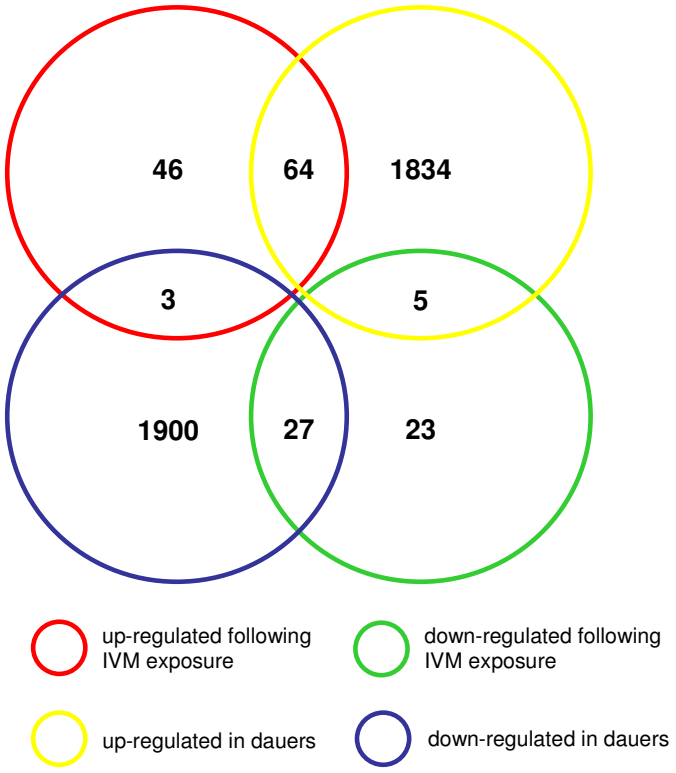


Figure 3-13: Comparison of genes enriched in dauers and those up-regulated in response to 4hrs exposure to 1µg/ml (1.14µM) IVM
Many of the genes enriched in the dauer stage are also up-regulated in response to ivermectin exposure. An overlap of ten genes up-regulated in both experiments would be expected by chance, but 64 are seen to be up-regulated in both IVM exposed and dauer stage larvae.

3.3.7 N2 exposure to 100ng/ml IVM for 4 hours results in an overlapping but distinct gene set compared to DA1316 exposed to the same dose

It is likely that many of the genes shown to be up-regulated in response to exposure of DA1316 to 1µg/ml (1.14µM) IVM are in fact genes up-regulated in response to fasting. However, some of the up-regulated genes may also be directly involved in detoxification pathways to eliminate ivermectin from the nematode. In order to identify candidate genes, microarray experiments were carried out using wild type *Caenorhabditis elegans*. Nematodes were exposed to 100ng/ml (114nM) IVM in an identical manner to the DA1316 experiments at the same dose. Phenotypically, wild-type *C. elegans* are completely paralysed after 4 hours exposure to this dose (data not shown) and pharyngeal pumping is

completely abolished (**Fig. 3-9**). Therefore, it was expected that genes involved in a fasting/ stress response would be more intensely up-regulated compared to the DA1316 strain, but that genes up-regulated in an ivermectin dose dependent manner would be up-regulated to a smaller degree.

In total, three biological replicates were sent for analysis and no chips were dropped following quality assurance. Analysis of the results using an empirical Bayesian t-test and Benjamini-Hochberg correction for false discovery rate revealed there to be no significantly changes in expression. Re-analysis using the rank products algorithm revealed fifteen probesets, equivalent to ten genes, to be significantly up-regulated and eight probesets to be significantly down-regulated (FDR <10%), see accompanying CD. The top 10 up-regulated genes are outlined in **Table 3-7**. The fact that so few probesets showed significantly altered expression in this comparison is remarkable given the dramatic phenotypic differences between the drug-exposed and control groups.

The small number of probesets showing significant changes in expression level meant that DAVID analysis was not undertaken. However, comparing the list of up-regulated genes in this experiment to those in the DA1316 100ng/ml (114nM) and 1µg/ml (1.14µM) IVM experiments revealed a subset of genes that were up-regulated in both DA1316 experiments but not in the wild-type nematode experiments (**Fig. 3-14**).

In total there were ten genes up-regulated in both of the DA1316 microarray experiments, but not in the wild-type experiment, see **Fig. 3-14**. These included *mtl-1, scl-2* and *cyp-37B1*, all of which are in within the top 10 up-regulated genes in the DA1316, 1µg/ml (1.14µM) IVM microarray experiment. In the wild-type experiment the $\log_2$ fold changes of these genes were 0.03, 1.12 and 0.64 respectively. In contrast, the $\log_2$ fold changes in the 100ng/ml (114nM) IVM experiment using strain DA1316 were 1.59, 1.31 and 1.27 respectively. The reason for the greater fold-change of *cyp-37B1* and *mtl-1* in strain DA1316 compared to N2 following exposure to 100ng/ml (114nM) IVM is unknown, but may be related to changes in gene regulation following the complete paralysis induced in N2 or due to strain differences. However, these three genes appear to be up-regulated in an IVM dose-responsive manner as the fold changes are

much higher in the 1µg/ml (1.14µM) IVM experiment. Therefore, *mtl-1*, *scl-2* and *cyp-37B1* were initially investigated further.

Probeset	Gene ID	Log_2 FC	BH FDR	RP FDR	Ontology
173729_at	T22F3.11	2.26	0.999807	0	permease of major facilitator family KOG
186971_at	C23G10.11	2.01	0.999807	0	uncharacterised
173550_at	F45D3.4	1.80	0.999807	0	uncharacterised
173558_at	ZC443.3	1.69	0.999807	0	uncharacterised
179272_at	C06B3.6	1.56	0.995349	0	uncharacterised
191581_at	B0564.3	1.54	0.944618	0	bestrophin- KOG
184913_s_at	T22F3.11	1.51	0.999807	0	permease of major facilitator family KOG
178900_s_at	F45D3.4	1.35	0.999807	0.076923	uncharacterised
186660_s_at	F46C5.1	1.32	0.999807	0.054545	uncharacterised
192181_at	T28H10.3	1.29	0.999807	0.071429	asparaginyl peptidase-KOG

Table 3-7: Top 10 up-regulated genes based on fold change following 4hrs exposure of N2 to 100ng/ml (114nM) IVM

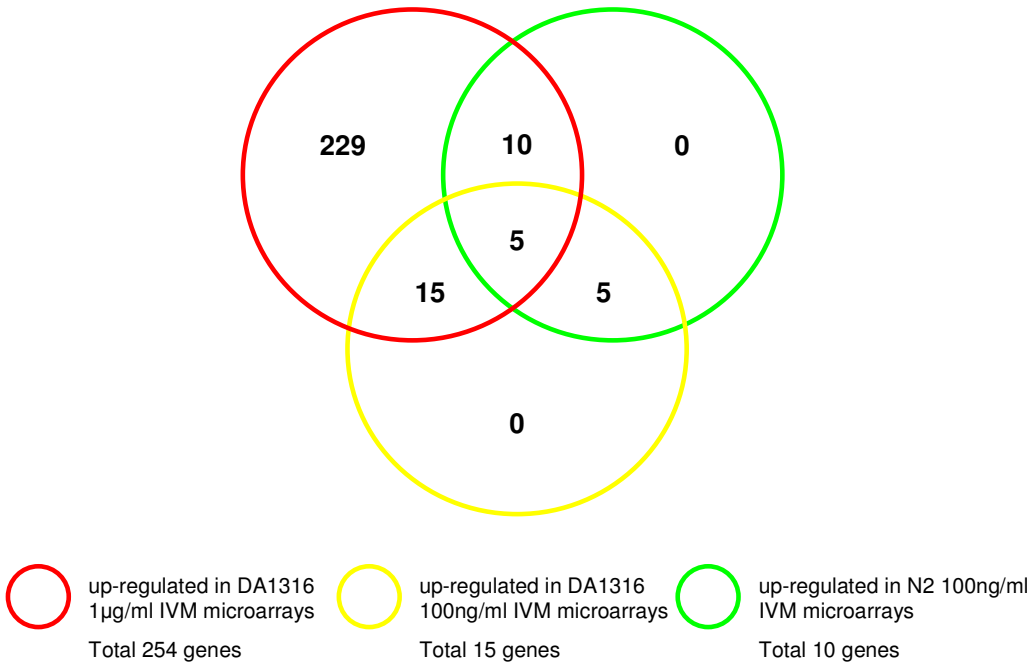


Figure 3-14: Comparison of up-regulated genes in all acute IVM response experiments There are a total of ten genes that are significantly up-regulated in both the DA1316 experiments, but not in the wild-type experiment. These were: *cyp-37B1, mtl-1, scl-2,* C35C5.8, C50F7.5, F09F7.6, F21C10.10, F53A9.8, F54F3.3 and T12D8.5.

3.3.8 cyp-37B1, scl-2 and mtl-1 are up-regulated in an ivermectin dose-dependent manner

1µg/ml (1.14µM) represents an extremely high concentration of ivermectin that parasitic nematodes are unlikely to come into contact with. In order to assess the response of *C. elegans* to more physiologically relevant concentrations of ivermectin, a concentration gradient experiment was designed. Strain DA1316 was exposed to ivermectin concentrations from 1-1000ng/ml (1.14-1140nM) in an identical manner to previous microarray and RT-QPCR replicates. Ivermectin (Sigma, I8898) was used and all groups, including controls, contained an identical volume of DMSO. Real-time QPCR was used to assess the fold induction of the candidate ivermectin specific genes: *mtl-1, scl-2 and cyp-37B1*. The results are summarised in **Fig. 3-15**.

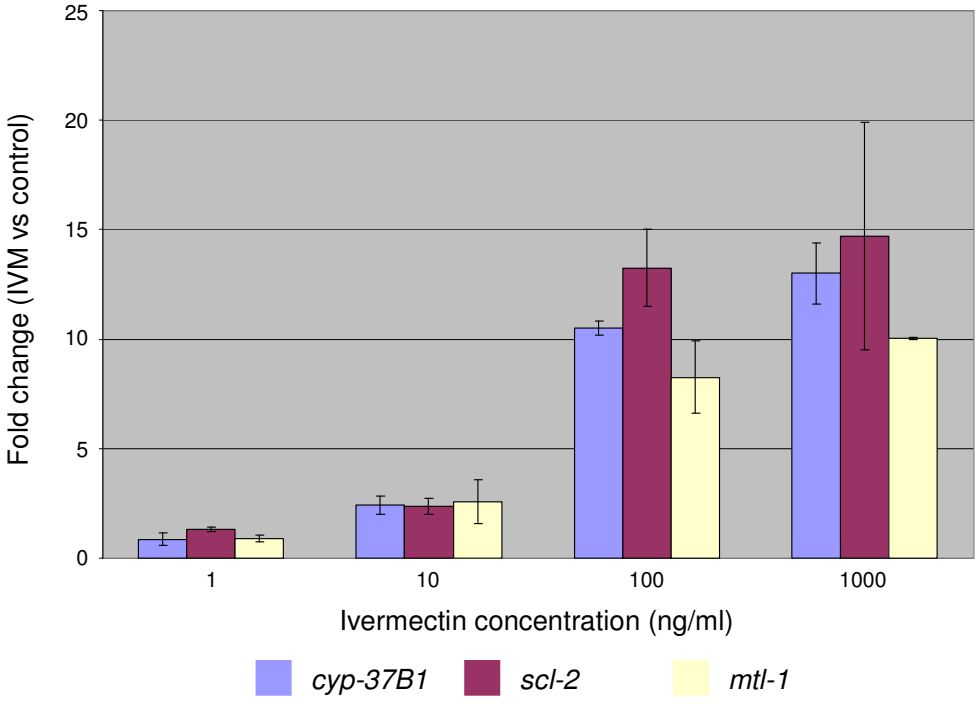


Figure 3-15: Up-regulation of *cyp-37B1, mtl-1* and *scl-2* in response to 4hrs exposure to varying concentrations of ivermectin
Up-regulation of the genes of interest appears to occur in a dose-dependent manner.

There is no apparent induction of any of the three genes assessed at 1ng/ml (1.14nM) ivermectin, but moderate induction is seen at 10ng/ml (11.4nM) IVM: *cyp-37B1* 2.4-fold increase; *scl-2* 2.37-fold increase; *mtl-1* 2.59-fold increase.

Large fold changes are seen at both 100ng/ml (114nM) and 1µg/ml (1.14µM) IVM. The fold changes of each gene are slightly different than those found when using Virbamec as the source of ivermectin. This likely reflects the different source of drug used.

3.3.9 GFP expression of cyp-37B1, scl-2 and mtl-1

In order to assess the possible function of the candidate genes, transcriptional GFP reporter constructs were created for *cyp-37B1, scl-2* and *mtl-1*, and assessed in a DA1316 background. Between three and five separate transgenic lines carrying extrachromasomal arrays were created and assessed for each reporter construct.

The expression pattern of *mtl-1* has previously been evaluated by other groups and was included here as validation of the PCR fusion technique used to create the reporter constructs. All three of the genes investigated showed intense GFP expression in the gut (**Fig. 3-16** to **3-18**), which is the proposed site for detoxification in nematodes (McGhee, 2007). *mtl-1* also showed expression in the terminal bulb of the pharynx (**Fig. 3-16**), as has been reported previously (Cui *et al.*, 2007; Freedman *et al.*, 1993). The fact that constitutive intestinal expression was observed for this reporter was unusual. Previous authors have reported that whilst expression in the terminal bulb of the pharynx was constitutive, intestinal expression was observed only after induction with heavy metals. This may represent a difference in regulation of this gene within the DA1316 strain as compared to wild-type or may simply be due to the fact that a transcriptional rather than translational reporter was used in this study.

The transcriptional reporter for *cyp-37B1* showed expression in two cells in the tail region in addition to the intestinal cells (**Fig. 3-17**). These were assumed to be the phasmid neurons. These are proposed to be chemosensory neurons involved in avoidance of noxious chemical stimuli (Bargmann, 2006). As can be seen in **Fig. 3-18**, expression of the *scl-2* transcriptional reporter was confined to the intestinal cells.

It was noted that *Caenorhabditis elegans* carrying reporter construct transgenes of each of the three genes of interest showed increased fluorescence on starved

plates compared to those with an excess of food. This suggested that the transcription of the genes was inducible. However, it also raised the possibility that these genes were not specific to ivermectin exposure and may be induced purely as a result of the phenotype of drug exposed nematodes. Attempts were made to quantify the induction of fluorescence using Image J analysis (http://rsbweb.nih.gov/ij/index.html) and an ELISA plate reader technique (Fluorostar software). However, the GFP reporter was found to be unstable in all of the transgenic lines created, other than that for *mtl-1,* and GFP fluorescence, both constitutive and following starvation and ivermectin exposure, diminished to a point where accurate quantification was not possible.

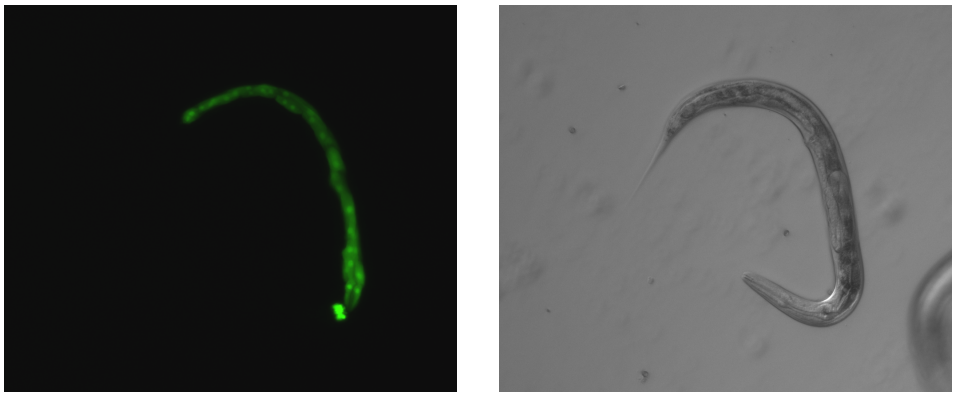


Figure 3-16: *mtl-1* GFP reporter (Genotype *[pRF4{rol-6(su-1006)}+mtl-1::GFP]; avr-14(ad1302); glc-1(pk54)*)

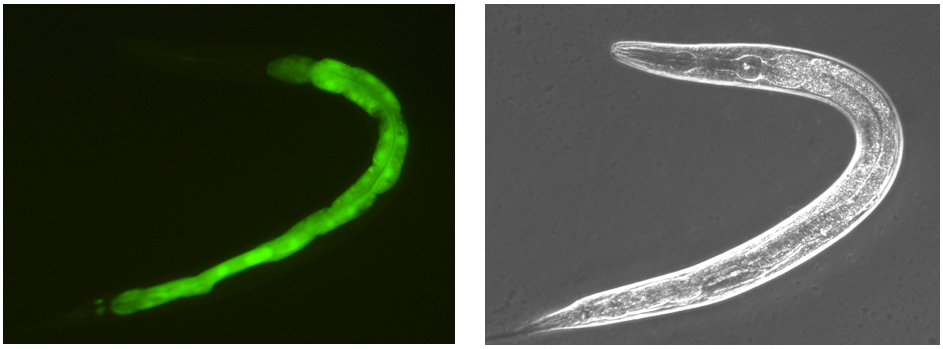


Figure 3-17: *cyp-37B1* GFP reporter (Genotype *[pRF4{rol-6(su-1006)}+cyp-37B1::GFP]; avr-14(ad1302); glc-1(pk54)*)

Figure 3-18: *scl-2* GFP reporter (Genotype *[pRF4{rol-6(su-1006)}+scl-2::GFP]; avr-14(ad1302); glc-1(pk54)*)

3.3.10 *cyp-37B1, mtl-1 and scl-2 are up-regulated in response to fasting in both DA1316 and N2 strains*

In order to assess the response of the genes of interest to fasting, real-time QPCR experiments were designed to compare the up-regulation of *cyp-37B1, scl-2* and *mtl-1* in response to 4hrs fasting and 4hrs ivermectin exposure. Synchronised L1 larvae of strain DA1316 or N2 were grown on standard NGM plates for 53hrs and 40hrs respectively (i.e. until they reached L4 stage). The larvae were then divided equally between three groups: ivermectin with food source (1µg/ml [1.14µM] for DA1316 and 100ng/ml [114nM] for N2), control plates with food source and control plates with no food source (fasting group). After 4hrs under these conditions worms were harvested and RNA extracted as previously described. Real time-QPCR was used to compare the change in expression of several genes of interest under the two experimental conditions. Two biological replicates were used in these experiments and each biological replicate was assessed in duplicate. All of the genes investigated showed up-regulation in both the ivermectin exposure and fasting group (**Fig. 3-19** and **3-20**). Statistical analysis showed no difference in the level of up-regulation between the two groups of *cyp-37B1, mtl-1* and *scl-2*, which strongly suggests that the induction of these genes following ivermectin exposure is entirely due to fasting caused by pharyngeal paralysis.

acs-2 and *gei-7*, genes known to be involved in the fasting response, were investigated in wild-type worms alongside the three genes of interest. The nematodes in this experiment were exposed earlier than those used for microarray replicates, at the L4 stage, so that they were biologically identical to the DA1316 used in this experiment and in microarray experiments. Interestingly, *acs*-2 and *gei*-7 showed a significantly greater induction following food withdrawal than ivermectin exposure ($p < 0.05$). In contrast, *mtl-1, scl-2* and *cyp-37B1* were intensely up-regulated in strain N2 in both the IVM exposure and fasting groups. Results are not shown for *mtl-1* and *cyp-37B1* due to the fact that expression of these genes in the control group was negligible and beyond the sensitivity of the RT-QPCR technique. However, attempts at quantification suggested huge up-regulation of near 300-fold. The reason for these genes'

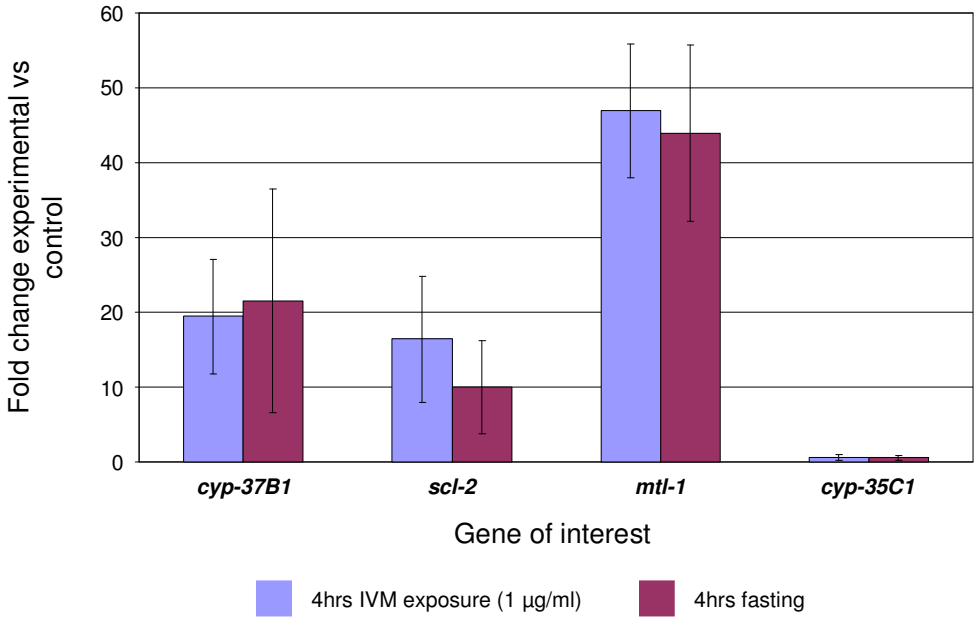


Figure 3-19: *mtl-1, scl-2, cyp-37B1* and *cyp-35C1* regulation following 4hrs exposure to 1µg/ml (1.14µM IVM) and 4hrs fasting in strain DA1316
There are no significant differences in the fold-up-regulation of the genes investigated following exposure to ivermectin and 4hrs fasting. *cyp-35C1* was unaffected by either treatment.

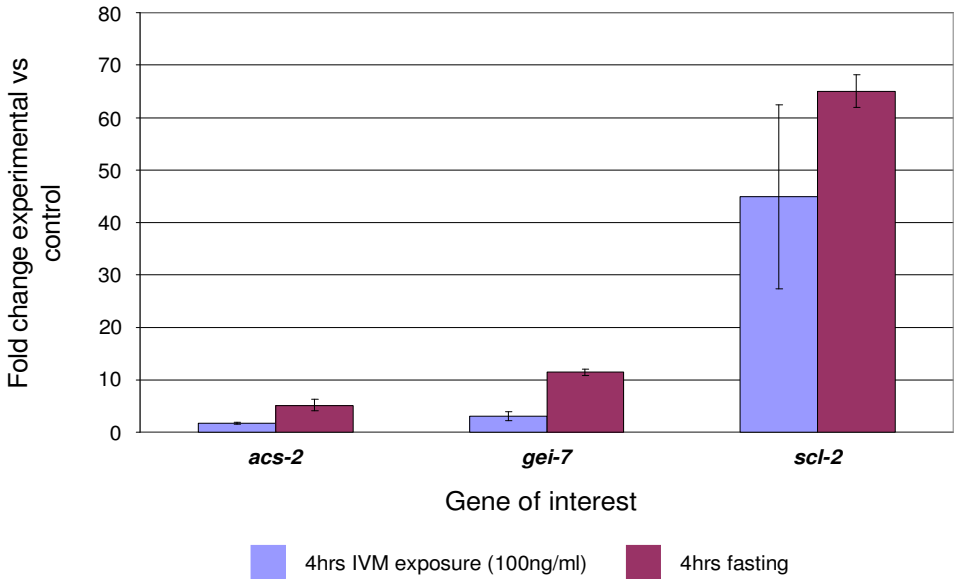


Figure 3-20: *acs-2, gei-7* and *scl-2* regulation following 4hrs exposure to 100ng/ml (114nM) IVM and 4hrs fasting in strain N2
***scl-2* is equally up-regulated in both conditions. However, *acs-2* and *gei-7*, genes known to be involved in the fasting response, show significantly higher up-regulation following fasting compared to ivermectin exposure. *mtl-1* and *cyp-37B1* are not included in this graph due to inefficient amplification of transcripts in control worms. However, both appear to be up-regulated under both conditions.**

absence in the list of significant genes from the N2 microarray experiment may be two-fold. First of all the up-regulation of *mtl-1, cyp-37B1* and *scl-2* may be stage specific and not occur in young adults, which were assessed by microarray experiments. Secondly, it appears that the expression of these genes is constitutively higher in strain DA1316 than in wild-type worms, perhaps due to a level of pharyngeal dysfunction causing a mild fasting response in this strain. If this is the case it may be that the low transcript number in control groups of wild-type worms affected microarray analysis in a similar manner to the problems encountered using RT-QPCR. Further experiments with wild-type worms prepared in an identical fashion to those for the microarray experiments (exposed to IVM at the young adult stage) would suggest that the latter may be the most important. However, similar issues with the detection of control transcript levels of these genes rendered these results unpresentable.

3.4 Discussion

Due to the potent nature of ivermectin, initial experiments were carried out using a very conservative dose of drug in combination with a resistant strain of *C. elegans*. At 0.5ng/ml IVM and 5ng/ml (0.57 and 5.7nM) IVM there were no phenotypic differences between drug exposed groups and control groups after 60hrs. Glutamate-gated chloride channel subunit double mutants are reported to be resistant to ivermectin concentrations of around 10ng/ml (11.4nM; Dent *et al.*, 2000). The reason for the initial spurious microarray results is unknown. Initial bioreplicates were exposed to drug plates prepared using a stock solution of IVM appropriately diluted in distilled sterile H_2O, rather than DMSO. This means that the drug-exposed and control plates differed by the addition of H_2O as well as drug. It is possible that introduction of impurities in this manner resulted in the initial results. It seems unlikely that *cyp-13A6* is involved in the response to ivermectin given that its induction has not been repeatable in further microarray or real- time QPCR experiments.

In most of the microarray experiments carried out, the low number of genes with significantly changed expression levels has been remarkable. The experiments were designed using a resistant strain, specifically to minimise changes in the transcriptome due to general stress. However, more dramatic

changes in gene expression were expected in the wild-type experiment, where phenotypic changes between the drug exposed and control groups were marked. This may be a result of the strict statistical analysis used to assess the microarray data. Many other published papers examining transcriptomic changes in *C. elegans* have used fold-change alone or a simple t-test to assign significance (Reichert *et al.*, 2005; Wang *et al.*, 2003; Custodia *et al.*, 2001). Therefore, follow-up of genes beyond the statistical cut off used in the current study may be appropriate.

Analysis of microarray data and real-time QPCR strongly suggests that the predominant response to ivermectin in this study is a fasting response. This is likely to be due to the pharyngeal paralysis induced by exposure to 1µg/ml (1.14µM) IVM. Many of the genes that were up and down-regulated have clear roles in fatty acid metabolism pathways and gluconeogenesis. There are no microarray studies in the literature comparing whole genome responses in fasted and control nematodes. However, comparison to dauer-stage transcriptome data revealed significant overlap of differentially expressed genes. The trend of gene expression changes between the current study and the fasting response data presented by van Gilst *et al.* (2005b) is compelling evidence of a similar fasting response in *C. elegans* exposed to IVM. van Gilst *et al.* (2005b) used real-time QPCR to monitor the expression of only a small subset of genes that were expected to change during fasting. Therefore, it is possible that several of the genes, whose expression was changed in response to ivermectin exposure, are novel fasting response genes. Alternatively, they may in fact be involved in the detoxification of ivermectin.

The wild-type ivermectin exposure microarray experiment was compared to the DA1316 experiments in an attempt to elucidate genes that may potentially be involved in a detoxification response. This analysis was hampered by the low number of genes with significantly up-regulated gene expression in the wild-type experiment. However, there were ten genes which were not up-regulated in the wild type experiment but that were in both of the DA1316 experiments. Of these ten genes *cyp-37B1, mtl-1* and *scl-2* were chosen for further analysis as they have undergone some level of previous characterisation and were in the top 10 list of up-regulated genes following exposure of strain DA1316 to IVM. In addition, *cyp-37B1* is a member of the cytochrome P450 family, which has been

proposed to be the major group of enzymes metabolising ivermectin in mammalian systems (Gonzalez *et al.*, 2009; Zeng *et al.*, 1998). Initial analysis of these genes suggested they may have a role in detoxification. Their regulation appeared to respond to ivermectin in a dose dependent manner and all appeared to be expressed in the intestine of *C. elegans*, which is thought to be the major organ involved in detoxification in nematodes (McGhee, 2007). However, further examination of the regulation of the three genes revealed that they were up-regulated to the same level following food withdrawal as following ivermectin exposure.

mtl-1 is a metallothionein gene, which is inducible in response to heavy metal intoxication and stress adaptation (www.wormbase.org). *mtl-1* may therefore be involved in protection of the nematode from stressors (Cui *et al.*, 2007). However, metallothioneins have also been proposed to be involved in zinc signalling pathways within mammalian cells (Cousins *et al.*, 2006). Up-regulation of the gene under fasting conditions may represent modulation of a similar signalling pathway in *C. elegans*. Interestingly, *mtl-1* has been noted to be up-regulated in response to several xenobiotics including progesterone, clofibrate and ß-naphthoflavone and was also up-regulated in nematodes grown in axenic culture (Szewczyk *et al.*, 2006; Reichert *et al.*, 2005; Custodia *et al.*, 2001). The phenotype of worms exposed to these xenobiotics does not appear to have been reported in the literature. However, it seems likely that induction of *mtl-1* occurs under many different circumstances and may represent part of a common signalling pathway rather than an effector protein in the response to xenobiotic intoxication.

There have been no citations for *scl-2* in the literature and its function remains largely unknown. However, the gene encodes a sterol carrier-like protein domain and may potentially be involved in the transport of lipid breakdown products. Up-regulation of a gene involved in such processes during fasting would be expected.

cyp-37B1 represents a cytochrome P450 gene which encodes a CYP4/ CYP19/ CYP26 domain. Again, this gene has been shown to be up-regulated in response to other xenobiotics, but the phenotype of the exposed worms was not reported (Menzel *et al.*, 2007; Reichert *et al.*, 2005; Custodia *et al.*, 2001). BLASTp

analysis reveals that isoform 1 of CYP4V2 is a homologue of *C. elegans* CYP37B1 in the *Homo sapiens* proteome (BLAST E-value 7.9 $x10^{-98}$, 90.6% length). Mutations of the gene encoding this protein have been associated with Bietti Crystalline Corneoretinal Dystrophy and the protein has recently been characterised as a fatty acid {omega}-hydroxylase (Nakano *et al.*, 2009). *cyp-37B1(RNAi)* suggests that this gene may have limited hydroxylase activity against eicosapentaenoic acid in *C. elegans* (Kulas *et al.*, 2008). Therefore it is possible that this cytochrome P450 is involved in fatty acid metabolism.

It is possible that several of the genes up-regulated following ivermectin exposure are involved in detoxification of the drug. However, given the overwhelming fasting response and the failure of the wild-type experiments to aid in identification of potential candidates, these genes may be difficult to define. It is important to note that many gene families potentially involved in xenobiotic metabolism; including cytochrome P450s such as *cyp-37B1* and members of the UGT and GST families that were down regulated in the current study; may also have constitutive functions such as involvement in fatty acid metabolism. Therefore, selecting genes based on membership of these families is unlikely to assist.

mtl-1, scl-2 and *cyp-37B1* did not show statistically significant changes in expression following microarray analysis of wild type worms exposed to ivermectin and controls. Follow-up real-time QPCR experiments suggest that this may be due to low constitutive expression of these genes in the wild-type worm. In addition, the *mtl-1* transcriptional GFP reporter construct showed constitutive expression in the intestine. Previous reported studies have suggested that whilst *mtl-1* can be induced in the gut, constitutive expression is only found in the posterior bulb of the pharynx (Freedman *et al.*, 1993). This would also suggest that *mtl-1* expression is higher in strain DA1316 than in strain N2. The reason for this may be due to a level of pharyngeal dysfunction noted in glutamate-gated chloride channel subunit mutants resulting in slight starvation and a chronic up-regulation of the pathways involved in this response (Dent *et al.*, 2000).

Strain DA1316 is phenotypically affected by high dose ivermectin exposure. However, despite the fact that the *avr-15(ad1051)* mutation is absent, this strain may still carry an uncharacterised functional null mutation of the *avr-15* gene

(*pers. comm.;* Dr. J. Dent). If this is the case then these results are potentially very interesting with regard to the mechanism of action of ivermectin on the pharynx. An *avr-15, avr-14, glc-1* triple mutant is being provided by the Dent lab. Confirmation of the three mutations will be undertaken and the phenotype following 4hrs exposure to 1µg/ml (1.14µM) IVM will be assessed. If this strain shows no reduction in the pharyngeal pumping rate then further microarray experiments using this strain may help to elucidate ivermectin detoxification pathways in *Caenorhabditis elegans*.

Chapter 4: *C. elegans* Transcriptomic response to albendazole

4.1 Introduction

Albendazole is a member of the benzimidazole class of drugs. It is used both in human and veterinary medicine to treat a variety of helminthoses and thus the pharmacokinetics of the drug in mammalian systems has been well documented (Mirfazaelian *et al.*, 2002; Marriner *et al.*, 1986; Prichard *et al.*, 1985; Marriner *et al.*, 1980). Albendazole is almost entirely converted to the active metabolite albendazole sulphoxide (ABZ-SO) during first-pass metabolism. This reaction is mostly catalysed by flavin monooxygenase and the CYP3A family (Moroni *et al.*, 1995; Delatour *et al.*, 1991; Souhaili-el *et al.*, 1988a; Fargetton *et al.*, 1986). Further sulphoxidation to the inactive albendazole sulphone (ABZ-SO_2) is thought to occur via the CYP1A family (Delatour *et al.*, 1991; Souhaili-el *et al.*, 1988b).

Albendazole and its metabolites are known to induce cytochrome P450 enzymes and other xenobiotic metabolising enzymes in many species (Velik *et al.*, 2005; Velik *et al.*, 2004; Bapiro *et al.*, 2002; Rolin *et al.*, 1989; Souhaili-el *et al.*, 1988a). This has been assessed using enzyme assays, protein analysis and RNA quantitation. Studies in rats suggest that the CYP1A family is the major cytochrome involved in the conversion of albendazole sulphoxide to the inactive sulphone (Souhaili-el *et al.*, 1988b). CYP1A activity was significantly increased in livers from rats exposed to ABZ as was the subsequent production of ABZ-SO_2 in the perfused livers. Specific CYP1A activities (ethoxyresorufin O-deethylase (EROD) activity) and/or mRNA levels appear to be increased following exposure to albendazole or albendazole sulphoxide in all species examined. These include human HepG2 cells; rat liver microsomes following *in vivo* exposure; and intestinal and liver microsomes of mouflon (*Ovis musimon*) following *in vivo* exposure (Velik *et al.*, 2005; Bapiro *et al.*, 2002; Souhaili-el *et al.*, 1988b). In addition, ABZ has shown to have some inductive effect on rat CYP 2A6, 2E1, 2B1, 2B2 and 3A4 (Asteinza *et al.*, 2000; Souhaili-el *et al.*, 1988a). The induction of these enzymes greatly affects the pharmacokinetics of the drug by increasing the speed of turnover of drug metabolism and reducing the area under the ABZ-

SO plasma concentration vs. time curve. The resulting metabolism of ABZ-SO by the host could effectively lower the drug concentration to which parasites are exposed or the duration of the exposure. It is widely accepted that exposure to suboptimal doses of anthelmintics predisposes to the development of resistance (Geerts *et al.*, 2000).

The mechanism by which ABZ and other benzimidazoles cause induction of xenobiotic metabolism enzymes is not fully understood. Cytochrome P450s have been shown to be induced via pathways involving nuclear hormone receptors (Wei *et al.*, 2000; Kliewer *et al.*, 1999). The structure of the drugs plays a major role in binding to these receptors and different members of the BZ group will induce CYPs to differing degrees. For example, studies carried out in H4IIE cultures, HepG2 cells and rabbit hepatocytes suggest that CYP1A appears to be induced by more planar molecules and those containing a sulphide atom or sulphoxide form of sulphur, as is the case with ABZ and ABZ sulphoxide (Velik *et al.*, 2004). However, this is not always the case as several non-sulphur containing benzimidazole drugs, such as carbendazim and mebendazole, are also potent inducers of CYP1A in these systems (Rey-Grobellet *et al.*, 1996). Work carried out with both albendazole sulphone and the sulphone metabolite of omeprazole, a proton pump inhibitor, suggest that the less planar and more polar structure abolishes the inductive effect on CYP1A (Velik *et al.*, 2004; Lewis *et al.*, 1998). It is likely there are several differences between the interaction of albendazole and nuclear hormone receptors between different species. Therefore, whilst these studies provide some insight into the mechanisms of induction it is unlikely that the interactions are the same in species as distantly related as *C. elegans*.

The mode of action of the benzimidazoles has been well documented. Members of this group act, in both nematodes and fungi, upon ß-tubulin by binding and inhibiting polymerisation to form microtubules. In nematodes the effect appears to predominate in the intestinal cells. The downstream effects of ß-tubulin disruption by BZ drugs have only been fully characterised in *H. contortus*. These include dissociation of apical vesicles from the apical membrane in the anterior gut and inhibition of erythrocyte digestion by six hours post-treatment with fenbendazole. By twelve hours post-treatment, tissue disintegration, DNA fragmentation and secretory antigen dispersal in the anterior intestine was

noted (Jasmer *et al.*, 2000). The eventual result is immobilisation and death of the worm, but the time to effect is much longer than for ivermectin exposure (O'Grady *et al.*, 2004). Microarray experiments comparing albendazole exposed and control populations of *Caenorhabditis elegans* are unlikely to show signs of fasting as pharyngeal paralysis is not a feature of the drug mode of action. In addition, there are several *Caenorhabditis elegans* ß-tubulin (*ben-1*) mutants available (www.wormbase.org). These confer high level resistance to the benzimidazoles, but *ben-1* is presumed to be functionally redundant as mutant worms remain phenotypically wild-type (Driscoll *et al.*, 1989).

In a similar manner to the ivermectin response microarray experiments (**Chapter 3**), the aim was to investigate which genes encode enzymes that may potentially be involved in the metabolism of albendazole. As *ben-1* mutants are phenotypically wild-type, but completely resistant to the effect of benzimidazoles, a strain carrying a mutation of this gene was used to compare the transcriptomes of ABZ exposed and unexposed worms. This study was expected to return a list of genes that were specifically up-regulated in response to the presence of albendazole and not those involved in general stress pathways or those associated with drug exposure phenotypes. The functional ontology and expression profiles of these genes were analysed to assess the hypothesis that these genes were involved in xenobiotic metabolism.

4.2 Methods

4.2.1 Preparation of nematodes for microarray analysis

Initial experiments were carried out on NGM plates using strain CB3474 exposed to 25µg/ml (94.22µM) albendazole (Sigma, A4673) for 48 hours, during the period of development between L1-L4/ young adult (**Section 3.2.1**). Further experiments were designed to assess the response to an acute, 4 hour, exposure to high dose albendazole (300µg/ml, 1.13mM). Due to the extremely insoluble nature of the benzimidazole drugs it was necessary to perform these experiments in liquid culture.

Standard liquid culture methods were used with the exception that water soluble cholesterol (Sigma, C1145) was used at a stock concentration of 25mg/ml. This appeared to increase the solubility of the drug compared to the use of standard cholesterol. CB3474 strain was grown on NGM plates and synchronised as per standard methods (**Chapter 2**). Approximately 10000 L1 larvae were then added to each of two 30ml S-basal cultures, containing 1ml concentrated OP50. The worms were grown at 20^{o}C, with shaking at 240rpm for 70hrs. 100µl samples were taken from each flask to ensure that they were accurately matched in developmental stage (adults). 450µl of 20mg albendazole/ml (Sigma, A4673) in DMSO stock solution was added to one flask (final concentration 300µg/ml [1.13mM] ABZ) and 450µl of DMSO (Sigma, D8418) to the other. The cultures were grown for a further 4 hrs, harvested by sucrose flotation and snap frozen in liquid nitrogen until RNA was extracted. Sucrose flotation, RNA extraction and microarray hybridisation were carried out as described in **Chapter 2**.

The final concentration of DMSO in the flasks was 1.5% v/v. This did not appear to have any phenotypic effect over the 4hr exposure time. The high dose of albendazole meant that the drug was not in a true solution but a suspension. However, due to the constant shaking of the cultures the worms were expected to have received a constant exposure to the drug.

4.2.2 Preparation of nematodes for RT-QPCR

Three separate biological replicates, independent from those sent for microarray analysis were used. The protocol used to prepare these replicates was identical to that described for the microarray experiments except that a commercial preparation of albendazole (Albex 10%, Chanelle) was used as the source of drug. RNA was extracted and cDNA synthesised from 5µg total RNA for each sample using a cloned AMV first strand synthesis kit (Invitrogen, 12328-032) and random hexamer primers. For each sample an identical reaction lacking reverse transcriptase enzyme was carried out. cDNA was purified using PCR purification columns (Qiagen, 28106).

Investigation of gene up-regulation following exposure to a gradient of albendazole concentrations was also undertaken. The method was essentially identical to that used in the microarray experiments, but five matched cultures of *C. elegans* (strain CB3474) were prepared. Cultures were exposed to 0.3, 3, 30, 300µg/ml (1.13, 11.31, 113.1, 1131µM) or no ABZ control for 4hrs. Sigma albendazole dissolved in DMSO (stock 20mg/ml [75.4mM]) was used and all cultures contained an identical volume of DMSO.

4.2.3 SAGE analysis

Serial Analaysis of Gene Expression (SAGE) is a technique by which the level of expression of many genes can be quantified. Libraries of expression data have been created for different larval stages of *C. elegans* as well as for individual organs of the nematode. Searching these libraries for genes with significantly changed expression levels, following exposure of CB3474 to 300µg/ml (1.13mM) ABZ, was undertaken to aid in the assessment of expression site. SAGE data were obtained from the Genome BC *Caenorhabditis elegans* Gene Expression Consortium http://elegans.bcgsc.bc.ca. SAGE tags were mapped to protein coding sequences derived from conceptual mRNAs from the WS190 mappings. Only unambiguous tags were assessed and all libraries were normalised to 100K tags to allow accurate comparison. A developmental series, FACS sorted gut cell and *glp-4* dissected gut library were compared.

4.3 Results

4.3.1 Microarray analysis

4.3.1.1 No statistically significant changes to gene expression were detected following exposure of *C. elegans* to 25µg/ml ABZ for 48 hours

Initial experiments, comparing exposure to 25µg/ml (94.22µM) albendazole to controls, used three biological replicates with matched controls (A-C and controls). Following quality control one chip, control A, was dropped from further analysis leaving two control chips and three ABZ exposure chips for analysis. There were no genes with significantly altered expression using either empirical Bayesian or rank products analysis. No probesets had a log-fold change of greater than 1. However, there were two genes with log-fold changes of less than -1: C06B3.7 and C08F11.13. The C08F11.13 sequence encodes an integral membrane O-acyltransferase and may be involved in fatty acid metabolism. C06B3.7 is completely uncharacterised.

Table 4-1 lists the top 10 genes, based on log-fold change, to be up-regulated following exposure to 25µg/ml (94.22µM) ABZ. Complete microarray data for this experiment is available on the accompanying CD.

Any genes potentially involved in albendazole metabolism would have been expected to be up-regulated following exposure to the drug. The top 10 genes listed in **Table 4-1** show only slight increases in expression level, but this may be due to the low dose of drug reaching the nematodes or the long period between initial exposure to the drug and RNA harvesting. The list contains only two genes which could be referred to as encoding "classical" xenobiotic detoxification genes: *cyp-35C1* and *ugt-41*. Regulation of *cyp-35C1* has been linked to the mediator subunit MDT-15 (Taubert *et al.*, 2008). The top gene on the list, *fat-7*, is known to be regulated by NHR-49 and MDT-15 as a coregulator (Van Gilst *et al.*, 2005b). Several of the genes; *fat-7*, C30G12.2, *cyp-35C1*, F42A8.1, *ttr-14*, T22B7.7; have also been linked to the innate immune response in studies investigating the response to *P. aeruginosa, P. luminescens* and *S. marcescens*

(Wong *et al.*, 2007; Troemel *et al.*, 2006). However, they are not consistently regulated in the same manner between or within experiments. Whilst there is some suggestion that these genes may truly be up-regulated, due to a linked regulation pathway, the lack of any statistical significance or of any genes showing a convincing fold change (greater than 2-fold) mean it is impossible to draw any conclusions.

Probeset	Gene ID	Log_2 FC	p-value	Adjusted p-value	Ontology
192578_at	*fat-7*	0.80	0.59	1	Fatty acid desaturase
185902_at	F21C10.9	0.56	0.53	1	Uncharacterised
190541_at	C30G12.2	0.50	0.25	1	Predicted short chain-type dehydrogenase KOG
183211_s_at	C04F12.7	0.47	0.39	1	Uncharacterised
191068_at	*ttr-14*	0.47	0.28	1	Transthyretin related family domain
181473_s_at	C15C6.2	0.45	0.21	1	Uncharacterised
189283_s_at	*cyp-35C1*	0.44	0.62	1	Cytochrome P450 (CYP 2 family)
173688_s_at	T22B7.7	0.42	0.56	1	Acyl-CoA thioesterase KOG
190849_at	*ugt-41*	0.42	0.43	1	UDP- glucuronosyl/glucosyl transferase KOG
186757_s_at	F42A8.1	0.42	0.30	1	Uncharacterised

Table 4-1: Top 10 up-regulated genes, based on log_2-fold change, following 48hrs exposure of strain CB3474 to 25µg/ml (94.22µM) ABZ
There were no statistically significant changes in gene expression as can be seen from the adjusted p-value (Benjamini-Hochberg) column. C30G12.2, *cyp-35C1* and *ugt-41* represent members of "classical" xenobiotic metabolism pathways.

4.3.1.2 Exposure of *C. elegans* to 300µg/ml ABZ for 4 hours results in significant up-regulation of a distinct set of genes

Given the lack of any significant changes in gene expression following a chronic exposure to 25µg/ml (94.22µM) albendazole, and previous ivermectin exposure assays, the experiments were repeated at a high dose of ABZ. Albendazole, like all benzimidazole drugs, is highly insoluble. When NGM plates were made containing albendazole at concentrations greater than 25µg/ml (94.33µM), the drug was seen to precipitate. Therefore, further experiments were carried out in liquid culture. A 4 hour exposure to 300µg/ml (1.13mM) ABZ was chosen as the mutant strain showed no phenotypic differences, as assessed by motility, following this exposure. In fact, wild type worms showed little sign of

intoxication over this period either. To assess that the drug was effective for microarray replicates, liquid cultures were set up with wild-type worms. However, these had to be exposed to albendazole for 72 hours (from L1 stage) before phenotypic differences between experimental and control flasks could reliably be seen.

Total RNA from the four independent albendazole exposure experiments and four matched controls were sent for microarray hybridisation. Two chips, one ABZ exposure and one control, were dropped from further analysis following quality control leaving three biological replicates for this experiment. Analysis of the remaining chips using empirical Bayesian methods revealed no significant changes. However, the fold change of many probesets was large. Re-analysis of the data using the rank products algorithm showed 33 probesets to be significantly up-regulated and three probesets to be significantly down-regulated with a false discovery rate of 5%. The top 10 genes, based on $\log_2$ fold change, to be up-regulated and down-regulated are identical by either method of analysis and are represented in **Tables 4-2** and **4-3** respectively. **Fig. 4-1** summarises the results and the full microarray data can be found in the accompanying CD.

Fig. 4-1A clearly shows that the majority of genes show no change in expression following exposure to 300µg/ml (1.13mM) albendazole for 4hrs. Whilst most microarray studies will return a large list of genes possibly affected by the experimental conditions these experiments were designed to minimise non-specific change and focus on the genes responding to the presence of albendazole. Unlike the ivermectin experiments, the nematodes were phenotypically wild-type following exposure to albendazole. Therefore, genes involved in general or non-specific stress response pathways were not expected to have been affected by the drug-exposure. The genes listed in **Table 4-2** are convincing as candidates involved in a potential drug detoxification pathway. There are a total of three cytochrome P450s, two UDP-glucuronosyl/ glucosyl transferases and one glutathione-s-transferase, see also **Fig. 4-1B**.

Probeset	Gene ID	Log_2 FC	FDR BH*	FDR rank products	Ontology
189282_at	*cyp-35C1*[+]	3.55	0.6514	0	Cytochrome P450 (CYP 2 family)
189394_at	*cyp-35A5*	3.29	0.6514	0	Cytochrome P450 (CYP 2 family)
189283_s_at	*cyp-35C1*[+]	3.27	0.6514	0	Cytochrome P450 (CYP 2 family)
189512_at	*cyp-35A2*	2.29	0.6514	0	Cytochrome P450 (CYP 2 family)
178316_at	C29F7.2	2.28	0.6514	0	Predicted small molecule kinase
178563_at	T16G1.6	1.98	0.6514	0	Predicted small molecule kinase
190744_at	*ugt-63*	1.92	0.6514	0	UDP-glucuronosyl/glucosyl transferase KOG
192820_at	*gst-5*	1.78	0.6514	0	Glutathione-s-transferase
191418_at	*ugt-16*	1.68	0.6514	0	UDP-glucuronosyl/glucosyl transferase KOG
177701_s_at	K08D8.6	1.62	0.6514	0	Uncharacterised

Table 4-2: Top 10 up-regulated genes, based on log_2-fold change, following 4hrs exposure of strain CB3474 to 300µg/ml (1.13mM) ABZ
The top ten up-regulated probesets represent a selection of xenobiotic metabolism pathway genes as was hypothesised. Whilst the top 10 list and fold changes are identical using empirical Bayesian analysis and the rank products analysis there are profound differences in the allocation of significance (FDR- false discovery rate) to these results between the two methods.

* ***BH = empirical Bayesian t-test with Benjamini- Hochberg correction***

\+ ***cyp-35C1* is represented by two different probes in the top 10 list**

Notably *cyp-35C1*, which was also up-regulated following 48hrs exposure to 25µg/ml (94.22µM) ABZ, is represented by two different probesets in this list. Of the three probesets not representing genes encoding xenobiotic metabolising proteins only one is completely uncharacterised. The other two both represent predicted small molecule kinases, which may be involved in the signalling cascade in response to albendazole.

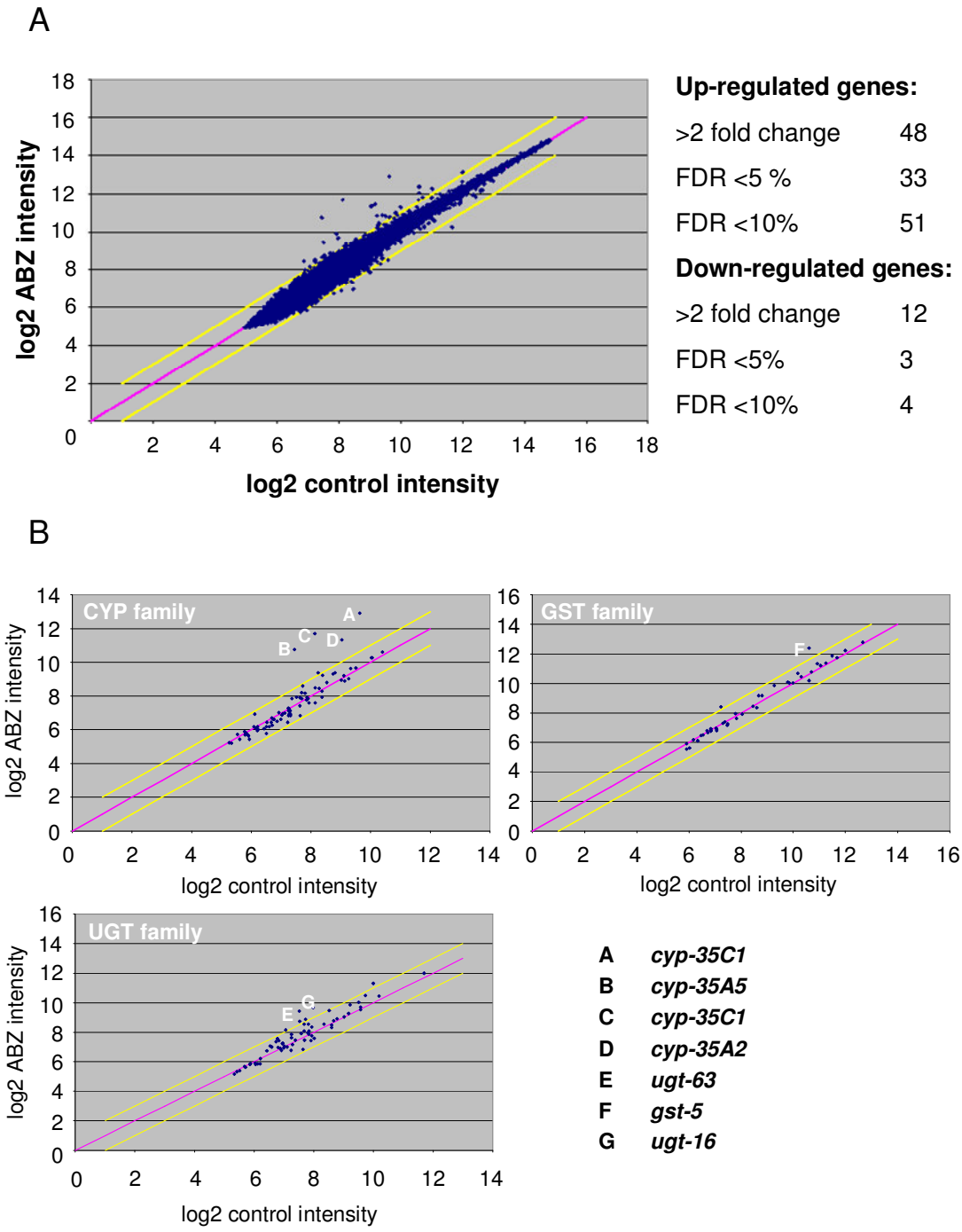


Figure 4-1: Scatter plot of whole genome microarray results following 4hrs exposure of strain CB3474 to 300µg/ml (1.13mM) ABZ
A. Model fitted expression levels of all 22625 probesets on control chips plotted against ABZ exposed chips. The upper and lower yellow lines represent a 2-fold increase and decrease in expression level respectively. Total number of probesets with significantly altered levels of gene expression are summarised based on rank products analysis.
B. Scatter plots containing only probesets specific to members of the cytochrome P450 (CYP), glutathione-s-transferase (GST) and UDP-glucuronosyl transferase (UGT) families. Members of these families present in the top 10 up-regulated genes are noted.

Probeset	Gene ID	Log_2 FC	FDR BH	FDR rank products	Ontology
183330_s_at	C09B8.4	-1.51	0.6514	0	Uncharacterised
188822_at	*acdh-1*	-1.39	0.7026	0.1	Acyl CoA dehydrogenase
190958_s_at	F44E5.4	-1.16	0.6514	0	HSP 70 superfamily
171941_s_at	F44E5.5	-1.14	0.6514	0	HSP-70 superfamily
173288_at	*spd-5*	-1.06	0.6514	0.3131	Involved in mitotic spindle formation and cell division
192195_at	*acs-2*	-1.05	0.6514	0.1643	Fatty acid CoA synthetase family
184054_at	ZK355.4	-1.04	0.6514	0.3123	Uncharacterised
182353_at	*ist-1*	-1.01	0.6514	0.2435	Insulin receptor substrate homologue
187441_at	Y110A2AL.2	-0.97	0.6514	0.2375	Uncharacterised
172397_x_at	K09E3.4	-0.96	0.6514	0.3534	C2H2-type Zn-finger protein KOG

Table 4-3: Top 10 down-regulated genes, based on log_2-fold change, following 4 hours exposure of strain CB3474 to 300µg/ml (1.13mM) ABZ
The down-regulated genes have varied ontologies. Many appear to be involved in fatty acid metabolism.

The down-regulated gene list contains several genes that are directly involved in or linked to fat metabolism. *acdh-1* and *acs-2* encode enzymes that are part of the fatty acid ß oxidation pathway (www.wormbase.org). *acs-2(RNAi)* and ZK355.*4(RNAi)* affects the fat content of *C. elegans*, although *acs-2* depletion causes increased fat content and ZK355.4 depletion causes decreased fat content (Ashrafi *et al.*, 2003). Whilst Y110A2Al.2 remains largely uncharacterised, the best BLASTp match against the *H. sapiens* proteome represents Prolow-density lipoprotein receptor-related protein 1 (BLAST E-value 6e -07, percentage length 87.0%). This receptor is involved in cellular cholesterol uptake.

ist-1 is an insulin receptor substrate homologue that negatively regulates lifespan and dauer development (www.wormbase.org; Wolkow *et al.*, 2002). Dauer larvae are a stress resistant life stage. Therefore, down-regulation of *ist-1* may be involved in promoting the response to certain stressors, such as exposure to a xenobiotic such as albendazole.

spd-5 is an essential gene involved in spindle formation, cell division and anterior posterior axis development during embryogenesis (www.wormbase.org; Hamill *et al.*, 2002). The mode of action of the benzimidazole drugs is to disrupt ß-tubulin polymerisation and formation of microtubules, which are also intimately involved in cell division. This represents an interesting coincidence of function, but it is difficult to draw any conclusions given that a *ben-1* mutant was used in these experiments and the strain was phenotypically normal at the experimental dose of drug.

Due to the small number of significantly down-regulated genes and the fact that these genes were unlikely to be directly regulated in ABZ metabolism, further analysis focussed only on the up-regulated gene list.

4.3.2 Real-time QPCR confirms up-regulation of genes in response to ABZ exposure

QPCR primers were designed for several of the most interesting up-regulated genes following exposure to 300µg/ml (1.13mM) ABZ. Analysis was carried out using three separate biological replicates independent to those sent for microarray analysis. Albendazole (Sigma, A4673), as was used for the microarray experiments, is estimated to be 90% pure. Therefore it was possible that the changes seen in the microarray were as a result of impurities rather than a response to albendazole itself. Albex (Chanelle) is a commercial preparation of albendazole licensed for use in cattle and sheep, and as such was presumed to be pure. However, the exact make up of the excipient was not detailed and experiments were carried out comparing nematodes exposed to Albex and those with no additional supplements to the standard liquid culture medium. Real-time quantitative PCR results are summarised in **Fig. 4-2**.

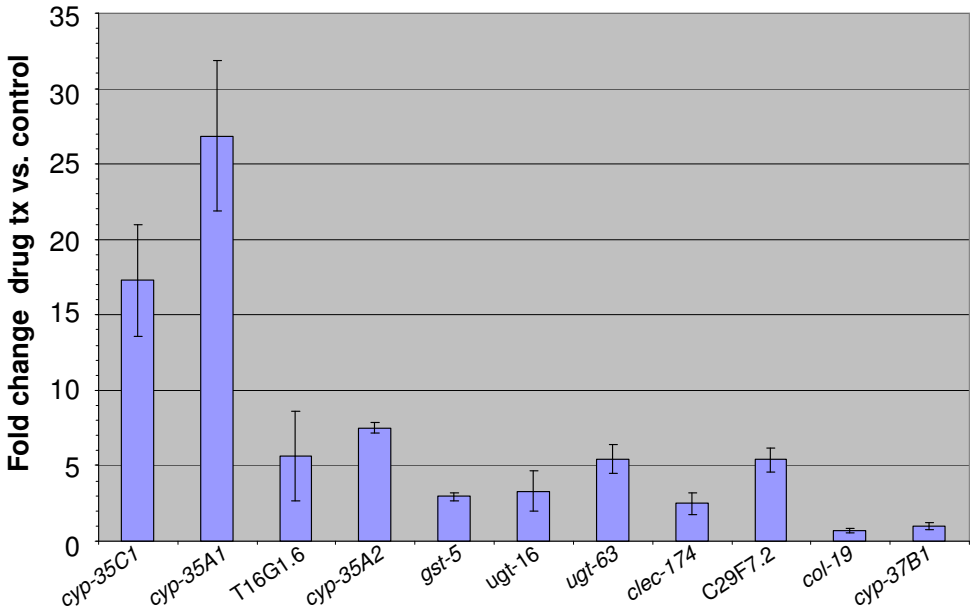


Figure 4-2: RT-QPCR results following 4hrs exposure of strain CB3474 to Albex (300ug/ml [1.13mM] ABZ)
The first nine genes are those suggested to be up-regulated following exposure to ABZ in microarray experiments. All of these also show up-regulation using Albex exposure and RT-QPCR. The last two genes of the chart were included as negative controls as both showed no change in expression on the arrays following albendazole exposure.*col-19* is an adult specific collagen and *cyp-37B1* is a gene up-regulated in response to ivermectin. Neither shows a change in expression following ABZ exposure.

All genes examined that were considered to be up-regulated in the microarray experiments were validated using RT-QPCR experiments. The fold change of specific genes was higher using RT-QPCR than that suggested by microarray experiments. This was likely due to RT-QPCR being much more sensitive than microarrays which compare many genes simultaneously. In addition, random hexamer primers were used in the reverse transcriptase step, which may exaggerate differences in expression. This was not considered problematic as these experiments were used only to confirm up-regulation of genes of interest. The absolute up-regulation was not important and may be biologically irrelevant. In addition, the lack of any change in expression level of *col-19*, an adult specific collagen gene, not only serves to confirm the accurate staging of the control and drug exposed populations but acts as a negative control for the RT-QPCR technique. Similarly *cyp-37B1,* which was up-regulated in response to ivermectin exposure, was not significantly up-regulated following ABZ exposure using microarray analysis or RT-QPCR.

4.3.3 DAVID analysis of up-regulated genes

Global analysis of function was carried out only with the 300µg/ml (1.13mM) ABZ data set. In order to broaden the scope of the analysis, up-regulated genes with a false discovery rate cut-off of less than 10%, as assessed by the rank products method, were analysed. This data set contained 51 probe sets, which represented 42 genes in the *Caenorhabditis elegans* genome.

4.3.3.1 Transferase and monooxygenase terms are enriched in ABZ responsive genes

The functional annotation of the list of up-regulated genes was analysed by looking for enrichment of gene ontology terms. **Fig. 4-3** shows the enrichment of several terms associated with transferase and monooxygenase enzymes. These classes of enzyme are common within xenobiotic metabolism pathways. However, 20 genes from the list had no gene ontology terms associated with them. Therefore, to increase the coverage of annotation the following terms were applied: gene ontology (GOterm_BP_all, GOterm_CC_all, GOterm_MF_all); protein domains (INTERPRO, PIR_SUPERFAMILY, SMART); KEGG pathways and functional categories (COG_ONTOLOGY, SP_PIR_KEYWORDS, UP_SEQ_FEATURE). Using this method only six genes were not annotated, all of which were uncharacterised hypothetical proteins. The resultant list of terms was highly redundant. Therefore, functional annotation clustering was carried out to group similar terms. Interestingly, only two clusters were formed and the genes associated with these clusters are outlined in **Fig. 4-4**. Enrichment scores for the clusters represent the geometric mean of the p-values associated with each of the terms in the cluster. A score of over 1.3 can be considered a significant enrichment. The genes in each of the clusters represent many potential xenobiotic metabolism genes including cytochrome P450s, an alcohol dehydrogenase, glutathione-s-transferases and UDP-glucuronosyl transferases. Other genes represented in these clusters include the predicted small molecule kinases and *jnk-1*, another kinase likely involved in signalling cascades, and the metallothionein encoding gene *mtl-1*, which was up-regulated in response to ivermectin exposure. Interestingly, regulation of several of these genes; including *cyp-35C1*, *ugt-25*, *ugt-63* and *gst-5*; has been associated with the

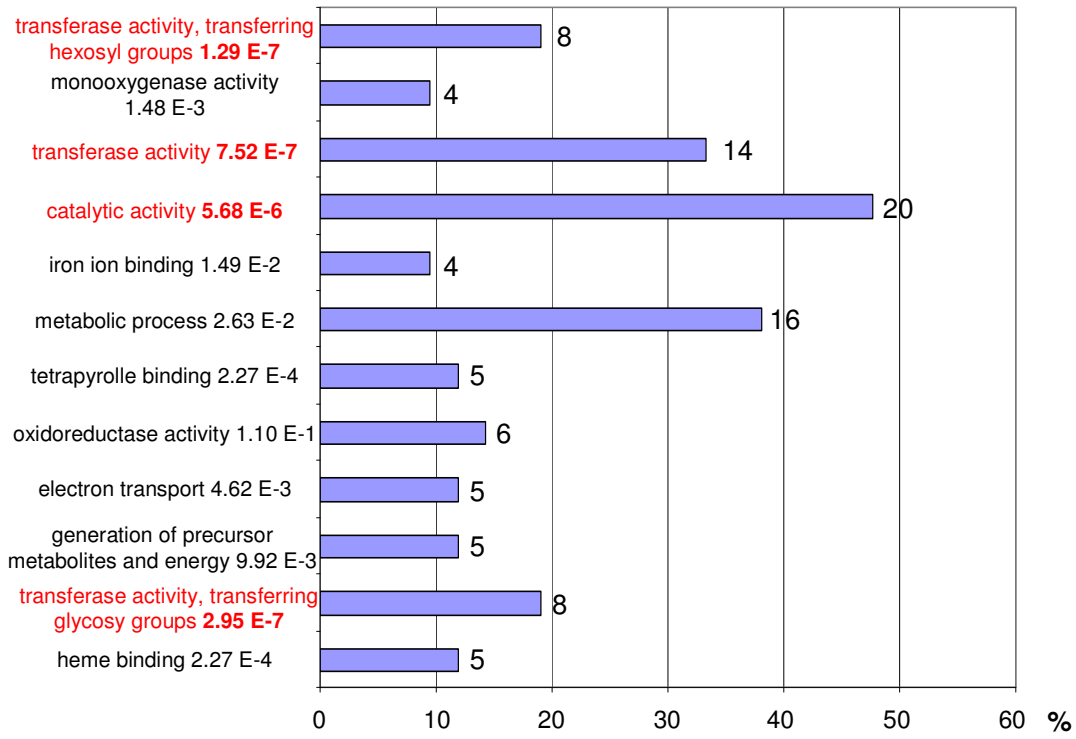


Figure 4-3: Ontology terms associated with genes up-regulated in response to 4hrs exposure of strain CB3474 to 300µg/ml (1.13mM) ABZ.
Ontology terms significantly enriched in the gene list are highlighted in red. Columns represent the percentage of up-regulated genes associated with each ontology term and the numbers at the end of the column are the absolute number of genes.

coregulatory element MDT-15 (Taubert *et al.*, 2008). As mentioned previously, *mdt-15* is also known to associate with *nhr-49* to regulate fatty acid metabolism pathways, which may explain the changes in expression of several genes involved in these pathways, such as *acdh-1* and *acs-2*.

Six terms were not clustered: INTERPRO- CUB-like region (ten genes); SMART-ShK Toxin domain (three genes); INTERPRO- Metridin-like ShK toxin (three genes); GOTERM_MF_ALL- kinase activity (three genes); GOTERM_MF_ALL- transferase activity, transferring phosphorous-containing groups (three genes); GOTERM_BP_ALL- response to stimulus (four genes). This includes a total of 13 individual genes from the initial list that were not associated with either of the annotation clusters.

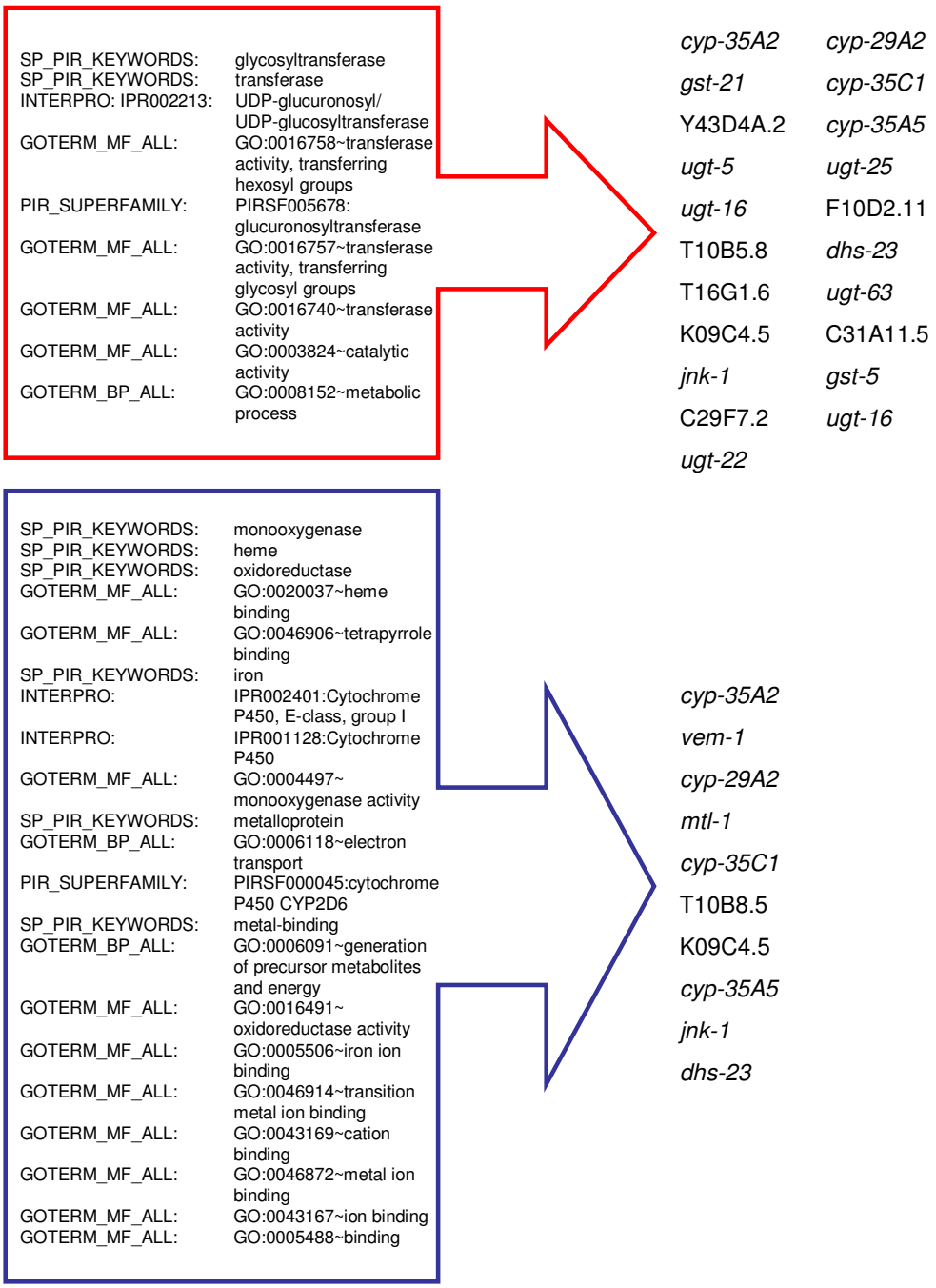


Figure 4-4: Clustering of all annotation terms associated with genes up-regulated in response to 4hrs exposure of strain CB3474 to 300µg/ml (1.13mM) ABZ
Clustering of all annotation terms resulted in only 2 significant groups. Cluster 1 (red box) has an enrichment score of 7.14 and assembles functional terms associated primarily with the UGTs and GSTs. Cluster 2 (blue box) has an enrichment score of 2.14 and associates terms more specifically associated with CYPS. In both cases several other genes, not associated with xenobiotic metabolism pathways, are also clustered that may be significant in the response to albendazole exposure.

4.3.3.2 UGTs and CYPs are enriched in the set of ABZ up-regulated genes

Further clustering of the up-regulated genes based on annotation term co-occurrence revealed there to be two gene families up-regulated. Cluster 1, enrichment score 8.66, represents eight genes which are confirmed or putative members of the UDP-glucuronosyl transferase family (**Table 4-4**). Cluster 2, enrichment score 2.64, represents five genes, four of which are members of the cytochrome P450 family (**Table 4-5**). The fifth gene in cluster 2 is *vem-1*, which represents a cytochrome b5-like transmembrane protein. This gene is thought to play an important role in neuron development (Runko *et al.*, 2004). However, it has also been reported to be induced in response to exposure to xenobiotics such as ß-naphthoflavone and clofibrate (Reichert *et al.*, 2005).

Affymetrix Probe(s)	Gene ID	Ontology
190879_at	*ugt-1*	UDP-glucuronosyl/ glucosyl transferase KOG
183703_s_at	Y43D4A.2	UDP-glucuronosyl/ glucosyl transferase protein domain
191066_s_at	*ugt-5*	UDP-glucuronosyl/ glucosyl transferase KOG
191418_at	*ugt-16*	UDP-glucuronosyl/ glucosyl transferase KOG
184602_at	*ugt-25*	UDP-glucuronosyl/ glucosyl transferase KOG
190849_at	*ugt-41*	UDP-glucuronosyl/ glucosyl transferase KOG
190744_at	*ugt-63*	UDP-glucuronosyl/ glucosyl transferase KOG
183703_s_at, 193604_at	*ugt-22*	UDP-glucuronosyl/ glucosyl transferase KOG

Table 4-4: ABZ up-regulated gene functional classification cluster 1 (enrichment score 8.66)

Affymetrix Probe(s)	Gene ID	Ontology
189512_at	*cyp-35A2*	cytochrome P450
189282_at, 189283_s_at	*cyp-35C1*	cytochrome P450
189394_at	*cyp-35A5*	cytochrome P450
189350_at	*cyp-29A2*	cytochrome P450
188031_s_at	*vem-1*	Putative steroid membrane receptor KOG

Table 4-5: ABZ up-regulated gene functional classification cluster 2 (enrichment score 2.64)

These results together with annotation clustering confirm the significant up-regulation of gene members putatively involved in xenobiotic metabolism pathways in response to ABZ exposure. Whilst several classes of these genes were up-regulated, the UDP-glucuronosyl transferases and cytochrome P450s predominate and metabolism of albendazole may be expected to occur via these enzyme systems.

4.3.4 Many ABZ up-regulated genes may be targets of mdt-15

Mediator is an evolutionary conserved co-regulator of RNA polymerase II. Different subunits of the mediator complex allow binding of regulatory elements to control the transcription of specific genes. The constitutive and induced expression of *cyp-35C1* has been shown to be dependent upon the *C. elegans* mediator subunit MDT-15 (Taubert *et al.*, 2008). Taubert *et al.* (2008) investigated the targets of the product of *mdt-15* by using whole genome microarrays to compare the transcriptomes of *mdt-15(RNAi)* worms to a control population. MDT-15 is thought to be a coactivator therefore genes that were down-regulated in this experiment could be expected to be regulatory targets of MDT-15.

To assess whether more of the ABZ responsive genes may also be regulatory targets of MDT-15, the list of ABZ up-regulated genes was compared to the *mdt-15(RNAi)* down-regulated genes. This represents a very different experiment to the ABZ exposure microarrays carried out in this study and $\log_2$ FC of the genes would not be expected to be similar. Therefore, Log_2 FC for both experiments were converted to a scoring system where 2= highly up-regulated, 1 = mildly up-regulated, 0= no change, -1= mildly down-regulated and -2= highly down-regulated.

As can be seen in **Fig. 4-5**, 21 of the up-regulated genes in the ABZ microarray appear to be regulated by MDT-15. Only eight appear to be regulated in opposite directions. Conspicuously, eight of the top 10 genes in the ABZ up-regulated microarray are regulated by MDT-15. Of the two that do not fit this pattern K08D8.6 showed no change in the *mdt-15(RNAi)* experiment and *ugt-16* was not represented at all in that experiment.

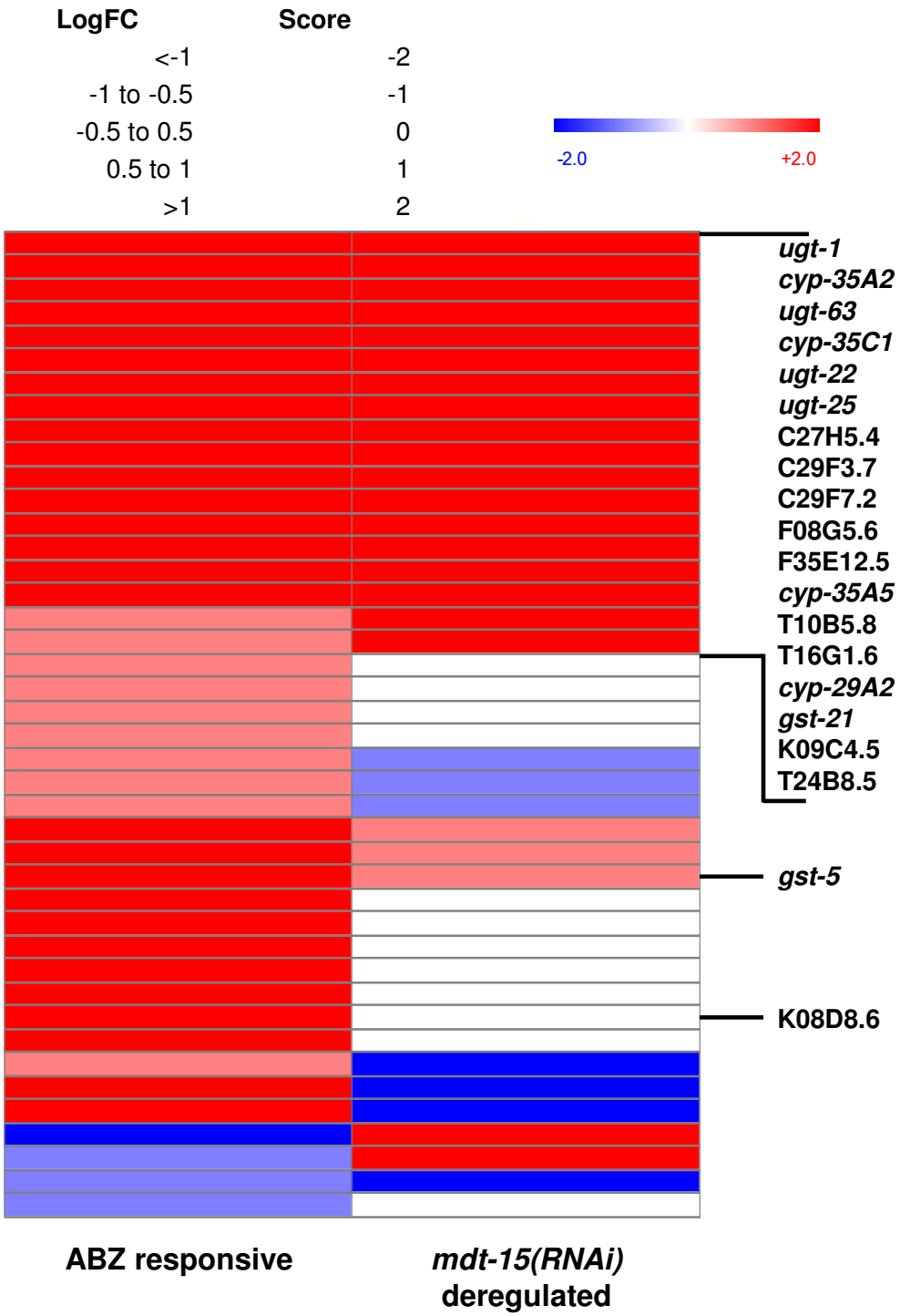


Figure 4-5: Comparison of genes up-regulated in response to ABZ exposure and those deregulated by *mdt-15(RNAi)*
Comparison of ABZ up-regulated/down-regulated genes (FDR< 10%) to the same genes in an *mdt-15(RNAi)* microarray experiment (Taubert *et al.*, 2008). Log_2 FC are not expected to be very similar due to the different nature of the experiments. Therefore, log_2 FC has been converted to a simple scoring system detailed above. As can be seen, many of the genes show similar changes in expression.

4.3.5 XME RNA induction is evident at low doses of ABZ

300µg/ml (1.13mM) ABZ represents an extremely high dose of albendazole. In order to investigate the response to albendazole over a range of concentrations RT-QPCR was used to assess the fold up-regulation of *cyp-35C1*, *cyp-35A2*, *cyp-35A5* and *ugt-16* over a range of ABZ concentrations. Albendazole (Sigma, A4673) was dissolved in DMSO and the same volume of drug or DMSO (in the case of the control) was added to each of four flasks to the final concentrations of 0.3, 3, 30 and 300µg/ml (1.13µM- 1.13mM).

Whilst *cyp-35A5* showed a 2-3 fold change at 0.3µg/ml (1.13µM) ABZ, the other three genes investigated did not show any convincing up-regulation. At 3µg/ml (11.31µM) both *cyp-35A5* and *cyp-35C1* showed up-regulation. Maximal fold-changes for all genes investigated occurred at 30µg/ml (113.1µM) ABZ. The fold-changes observed for all genes were significantly higher than in previous RT-QPCR experiments. This may be partially due to the different source of albendazole used. However, in these biological replicates all of the reverse transcriptase minus controls also reached threshold and the dissociation curve of these wells showed a melting point identical to the experimental wells. The no template controls did not reach threshold so it was assumed that the samples themselves must be contaminated. Further DNase digests of the RNA samples and new cDNA syntheses were carried out, but the problem remained. Whilst these experiments will ideally be repeated, the trend seen over the concentration gradient is still valid as all RT minus controls (both ABZ exposed and control samples) were equally affected.

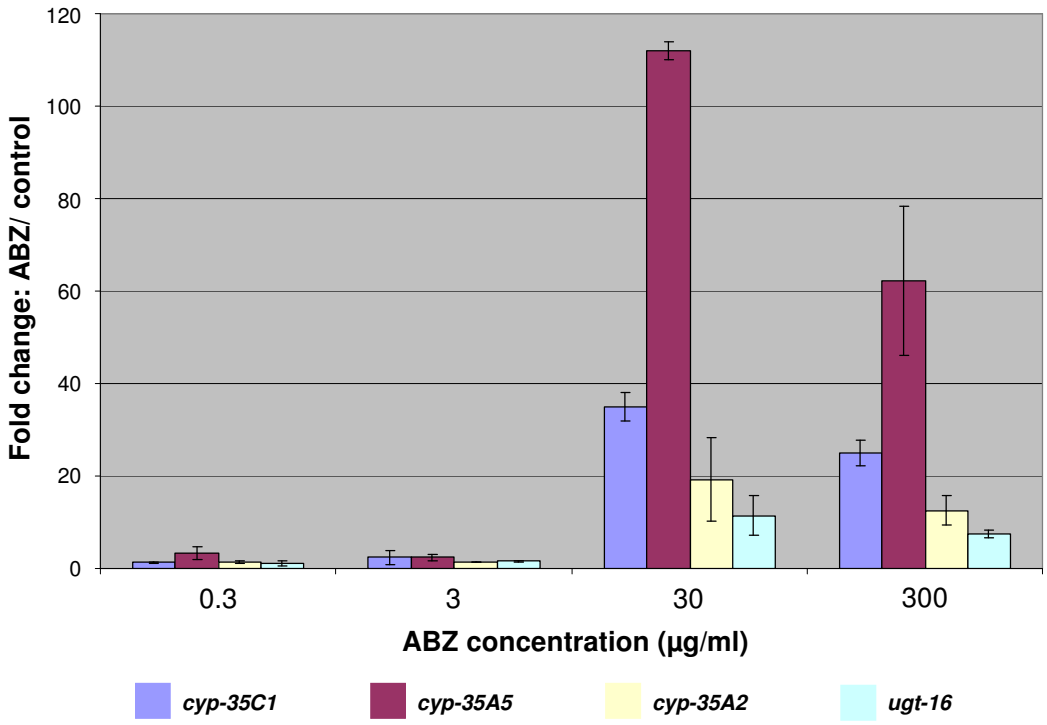


Figure 4-6: Response of four genes of interest to 4hrs exposure of strain CB3474 to gradient of ABZ concentrations
All genes analysed showed their maximal fold changes at 30µg/ml (113.1µM) ABZ. Modest up-regulation of *cyp-35A5* was apparent at 0.3µg/ml (1.13µM) ABZ and of both *cyp-35A5* and *cyp-35C1* at 3µg/ml (11.31µM) ABZ.

4.3.6 cyp-35C1 is expressed in the gut

A GFP reporter fusion construct was prepared containing the promoter of cyp-35C1 fused to a GFP fragment amplified from plasmid pPD95.67 (**Section 2.2.12.1**). Transmitting lines were obtained upon microinjection into strain DA1316 [*avr-14(ad1302); glc-1(pk54)*]. Minimal fluorescence was seen under standard conditions. However, upon exposure to ABZ, fluorescence could be seen throughout the entire length of the gut at all stages (**Fig. 4-7**). In a similar manner to the IVM responsive gene reporter constructs, the GFP production was not stable. Following freezing the GFP signal was significantly diminished and attempts to quantify GFP induction by ABZ, using Image J software (http://rsbweb.nih.gov/ij/index.html) analysis and direct fluorescence quantification with Fluorostar software, were unsuccessful.

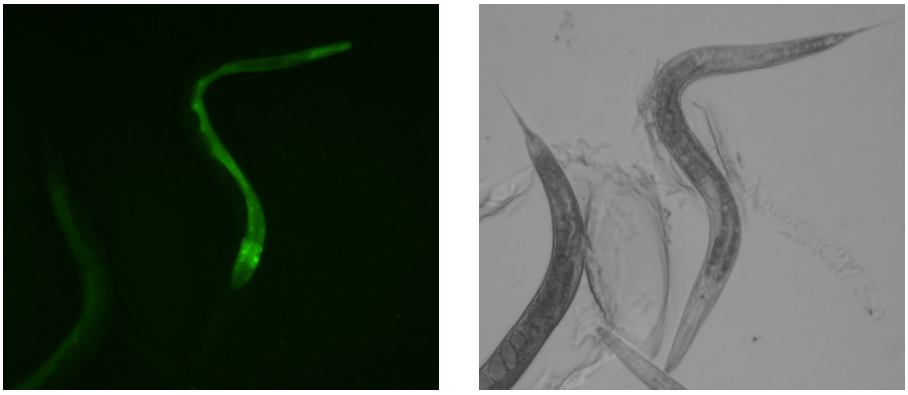
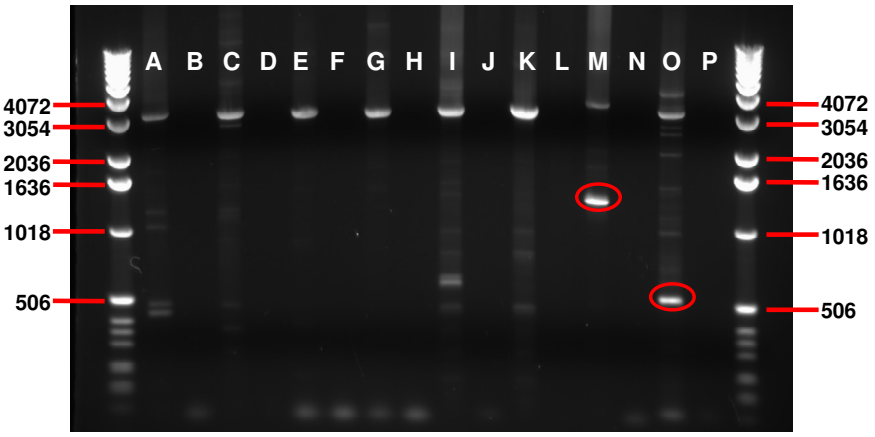


Figure 4-7: *cyp-35C1* transcriptional GFP reporter fusion (Genotype: *[pRF4{rol-6(su-1006)}+cyp-35C1::GFP]; avr-14(ad1302); glc-1(pk54)*)
The 3kb upstream segment of the transcriptional start site of *cyp-35C1* was fused to the GFP gene amplified from fire vector pPD95.67. GFP fluorescence was evident throughout the intestine at all life stages of transgenic worms. Intensity of fluorescence was subjectively enhanced following ABZ exposure.

4.3.7 PCR-fusion GFP reporters appear to be unstable for genes with low expression

The GFP expression of several of the transgenic lines created for both ABZ and IVM responsive genes appeared to diminish with time. The strains carrying the constructs *[pRF4{rol-6(su-1006)}+cyp37B1::GFP], [pRF4{rol-6(su-1006)}+scl-2::GFP]* and *[pRF4{rol-6(su-1006)}+cyp35C1::GFP],* showed strong GFP expression in the F2 generation. Following maintenance by selection for the roller phenotype for several generations and subsequent freezing at -80^{o}C, GFP expression was much less bright and for *cyp-37B1* and *cyp-35C1* was completely absent. In comparison, the strains carrying the construct *[pRF4{rol-6(su-1006)}+mtl1::GFP],* continued to show strong GFP expression.

Analysis of the GFP reporter strains was carried out using transgene specific primers. These consisted of a promoter specific primer (primer A* used in the original fusion PCR) and a common reverse primer within the *gfp* gene (GFP_R). Primer sequences can be found in **Appendix 7.2** and on the accompanying CD. These primers were expected to produce products of 3186bp, 3167bp, 3237bp and 3219bp for the reporter strains of *cyp-37B1, scl-2, cyp-35C1* and *mtl-1* respectively. Appropriate sized bands were amplified from worm lysates of each of two lines of worms carrying the constructs *[pRF4{rol-6(su-1006)}+cyp37B1::GFP], [pRF4{rol-6(su-1006)}+scl-2::GFP*] and [pRF4{rol-6(su-1006)}+cyp35C1::GFP], see **Fig. 4-8**. This suggests that the constructs are present within the worms, but for some reason are not being expressed. Interestingly, the transgenic line carrying construct *[pRF4{rol-6(su-1006)}+mtl1::GFP]*, which fluoresces as expected, has a faint band of the appropriate size, but a far brighter one between 1018 and 1636bp. The sequences of the primers used were subject to a BLASTn search against the *C. elegans* genome, but could not explain the appearance of this band. These PCR products will require sequencing in order to further investigate the cause of decreased GFP expression. There do not appear to be any reports in the literature of a similar phenomenon.



A	*cyp-37B1* GFP line 1	**I**	*cyp-35C1* GFP line 1
C	*cyp-37B1* GFP line 2	**K**	*cyp-35C1* GFP line 2
E	*scl-2* GFP line 1	**M**	*mtl-1* GFP line 1
G	*scl-2* GFP line 2	**O**	*cyp-35C1* promoter region (positive control)

B,D,F,H,J,L,N,P No template controls for preceding lane

Figure 4-8: Amplification of promoter-GFP sequence from transgenic worms displaying the roller phenotype but which no longer fluoresce.
The combined promoter and GFP sequence for each of the transgenic lines was amplified using a common reverse primer within the GFP sequence and the gene specific forward primers used to create the fusion construct. Two separate GFP reporter strains for *cyp-37B1, scl-2* and *cyp-35C1* all had bands of the expected size. The GFP reporter strain for *mtl-1* had an unexpected band between 1018 and 1636bp and the positive control (*cyp-35C1* promoter region- expected 3120bp) at just over 506bp, both circled red. BLASTn analysis of the *C. elegans* genome could not explain these bands.

4.3.8 SAGE analysis reveals enrichment of ABZ up-regulated genes in the intestine

Intestinal enrichment was assessed by comparing the number of SAGE tags for each of the up-regulated genes in both a whole adult and dissected gut library. Only tags that unambiguously associated with each of the genes of interest were used and the libraries were normalised to 100000 tags total. The analysis shows that many of the tags are expressed at a very low level. This is not unexpected in a set of genes that are expected to be induced in response to specific conditions. Additionally, the SAGE library confirms the over-representation of genes that are expressed in the intestine (**Fig. 4-9**).

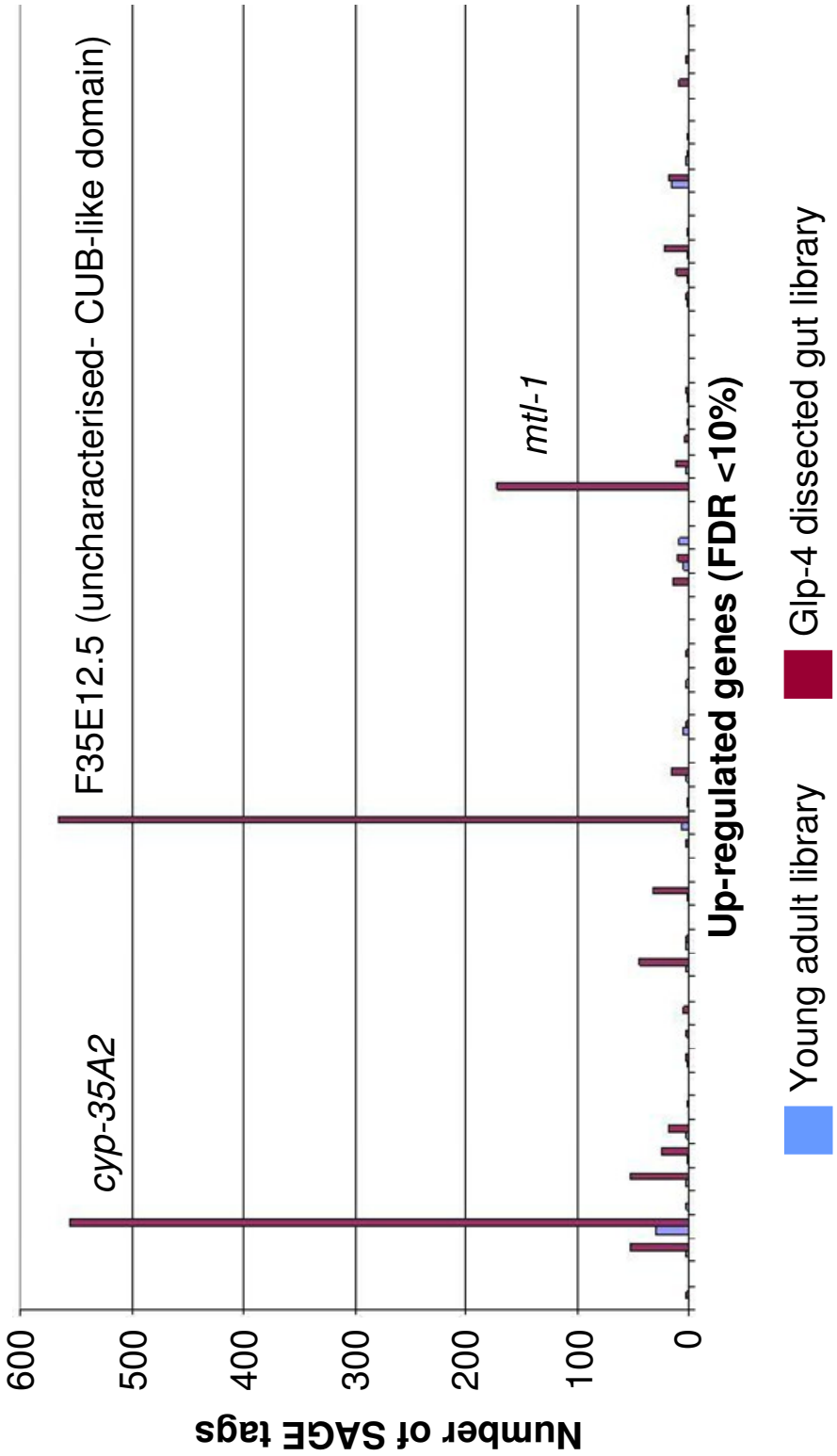


Figure 4-9: No. of SAGE tags for ABZ responsive genes in young adult and intestinal libraries
Most of the ABZ responsive genes have very low expression levels and as such have a low number of tags in both libraries. However, as a general trend, the genes are represented by higher numbers of tags in the intestinal library suggesting this is an area of increased expression.

4.4 Discussion

Initial experiments in which *C. elegans* were exposed to 25µg/ml (94.22µM) albendazole for 48hrs did not show any significant changes in gene expression. No changes in gene expression associated with drug phenotype were expected as strain CB3474 is completely wild type at this dose and it is possible that any dramatic changes in gene expression in response to the drug may have occurred earlier in the exposure. Analysis of concentration gradient experiments with a four hour exposure time would suggest that at least some of the genes up-regulated in response to 300µg/ml (1.13mM) ABZ could also be up-regulated in response to lower doses of drug. Parasites of sheep are likey to be exposed to albendazole sulphoxide at concentrations between 3.2 and 26.2µg/ml, the peak plasma and abomasal concentrations of drug respectively (Marriner *et al.,* 1980). The use of albendazole sulphoxide rather than albendazole in these experiments may have been more applicable as a model of parasite drug exposure, but the cost of sourcing albendazole sulphoxide was prohibitive. Regardless of these caveats, the intention of these experiments was to maximise the number of genes returned as significantly changed in expression level in response to albendazole exposure, whilst minimising the identification of genes involved in drug phenotype/ worm death. Thus by using the BZ resistant strain, CB3474, in combination with a short exposure to an artificially high dose of albendazole we hoped to identify a list of genes that could be further investigated at more physiologically relevant concentrations of drugs. The small number of genes with significant changes in expression levels is perhaps unusual for most microarray experiments, but is testament to the success of this approach.

Four hours of exposure to 300µg/ml (1.13mM) ABZ resulted in significant changes in the expression intensity of 33 genes (FDR <5%), as assessed by the rank products algorithm. Many of genes in the list showed very low p-values following t-test analysis, but following correction using a Benjamini-Hochberg technique none of the changes were considered significant. This may be due to the small number of genes showing large changes in expression level, which was the aim of the approach outlined above. Only 48 genes were up-regulated more than 2-fold and only 12 were down-regulated more than 2-fold. Huang *et al.* (2009) report that a list of at least 100 genes is optimal for analysis with DAVID software. The

number of probesets with significantly altered expression levels is significantly smaller in this study. However, up-regulation of these genes has been confirmed by real-time QPCR and analysis of both individual genes in the top 10 and analysis of function with DAVID software agree that there is an enrichment of genes encoding potential xenobiotic metabolising enzymes. Considering the question that these studies set out to answer, the results make biological sense which is an important aspect of analysing microarray data.

The genes showing the greatest fold-change in these experiments were three cytochrome P450s of the *cyp-35* family. CYPs encode ubiquitous haem-containing monooxygenase enzymes, which are involved in the metabolism of many drugs and xenobiotics in mammals and other species. The *Caenorhabditis elegans cyp-35* family has previously been reported to be inducible by several other xenobiotics including ß-naphthoflavone and atrazine (Reichert *et al.*, 2005); PCB52, fluoranthene and lansoprazole (Schafer *et al.*, 2009; Menzel *et al.*, 2005); and ethanol (Kwon *et al.*, 2004). However, up-regulation of the cyp-35 family does not appear to be a general response to all xenobiotics as it was not noted in response to acrylamide (Hasegawa *et al.*, 2008) or clofibrate and diethylstilbestrol (Reichert *et al.*, 2005). In addition, no members of the *cyp-35* family were induced following exposure to ivermectin, see **Chapter 3**. Other genes clustered with the cytochrome P450s by DAVID analysis, cyp-29A2 and vem-1, have also been reported to be up-regulated in response to PCB52, ß-naphthoflavone and diethylstilbestrol.(Menzel *et al.*, 2007; Reichert *et al.*, 2005).

The CYP35 family in *Caenorhabditis elegans* is most closely related to the CYP2 family of humans and other mammals. CYP35C1 has closest homology to *H. sapiens* CYP2B6 (BLASTp E-value: 1.1 e-56, 93.9% length). Both CYP35A5 and CYP35A2 bear closest homology to *H. sapiens* CYP2C8 (BLASTp E-value: 4.2 e-53, 93.9% length and 4.6 e-57, 97% length respectively). Li *et al.* (2003b) used both HLM and recombinant CYPs to assess the percentage contribution of different human CYP isoforms to the metabolism of several antiparasitic drugs. In this study they found that rCYP2B6 had no activity against albendazole, whilst rCYP2C8 had only contributed to 0.3% of total albendazole depletion noted in human liver microsomes. The major CYP isoform involved was CYP1A2 (53% of HLM clearance). However it should be noted that the rCYP isoforms used in this

experiment could only account for 65% of the total clearance in HLM (measured by substrate depletion). Despite the seemingly low contribution to albendazole metabolism of the human homologues of the *cyps* up-regulated in response to albendazole in the current study, both CYP2B6 and CYP2C8 are thought to play important roles in drug metabolism and are highly inducible (Mo *et al.*, 2009; Chen *et al.*, 2009; Wang *et al.*, 2008a). In addition, ß-naphthoflavone and lansoprazol are generally accepted as strong inducers of the mammalian CYP1A family, but have been shown to induce CYP35A2 in *C. elegans* (Menzel *et al.*, 2001). There is marked species variation in the induction of xenobiotic metabolism pathways even within mammals, so variation in the responsive CYP members between mammals and nematodes is to be expected.

The UDP-glucuronosyl/ glucosyl transferases are an important group of phase II xenobiotic metabolising genes. By conjugating glucuronate or glucose onto drugs directly or following functionalisation by phase I enzymes they render drugs more hydrophilic so that they can be excreted from the cell and organism. This group of enzymes is the most over-represented class of gene up-regulated in response to ABZ and may be very important in detoxification of this drug. Similarly to the up-regulated CYP genes, many of these proteins have been implicated in the response to other xenobiotics including atrazine, clofibrate and ethanol (Reichert *et al.*, 2005; Kwon *et al.*, 2004). Of the two UGTs represented in the top 10 up-regulated genes following ABZ exposure, the predicted polypeptide encoded by *ugt-63* has closest homology to mammalian UGT1A1 and that of *ugt-16* has closest homology to UGT2B7, both of which are involved in xenobiotic conjugation. Up-regulation of UGT type 1 activities following exposure to ABZ has been noted in the rat and is thought to speed the biological inactivation of the drug in this species (Rolin *et al.*, 1989; Souhaili-el *et al.*, 1988a). This represents an interesting coincidence, but again species differences in the affinity of specific classes of XME for substrates are common.

The cytochrome P450s and UDP-glucuronosyl transferases were the only two gene families to be enriched in the list of ABZ up-regulated genes. This in itself is suggestive of *C. elegans* mounting a specific response to metabolise albendazole. In addition, GFP reporter and SAGE library analysis has shown that many of these genes are highly expressed in the intestine, which is thought to be the major site of detoxification in the nematode (McGhee, 2007). In the case of

both CYPs and UGTs substrate induction of a particular gene does not mean that the enzyme encoded by that gene is the only one involved in the substrate's metabolism. ABZ is metabolised by several CYP genes in humans despite only inducing CYP1A1 and CYP3A4 (Li *et al.*, 2003b). Up-regulation of *cyp-6g1* in insecticide resistant *Drosophila melanogaster* appears to be present in most field strains (Daborn *et al.*, 2002). However, Daborn *et al.* (2007) more recently reported that constitutive up-regulation of several CYPs could induce a resistant phenotype. Whilst several members of the UGT family were only modestly up-regulated in the current study, it is likely that they have overlapping substrate specificities and several or all may be involved in the metabolism of ABZ. Up-regulation of any one of the CYPs or UGTs reported in this study may significantly increase the tolerance of nematodes to ABZ.

Functional annotation clustering of the ABZ up-regulated genes was used to aid in the identification of other genes that may be involved in metabolism pathways. In the same cluster as the many UGTs there are also two GST genes (*gst-5* and *gst-21*) and a short-chain dehydrogenase (*dhs-23*), all which could possibly be involved in ABZ metabolism. Additionally, three protein kinase-like genes were also clustered due to their transferase activity. *jnk-1* represents the sole member of the c-Jun N-terminal kinase subgroup of mitogen activated protein kinases in the *C. elegans* genome. This gene has been implicated in the response to heat and oxidative stress and also in the response to cadmium (Wang *et al.*, 2008b). The putative small molecule kinases C29F7.2 and T16G1.6 were markedly up-regulated in response to albendazole and have also been implicated in the response to cadmium (Cui *et al.*, 2007). Additionally, the metallothionein gene, *mtl-1*, which is important in the heavy-metal response, is also up-regulated in response to ABZ. All of these genes may potentially be involved in a signalling cascade in response to ABZ exposure.

The remaining clustered genes represent a predicted acyl-transferase (*oac-6*), a gene with an NADH: flavin oxidoreductase KOG and an uncharacterised gene with a predicted transport domain, which when knocked down by RNAi, results in a “fat increased” phenotype. All of these genes likely have function in fat metabolism. Interestingly, several of the few significantly down-regulated genes may also be involved in fat metabolism pathways. Aberration of these pathways has been noted in response to many lipophilic xenobiotics. This may be non-

specific, but could also represent part of a whole organism response to minimise toxin ingestion by utilising internal energy stores (Taubert *et al.*, 2008).

Of the completely unclustered annotation terms the CUB-like domain is highly enriched in the list of ABZ responsive genes (p-value 1.1 E-14). The function of this domain or any of the genes containing it is unknown. However, these genes have also been shown to be inducible in other conditions. All of the genes containing the CUB-like domain which are up-regulated in response to ABZ are also up-regulated in response to infection with *Pseudomonas aeruginosa* and may be involved in the innate immunity pathways (Shapira *et al.*, 2006). This is true of many of the other ABZ up-regulated genes including members of the CYP and UGT families. It is worth noting that *P. aeruginosa* secretes several toxins including phenazine, which has been shown to be involved in "fast killing" (4-24 hrs) of infected *C. elegans* (Mahajan-Miklos *et al.*, 1999). Therefore, up-regulation of genes in response to bacterial infection may also represent a detoxification response.

It is unlikely that the CUB-like domain containing genes, *mtl-1* and those involved in fat metabolism are directly involved in xenobiotic metabolism. However, it appears that the regulation of these genes and those involved in xenobiotic detoxification may occur through similar pathways. The mediator subunit MDT-15 has been implicated in the regulation of many of the genes up-regulated in this study. Induction of *cyp-35C1* in response to fluoranthene appears to be MDT-15 dependant, as does the induction of *mtl-1* in response to cadmium intoxication. However, it does not appear to be necessary for the response to heat shock (Taubert *et al.*, 2008). As previously discussed, MDT-15 is also involved in the regulation of fatty acid metabolism in both NHR-49 dependent and independent pathways (Taubert *et al.*, 2006). It is clear that MDT-15 must interact with several metabolic regulatory factors in order to produce specific responses to metabolic or toxic stimuli. *nhr-8* encodes a *C. elegans* nuclear hormone receptor that has previously been associated with xenobiotic responses to colchicine and chloroquine (Lindblom *et al.*, 2001). However, Taubert *et al.* (2008) report that *nhr-8(RNAi)* had no effect on the induction of *cyp-35C1* and other MDT-15 regulated detoxification genes in response to fluoranthene. Similarly, the nuclear hormone receptors encoded by

nhr-49 and *sbp-1* do not appear to be involved in this response, despite being associated with MDT-15 in other pathways.

The regulatory pathways involved in the response to specific xenobiotics are likely to be complex. The *C. elegans* genome contains 288 predicted nuclear hormone receptors, the function of most of which is unknown (www.wormbase.org). Whilst, MDT-15 appears to be a central node in many pathways, specificity of response may be accounted for by the NHRs or other co-regulatory factors that MDT-15 associates with. It is also likely that MDT-15 independent pathways are involved. The up-regulation of the CUB-like domain genes in this study, which appear to be repressed by MDT-15, is consistent with this hypothesis (Taubert *et al.*, 2008). Investigation of the promoter regions of the genes shown to be up-regulated in response to albendazole may help uncover coincident regulator binding sites and further elucidate the regulation of the xenobiotic response.

Chapter 5: Analysis of anthelmintic metabolism by nematode extracts

5.1 Introduction

In order to completely evaluate *Caenorhabditis elegans'* use as a model organism to investigate anthelmintic metabolism, it was necessary to prove that the nematode could metabolise drugs and to define the metabolites produced. High-Performance Liquid Chromatography with tandem Mass-Spectrometry (HPLC-MS/MS) is a standard technique in drug metabolism studies (Holcapek *et al.*, 2008). Following incubation with whole cells or extracts, the compound and any metabolites are dissolved in an organic solvent. A small volume of this solution is isolated on a chromatography column and then subject to washing with an aqueous to organic gradient of mobile phase. In this manner metabolites are separated from the column based on their solubility. Throughout the mobile phase gradient a fraction of the effluent from the column is directed into the ion source of a mass spectrometer. In the current study an electrospray in positive ion mode was utilised. This serves to produce gas phase ions which are then separated based on the mass/charge (m/z) ratio of the ion. There are several different types of mass spectrometer which identify m/z in slightly different manners. Both quadrupole and time of flight analysis were used in this study (Willoughby *et al.*, 1998).

A quadrupole mass spectrometer contains 4 parallel charged poles in a vacuum. Ions are introduced along the central axis between these poles and by varying the voltage to the opposing poles are filtered out based on m/z. A triple quadrupole contains three sets of these systems and allows MS/MS capability. The first quadrupole screens the parent ions from the electrospray; the second has a nitrogen atmosphere and is used to fragment the ions from the first; the third, again in a vacuum, filters the fragments based on m/z.

Time of flight (TOF) spectrometry relies on the fact that smaller mass ions will travel faster than larger ones. From the ion source, the ions are directed into a flight tube which has a pulse of high voltage applied across it. The time it takes

for an ion to cross the flight tube from the source to the detector is directly proportional to m/z.

HPLC-MS analysis of drug metabolites has most commonly been used in human and mammalian studies to examine pharmacokinetics. Both ivermectin and albendazole are anthelmintics used in human and animal medicine and as such the pharmacokinetics and pharmacodynamics of these drugs by mammals has been well documented. Ivermectin is metabolised to ten metabolites by human liver microsomes (Zeng *et al.*, 1998). However, the turn over is relatively low. The plasma half-life of ivermectin following subcutaneous injection is approximately 2.04 days in sheep and 4.95 days in cattle (El-Banna *et al.*, 2008). Following oral administration to sheep the half life is approximately 3.7 days (Mestorino *et al.*, 2003). In both cases the major route of clearance is in the faeces with minimal biotransformation. In contrast, albendazole appears to be metabolised to only 3 main metabolites: the pharmaceutically active albendazole sulphoxide (ABZ-SO) and inactive albendazole sulphone (ABZ-SO_2) and albendazole amino sulphone (Mirfazaelian *et al.*, 2002). Turnover of albendazole is rapid. The half-life of albendazole upon incubation with human liver microsomes is around 39.2 minutes (Li *et al.*, 2003b). Albendazole cannot be measured in serum following oral dosing in humans, sheep or cattle, due to the rapid first-pass metabolism of the parent molecule (Marriner *et al.*, 1986; Prichard *et al.*, 1985; Penicaut *et al.*, 1983; Marriner *et al.*, 1980). Albendazole sulphoxide can be measured in high concentrations and is thought to be responsible for the effect of the drug in the host.

There have been relatively few publications documenting the major metabolites of benzimidazoles and macrocyclic lactones in parasitic nematodes. There are no published papers examining the metabolism of ivermectin by nematodes. Alvinerie *et al.* (2001) reported the presence of an undefined moxidectin metabolite following incubation with homogenates of *Haemonchus contortus* adults. This work did not include mass spectrometry so the identity of the metabolite remains undefined. However, production of the metabolite was inhibited in the presence of carbon monoxide suggesting that the metabolite was the result of cytochrome P450 metabolism. Metabolism of albendazole by parasites has been reported in the literature (Cvilink *et al.*, 2009b; Cvilink *et al.*, 2008; Solana *et al.*, 2001). The most recently published data revealed that

Haemonchus contortus produces both albendazole sulphoxide and two glucose conjugates of albendazole *in vitro* (Cvilink *et al.*, 2008). Studies with *Dicrocoelium dendriticum* revealed only the oxidation metabolites ABZ-SO and ABZ-SO_2. Glucosylation is a much less common pathway of metabolism in mammals compared to glucuronidation, but is common in invertebrates where glucuronidation is not encountered (Hamamoto *et al.*, 2009; Huber *et al.*, 2009; Erve *et al.*, 2008; Gessner *et al.*, 1973; Dutton, 1966).

Caenorhabditis elegans has not previously been used as a model for anthelmintic metabolism. However, microsomal extracts have been extracted from *C. elegans* and used to assay the metabolism of endogenous fatty acids (Kulas *et al.*, 2008; Zhang *et al.*, 2003). In addition, metabolites of several potential environmental toxins have been shown to be produced upon incubation with the free-living nematode (Schafer *et al.*, 2009)

Cytochrome P450s are haem-containing enzymes that have been associated with insecticide resistance in many different insects (Amenya *et al.*, 2008; Djouaka *et al.*, 2008; Zhu *et al.*, 2008b; Daborn *et al.*, 2002; Berge *et al.*, 1998). *Caenorhabditis elegans* and many parasitic nematodes do not have functional haem synthesising pathways and rely on exogenous sources of haem (Rao *et al.*, 2005). However, it is recognised that haem containing enzymes, such as cytochrome P450s, are both present and functionally necessary in these organisms. Cytochrome P450 enzymes can be found in high concentrations in microsomal protein preparations along with UDP-glucuronosyl transferases and flavin monooxygenases. The microsomal fraction is an operational definition of the subcellular fraction sedimented following the prior removal of mitochondria by centrifugation at 10000g (DePierre *et al.*, 1976). It consists mostly of the endoplasmic reticulum of the cell and the enzymes which are bound to these membranes. However, there may be some contamination with lysosomes and peroxisomes. Microsomes are routinely used to investigate metabolism of drugs both in the development and post-development phase of drug design. Using microsomes allows more accurate control of the experimental conditions and removes the confounding factor of drug uptake into cells or in this case the nematode. However, in mammals and presumably in nematodes, microsomes may not evaluate metabolism through other enzymatic pathways, including short chain dehydrogenases, carboxyl esterases and some glutathione-s-transferases,

which are likely found in the cytosol of cells (Pfizer PDM/SOP/20 Version 2.0; Brodie *et al.*, 1955). Therefore, this study made use of whole worm- drug incubations (*ex vivo* exposures), in addition to microsome- drug incubations, to assess a broader scope of potential metabolic pathways.

These experiments were designed to confirm metabolism of ivermectin and albendazole by *C. elegans* and *H. contortus* and to compare the metabolites produced to those previously discovered in parasitic helminths. This will allow validation of the techniques used to analyse the mechanisms of metabolism of any anthelmintic drug.

5.2 Materials and Methods

5.2.1 Materials

5.2.1.1 *Caenorhabditis elegans* strains

Bristol N2: *C. elegans* wild type, DR subclone of CB original (Tc1 pattern I). Gift from the CGC.

CB3474: *ben-1(e1880) III*. Mutation of the ß-tubulin gene resulting in high level resistance to benzimidazoles. Dominant at 25°C, recessive at 15°C. Gift from CGC.

DA1316: *avr-14(ad1302)*; *glc-1(pk54)*. Mutations of two major subunits of glutamate-gated chloride channels resulting in high level resistance to ivermectin. Gift from CGC

5.2.1.2 *Haemonchus contortus* strains

MHco3 (ISE): susceptible inbred strain used for the *Haemonchus contortus* genome project (Roos *et al.*, 2004; Otsen *et al.*, 2001)

MHco4 (WRS): White River Strain. Ivermectin and benzimidazole resistant strain isolated in South Africa and maintained by experimental passage (van Wyk *et al.*, 1988).

MHco10 (CAVR): Chiswick Ivermectin Resistant Strain. Ivermectin resistant strain originally isolated in Australia and maintained by experimental passage (Le Jambre *et al.*, 1995)

All *H. contortus* strains were received from the Moredun Institute, Edinburgh.

5.2.1.3 Human Liver microsomes

Pooled donor Human Liver Microsomes from Gentest

5.2.2 Preparation of microsomes

5.2.2.1 *Caenorhabditis elegans* culture conditions

Large numbers of nematodes were grown in standard liquid culture medium containing 100 units/ml nystatin (Sigma, N3503). Each 250ml culture was started with either synchronised L1 worms or worms were washed from 20 x 5cm diameter NGM plates containing many adult worms (3-4 days growth at 20°C).

Several compounds were added to cultures in an attempt to improve the yield of microsomal protein/ cytochrome P450s:

Ivermectin (Sigma, I8898), final concentration 100ng/ml (114nM), for 12-16 hrs before harvesting (cultures of strain DA1316 only).

Fenofibrate (synthesised "in-house" at Pfizer Animal Health, Sandwich), final concentration 20µg/ml (55.43µM), for 24-60hrs before harvesting.

delta-Aminolevulinic acid (Sigma, A3785), final concentration 167.5µg/ml (1mM), for the duration of the culture.

The cultures were allowed to grow at 20°C for 4-5 days until many adult worms were present in a 200µl sample. Culture flasks were rested on ice for 15-20min to allow the worms to settle. Using a 50ml pipette the supernatant was removed to approximately 50ml, the worm pellet resuspended and transferred to a 50ml falcon tube. Samples were centrifuged at 2500rpm, 4°C for 3min in a table top centrifuge. The supernatant was removed using a pipette and the pellet resuspended in ice cold M9 buffer. This process was repeated twice to remove bacterial contamination.

5.2.2.2 *Haemonchus contortus* culture conditions

2.5-3 million infective stage larvae (L3) in tap water were rested on ice for 15-20 min to allow them to settle. The supernatant was removed to 200ml, using a 25ml pipette, and the resulting pellet resuspended and transferred to 4 x 50ml falcon tubes. The worms were pelleted by centrifugation at 2500rpm, 4°C for 3 minutes. The supernatant was removed to 10ml in each of the four falcon tubes.

The L3 were exsheathed by adding 200µl of Milton sterilising fluid (1% sodium hypochlorite) to each of the falcon tubes and shaking at 150rpm, 37°C. 20µl was removed from each tube every 2-3 minutes and examined under 40x magnification until the majority of the worms had exsheathed. Each of the falcon tubes were then filled to 50ml with ice cold M9 and centrifuged at 2500rpm, 4°C for 3min. The supernatant was removed and the tube filled to 50ml with ice cold M9 buffer again. The larvae were washed in M9 a further three times.

5.2.2.3 Homogenisation of Nematodes and Microsome isolation

Following culture and isolation, *C. elegans* pellet size varied between 2-3ml. The pellet was suspended in two volumes of TRIS-buffer (50mM, pH7.5) supplemented with 0.25M sucrose, 2mM EDTA, 0.15M KCl, 0.5M dithiothreitol (DTT), 0.25mM phenylmethylsulphonylfluoride (PMSF) and complete protease inhibitor cocktail (Roche, 04 693 124 001). Alternatively, the pellet was suspended in simple phosphate buffer (pH 7.5) with the addition of complete protease inhibitor cocktail. The suspension was split between 1ml glass homogenisers and homogenised for 15-20min. The homogenate was subject to secondary homogenisation using either 3 x 30 second pulses of an Ultra Turrax T8 homogeniser (IKA-Werke) at full speed or 3 x 30 second pulses of sonication using a Soniprep 150 (Sanyo). All steps were carried out on ice. Homogenates were subsequently used for microsome preparations or were incubated directly with the drug.

H. contortus L3 pellets were suspended in buffer as above. The small L3 larvae proved difficult to homogenise even following exsheathment. Several methods were undertaken including the use of 1ml glass homogenisers; freezing in liquid

nitrogen followed by grinding using a mortar and pestle or tissue grinder; high speed vortexing with glass beads; homogenisation with Ultra Turrax and sonication. The most successful microsome preparations followed homogenisation with Ultra Turrax T8 for 4 x 30 seconds with intervals of 1min followed by sonication for 4 x 30sec with intervals of 1min. All steps were carried out on ice.

Homogenates of both *C. elegans* adults and *H. contortus* L3 were immediately subject to differential centrifugation in a Sorval Discovery 100 ultracentrifuge. The homogenates were first centrifuged at 3000g for 5 minutes to remove cuticle and debris. The supernatant was removed and centrifuged at 10000g for 10 minutes and the supernatant of this step was subject to centrifugation at 100000g for 1hr. The supernatant of the final spin, containing the cytosol fraction, was removed and stored at -80^{o}C. The microsome pellet was resuspended in TRIS-buffer (50mM, pH 7.5) supplemented with 20% glycerol, 5mM EDTA, 0.5mM DTT, 0.25mM PMSF and the complete protease inhibitor cocktail (Roche, 04 693 124 001). If possible the microsomes were analysed and used the same day. Alternatively, the samples were stored at -80^{o}C until used.

5.2.2.4 Analysis of microsomal protein

5.2.2.4.1 Protein concentration

The protein concentrations of microsome and cytosol fractions were analysed using a BIORAD protein 96-well plate assay, based on the protocol described by Lowry *et al.* (1951). A 10µl sample of the microsomal or cytosolic preparations was diluted 1:100 in MQ H_2O and stored on ice until analysis. A standard curve was made using bovine serum albumin (BSA), also from BIORAD, at the following concentrations:

	Final protein concentration (mg/ml)	Volume MQ H_2O (µl)	Addition
A	0.77	100	100µl stock BSA
B	0.38	100	100µl solution A
C	0.19	100	100µl solution B
D	0.44	100	100µl solution C
E	0.22	100	100µl solution D
F	0.11	100	100µl solution E
G	0	100	100µl MQ H_2O

25µl of each standard solution and each of the diluted experimental samples was transferred in triplicate into a flat bottomed microtitre plate. 25µl of BIORAD reagent A was then added to each well. Finally 200µl of Biorad reagent B was added to each of the wells and the plate was allowed to stand at room temperature for at least 15min.

Absorbance at 750nm of each well was measured using a Spectramax plus 384 (Molecular Devices) and Softmax Pro 4.7.1 software. The mean absorbance of each of the standard solutions was calculated and plotted against protein concentration using Microsoft Excel. A line of best fit and associated Peterson coefficient (R^2) was calculated. Assuming R^2 was greater than 0.99, this was used to calculate the protein concentration of the experimental samples.

In most experiments the absorbance was not linear over the entire range of BSA concentrations. As the experimental samples were always very dilute the absorbance data of the most concentrated standard was removed from the analysis, which gave a linear relationship between protein concentration and absorbance.

5.2.2.4.2 Cytochrome P450 concentration

Human liver microsome samples were analysed by diluting 1:100 in phosphate buffer, pH 7.0. However, all nematode samples were more dilute and a dilution of 1:10 was used. Analysis was carried out as per Pfizer PDM SOP 20 (version 2.0):

The preparations were exposed to carbon monoxide by a stream of bubbles at approximately 1 bubble/ second for 1min. Spectral analysis was carried out using a Jasco V-650 spectrophotometer and Jasco spectra manager software. A baseline reading was taken by measuring the absorbance of phosphate buffer alone between 400 and 500nm. The experimental sample was then split between two matched cuvettes (Hellma Worldwide), i.e. reference and sample, and the reading was repeated. Approximately 1mg of sodium dithionite was added to the reference sample and the cuvette was inverted repeatedly for 1min. Both cuvettes were then subject to a final scan between 400-500nm. The absorbance data from the final reading was overlaid on the non reduced reading and

subtracted. After smoothing the subtracted spectrum was used to calculate cytochrome P450 concentration using the following formula:

$$\text{cytochrome P450 (nmol/ml)} = \frac{\text{abs. diff. x 1000}}{91} \text{ in diluted sample}$$

Where, abs. diff. = Absorbance difference (450-490nm) from trace

Extinction coefficient= $91 mM^{-1}cm^{-1}$

Cuvette path length= 1cm

5.2.3 Drug- Microsome Incubations

5.2.3.1 Human Liver Microsomes

Human liver microsome (HLM)- drug incubations were carried out in a total volume of 800µl in 50mM phosphate buffer pH 7.0. A NADPH generating system was used consisting of 5mM $MgCl_2$, 5mM isocitric acid, 1mM $NADP^+$ and 1IU/ml isocitrate dehydrogenase. HLM were added to a final P450 concentration of 400 pmoles/ml and the drug was added to 1µM-10µM. No NADP, no microsome and no compound controls were included in the experiments. Reactions were kept in a waterbath at 37°C with shaking at 200rpm for 1 hour before being terminated by the addition of 5 volumes of ice-cold acetonitrile (MeCN). The samples were centrifuged at 3000rpm, 4°C for 40min; the supernatant decanted and evaporated to dryness under nitrogen at 40°C using a Turbovap LV (Zymark). Samples were stored at -20°C until further analysis by HPLC-MS.

5.2.3.2 Nematode Microsomes

Cytochrome P450 concentrations could not be accurately defined for nematode microsome preparations. Therefore, a final microsome protein concentration of 0.5-1mg/ml was used. Nematode microsome incubations were either carried out as HLM incubations or using the incubation protocol used by Kulas *et al*. (2008): 100mM potassium phosphate buffer pH 7.2 with 0.1mM EDTA and 0.5µM flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). NADPH (1mM) was

used as the hydrogen source due to the lower temperature at which the *C. elegans* reactions were incubated. *C. elegans* microsome incubations were carried out at 25°C with shaking at 200rpm for between 24-72hrs. *Haemonchus contortus* microsome incubations were carried out at 37°C, with shaking at 200rpm for between 24-72hrs. In both cases the incubations were terminated and further treated as per HLM incubations.

5.2.4 Ex-vivo drug exposure

5.2.4.1 *C. elegans ex-vivo* drug exposures

Nematodes were washed from 5 x 10cm diameter NGM plates with M9. The worms were pelleted and washed twice in M9. The pellet of worms was split equally between two 250ml volume of standard liquid culture medium plus 100 units/ml nystatin and 3mls concentrated OP50 suspension. The flasks were placed in a shaking incubator at 20°C, with shaking at 240rpm for 4-5 days. Fenofibrate, a PPARα agonist known to induce CYPS and UGTs, was added to three biological replicates (experimental and heat-control cultures) for 12hrs prior to the addition of the anthelmintic. A further three biological replicates were not exposed to fenofibrate.

On the final day of culture, when many adult worms were present and the cultures were almost starved of OP50, one of the paired cultures was killed by heating to 50°C for 30min in a waterbath. A 200µl sample was taken from both of the cultures to ensure that the experimental flask contained many healthy adult worms and that the worms in the heat exposed flask were dead. 0.5ml of concentrated OP50 was added to each of the cultures. Drug was added to both cultures: albendazole was added to a final concentration of 15µg/ml (56.53µM); ivermectin was added to a final concentration of 100ng/ml (114nM). Cultures were maintained in the shaking incubator (240rpm, 20°C) for a further 7hrs. To harvest, the cultures were placed on ice for 15min to allow the worms to settle. The supernatant was removed to approximately 50ml and the suspension from each culture was transferred to a 50ml falcon tube. The worms were centrifuged at 2500rpm, 4°C and washed three times in ice-cold M9 buffer to remove bacteria and excess compound. Finally, the supernatant was removed and the worm pellet snap frozen in liquid nitrogen and stored at -80°C until analysis.

Bacterial controls were carried out in a similar way using cultures containing no nematodes and 3ml concentrated OP50 suspension per 250ml culture.

5.2.4.2 *H. contortus ex vivo* drug exposures

2.5-3 million L3 larvae were exsheathed as per **Section 5.2.2.3** and were split between 2 x 50ml falcon tubes. One tube was subject to heating to 50^{o}C for 30min to kill the nematodes. This was confirmed by analysis of 100µl sample under 40x magnification. Both samples were suspended in 10ml of M9 buffer, and albendazole or ivermectin was added to both of the tubes to a final concentration of 100ng/ml (114nM) IVM or 15µg/ml (56.53µM) ABZ. The samples were incubated at 37^{o}C, with shaking at 150rpm for 7hrs. The L3 larvae were then washed three times in M9 and either snap frozen in liquid nitrogen until analysis or analysed immediately.

5.2.4.3 Homogenisation and extraction of metabolites

Pellets of both *Caenorhabditis elegans* and *Haemonchus contortus* were homogenised using both an Ultra Turrax homogeniser and sonication. Ten volumes of methanol: Tris pH9, 9:1, or acetonitrile were added to the resulting homogenates and allowed to stand at room temperature for 30min. The samples were then centrifuged at 4000rpm, 4^{o}C for 40min to remove solid debris and the supernatant removed and evaporated to dryness under nitrogen. The samples were stored at -20^{o}C until further analysis by HPLC-MS.

5.2.5 HPLC-MS methods

5.2.5.1 Ivermectin

Both microsome and whole nematode incubations with ivermectin were analysed identically. Dried samples were first resuspended in 200µl 50:50 methanol: H_2O and transferred to a tear drop microtube. The samples were then centrifuged at 10000rpm, 4^{o}C for 10min and the supernatant transferred to a fresh tear-drop microtube. Samples derived from *ex vivo* drug incubations required several centrifugation steps to remove solid debris and prepare them for HPLC-MS analysis.

20µl of experimental samples were injected onto a Phenomenex Onyx monolithic C18 column (100 x 3mm) using an HTS PAL autosampler from CTC Analytics. An Agilent 1100 series HPLC pump system was used to provide the mobile phase gradient at a flow rate of 1ml/min. The wash from the column was analysed using an Applied Biosystems 4000 QTrap, LC/MS/MS system with electrospray ion source in positive ion mode. Analysis was carried out by a combination of Q1 analysis, MS/MS analysis and MRM methods. Results were analysed using Analyst version 1.4.1 software (Applied Biosystems).

5.2.5.2 Purification of ivermectin

Initial analyses of ivermectin using the system described in **Section 5.2.5.1** revealed low level impurities to be present in a standard solution of ivermectin. A prep liquid chromatography system was used to remove these impurities, which would have confounded analysis of low level metabolism. Stock ivermectin dissolved in 50:50 methanol: H_2O was injected onto a HICHROM HIRPB, base deactivated C18 column with 5µm packing (250 x 7.75mm). Agilent 1200 series pumps and an Agilent 6110 quadrupole LC/MS system were used. Prep LCMS software was used to analyse and collect the ivermectin fraction based on UV absorption. Reanalysis as per **Section 5.2.5.1** demonstrated the successful removal of impurities.

5.2.5.3 Albendazole and midazolam

Albendazole and midazolam incubations were resuspended in 200µl of 50:50 acetonitrile: MQ water supplemented with 0.1% formic acid. Sample preparation prior to HPLC-MS analysis was otherwise identical to ivermectin incubations.

5µl of experimental sample was injected onto a Waters HSS 1.8µm C18 column (100 x 1mm), using a Waters Acquity Ultra Performance LC system. Mobile phase was applied to the column at 200µl/min. MS analysis was carried out using a Micromass MS Technologies Q-Tof Premier™. A reference spray using Leucine Enkephalin was used to provide accurate mass data. Analysis was carried out by Q1 and MS/MS methods using Mass Lynx 4.1 software (Waters).

5.3 Results

5.3.1 Microsomal extract incubations

5.3.1.1 Microsome preparations from *C. elegans and H. contortus*

Kulas *et al.* (2008) reported the successful preparation of microsomes containing active cytochrome P450 enzymes from *C. elegans* strains. A modification of the protocol described in this paper was used to extract microsomes from both *C. elegans* and *H. contortus*. The yield of microsomal proteins per gram of nematode tissue was extremely small. However, by growing large numbers of *C. elegans* in liquid culture for four days approximately 6-9mg of protein could be extracted. Yields from L3 *H. contortus* were consistently poor (0.5-3mg). This is likely to be due to the difficulty in homogenising the larvae in an environment conducive to extracting active proteins (see **Section 5.2.2.3**).

Assessment of the presence and concentration of cytochrome P450 enzymes relies on their characteristic peak absorption at 450nm when in the reduced form, following treatment with sodium dithionate, and saturated with carbon monoxide. None of the prepared nematode microsomal extracts showed a convincing soret peak at 450nm. Preparations from all *C. elegans* cultures had intense peaks with maxima at approximately 421nm. Preparations from *H. contortus* had maxima at approximately 427nm. However, the concentration of microsomal protein was extremely low and only two spectral readings were carried out so this may not be accurate. Addition of fenofibrate, a known cytochrome P450 inducer (Kulas *et al.*, 2008) or ivermectin to *C. elegans* cultures prior to microsome preparation did not result in any change to the spectra. Delta- aminolevulinic acid, a haem precursor, has been used to improve the yield of P450 extractions from *Caenorhabditis elegans* (*pers. comm.* Dr. R. Menzel). However, supplementation of cultures with 1mM d-aminolevulinic acid did not alter the spectrum. In addition, microsome preparations from *Caenorhabditis elegans* were prepared in simple phosphate buffer supplemented with complete protease inhibitor cocktail (Roche, 04 693 124 001). The absorbance spectrum from microsomes prepared in this manner was identical to that of those prepared in the buffer described by Kulas *et al.* (2008).

To control for error in the spectral analysis of the extracts, commercially available human liver microsomes (HLM) were also analysed. Typical spectra for *C. elegans*, *H. contortus* and HLM are shown in **Fig.5-1** to **5-3**.

A spectral peak at 420nm is thought to be indicative of denatured P450 enzymes. However, it has also been suggested that an intense soret peak at 421nm, seen commonly in invertebrates may in fact be a functional haemoprotein (Rocha-e-Silva TA *et al.*, 2001). Therefore, drug incubations were carried out with the microsomal preparations despite there being no measurable P450 content.

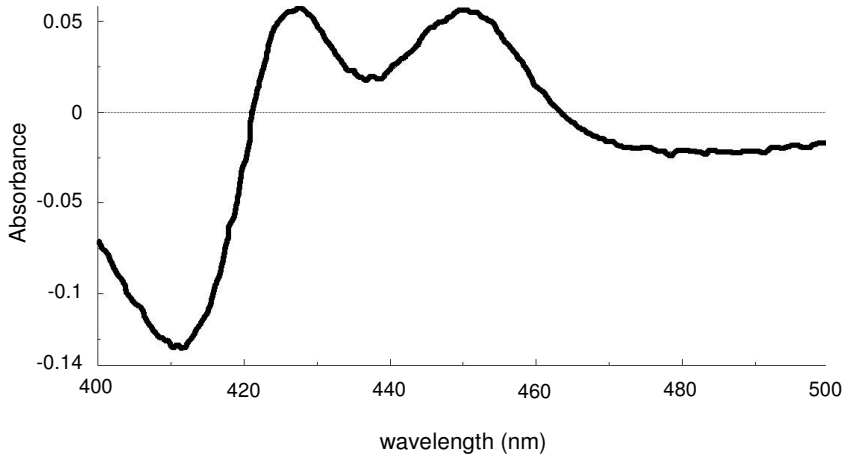


Figure 5-1: HLM absorbance spectrum

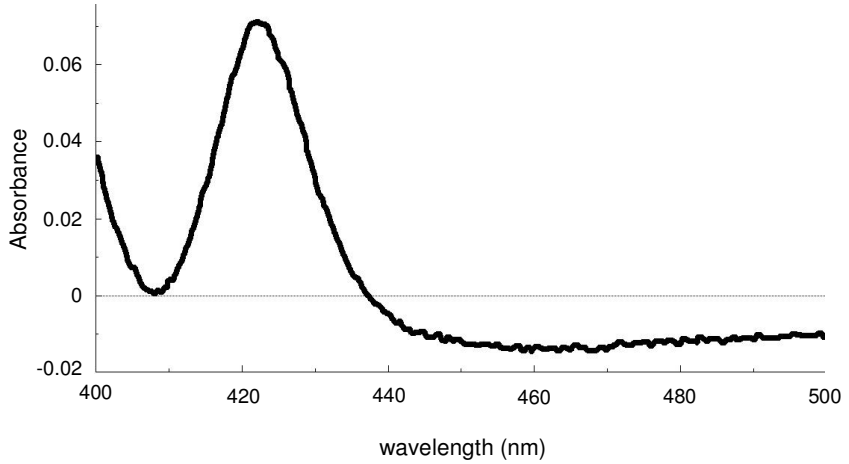


Figure 5-2: *C. elegans* strain DA1316 microsomal absorbance spectrum

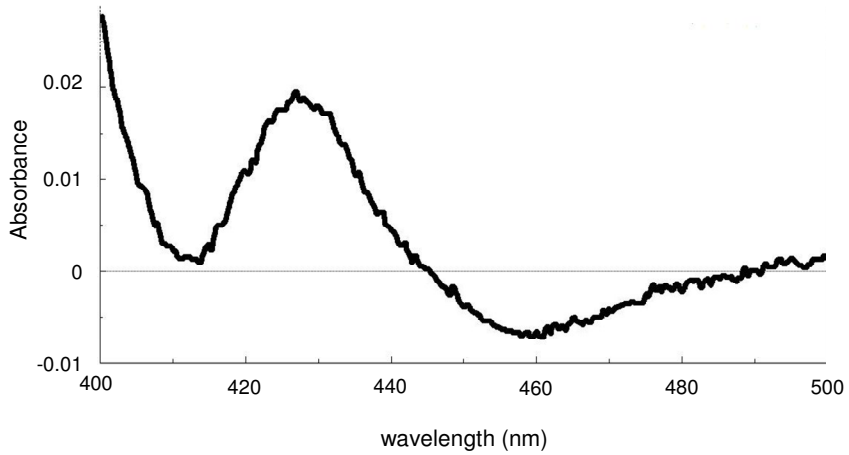


Figure 5-3: *H. contortus* strain CAVR microsomal absorbance spectrum

The absorbance spectrum of human liver microsome preparations shows a peak at 450 nm representing the cytochrome P450 enzyme content. Microsomal preparations from both *Caenorhabditis elegans* and *Haemonchus contortus* do not show a peak at 450nm.

5.3.1.2 Analysis of absorbance spectra of nematode culture medium

Liquid culture medium supplemented with delta-aminolevulinic acid was notably darker than that with no supplementation. This suggested that there was an increase in haem, presumably synthesised by the *E. coli* food source. A sample of this medium was taken and subjected to carbon monoxide exposure and reduction with sodium dithionite in the same manner as the microsomal preparations. However, this sample did not go through any of the homogenisation, sonication or centrifugation steps which were presumed to be the likely stages at which P450 enzymes could be denatured.

The absorbance spectrum of this sample showed a soret peak at exactly the same wavelength as the microsomal preparation of the worms grown in it. This result suggests that the P421nm soret peak does not represent that of a denatured cytochrome P450. The predominant haem-containing protein in the nematode is likely to be derived directly from *E. coli*. It is possible that the absorbance spectrum of this protein is masking that of smaller concentrations of modified haem-proteins in the nematode.

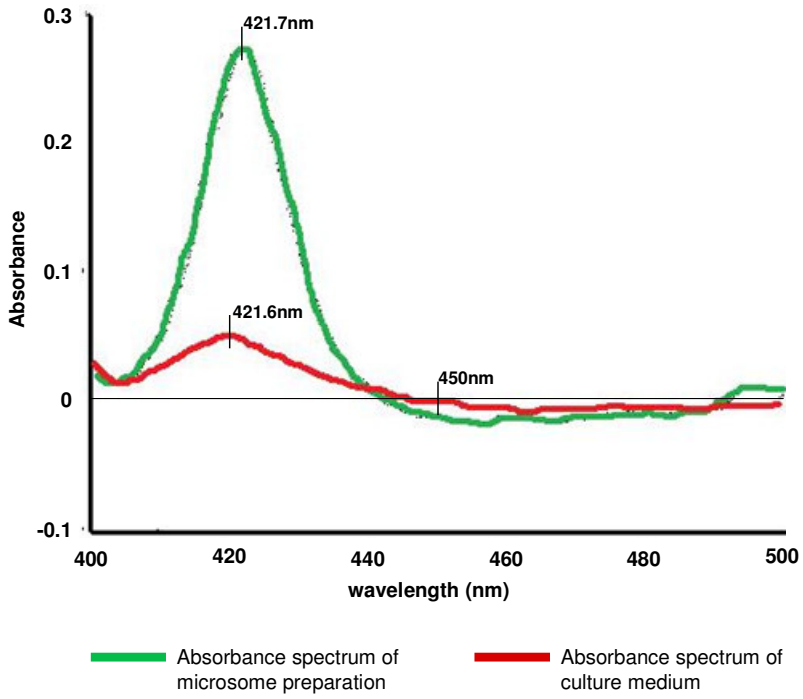


Figure 5-4: Absorbance spectrum of DA1316 microsomal preparation and of culture medium The soret peak of the both the microsomal preparation and the culture medium are almost identical.

5.3.2 HPLC-MS analysis of anthelmintic- microsome incubations

5.3.2.1 Development and validation of HPLC-MS method for ivermectin and metabolites

The HPLC-MS method used was based on that presented for the analysis of human liver microsome- ivermectin incubations (Zeng *et al.*, 1998). Using the equipment available (see **Section 5.2.5.1**) the H_2O- acetonitrile gradient described by Zeng *et al.* (1998) did not adequately separate the elution times of ivermectin and metabolites. Therefore, the commercially available mobile phase mixes MF5 and MF4 were used, see **Chapter 2** for details. The gradient began at 60% MF4 (organic): 40% MF5 (aqueous) and proceeded to 100% MF4 over 27min.

A multiple reaction monitoring (MRM) technique was used which allows sensitive detection of predefined metabolites. This was an appropriate method for this analysis as previous work has shown ivermectin undergoes low level metabolism resulting in low metabolite signal. In addition, both ivermectin and its known phase I metabolites are highly lipophilic resulting in a late elution time from the column in combination with non specific residue. This makes it extremely difficult to pick out specific metabolite peaks amongst the general increase in the total ion chromatogram signal.

In order to optimise the mass spectrometry method, a standardised solution of ivermectin was injected directly into the mass spectrometer at 1ml/min. Scanning between 200-1000Da identified all ivermectin ions. The declustering potential was then optimised to maximise the signal for each of these ions. Fragment ion spectra were analysed and the collision energy optimised for each of the parent ions to maximise the fragment signals.

Two significant parent ions were found for ivermectin: a sodium adjunct (897.5Da) and an ammonium adjunct (892.4Da), as per Zeng *et al.* (1998). Whilst the sodium adjunct had an intense maximal signal at a declustering potential of 230V, this ion was unstable when fragmented resulting in low intensity fragment ion spectra. The ammonium adjunct had a maximal signal at a declustering potential of 70V and provided consistent fragment ion spectra with collision energy of 35V. These parameters were used for the rest of the analyses. The

major fragment ions noted for ivermectin were as reported in Zeng *et al.* (1998). A peak at approximately 551Da was consistent with two dehydrations of the aglycone fragment of ivermectin; a peak at approximately 145Da was consistent with a single saccharide ion and a peak at approximately 307Da was consistent with the spirokeital moiety of the aglycone fragment (**Fig. 5-5A**).

In order to confirm the validity of the HPLC-MS method, ivermectin was first incubated with human liver microsomes (400pmoles cytochrome P450). Q1 scans were used to identify potential metabolites based on mass changes detailed by Holcapek *et al.*, (2008). Fragment ion analyses of each identified metabolite revealed the 307Da fragment ion, or modulations of this moiety, to be consistently the most intense. Therefore, transitions of the parent molecule and this fragment were used to identify ivermectin and metabolites in further drug incubations, see **Table 5-1**. This MRM method clearly identified all but two of the metabolites published by Zeng *et al.* (1998), see **Fig. 5-5B**.

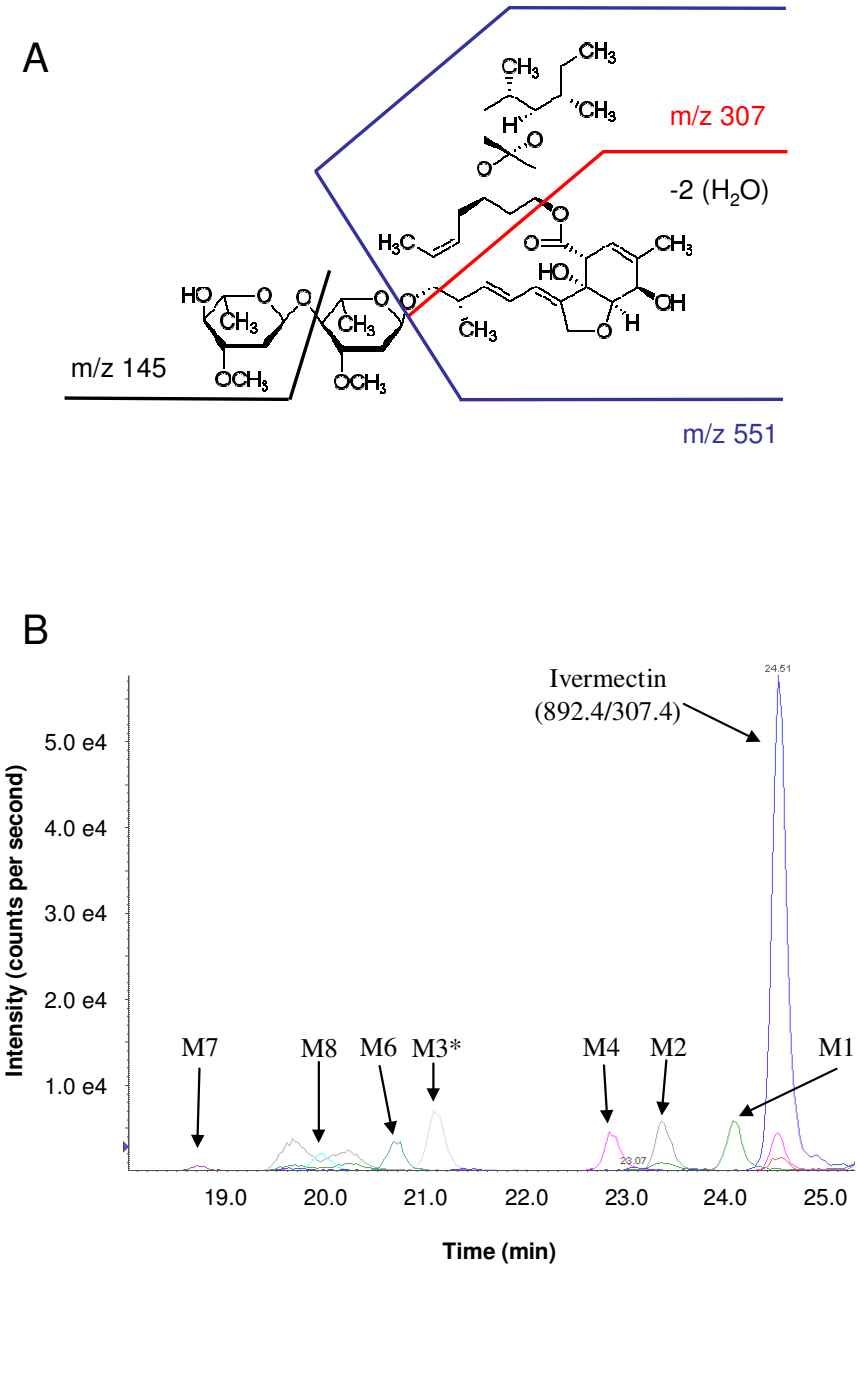


Figure 5-5: Major fragment ions of ivermectin and MRM chromatogram of HLM-ivermectin incubations
A: Adapted from Zeng *et al.* (1998). The major fragment ions of ivermectin following MS-MS analysis. The fragment of m/z 307.4 was consistently the most intense in both ivermectin and its metabolites. B: Typical MRM chromatogram of an HLM-ivermectin incubation. Note the large peak representing unmetabolised ivermectin. Metabolites (M1-M8) are labelled as per Zeng *et al.* (1998).M1 transition- 878.4Da/ 307.4Da; M2 transition- 908.4Da/ 307.4Da; *M3 transition- 908.4Da/ 323.4Da (identical transition to Zeng *et al.* M9 also); M4 transition- 894.4Da/ 307.4Da (peak at same elution time as ivermectin due to naturally occurring 2 x C14 isotope); M6 transition-894.4Da/ 323.4Da; M7 transition- 764.4Da/ 323.4Da; M8 transition- 924.4Da/ 323.4Da.

Transition	ID (assigned by Zeng *et al*. (1998))	MS1 (Da)	MS2 (Da)
1	Ivermectin B_{1a}	892.4	307.4
2	Metabolite 1 (3"-*O*-desmethyl-IVMB_{1a})	878.4	307.4
3	Metabolite 2 (4-OHMe-IVMB_{1a})	908.4	307.4
4	Metabolite 3 & 9 (26-OHMe-IVMB_{1a} and 24-OHMe-IVMB_{1a})	908.4	323.4
5	Metabolite 4 (3"-*O*-desmethyl, 4-OHMe-IVMB_{1a})	894.4	307.4
6	Metabolite 5	764.4	307.4
7	Metabolite 6 (3"-*O*-desmethyl, 26-OHMe-IVMB_{1a})	894.4	323.4
8	Metabolite 7 (26-OHMe-IVMB_{1a} monosaccharide)	764.4	323.4
9	Metabolite 8 (4,26-dihydroxymethyl-IVMB_{1a})	924.4	323.4

Table 5-1: MRM transitions for ivermectin and metabolites
MRM transitions initially used to identify ivermectin and its metabolites in HLM, nematode microsome and whole worm IVM incubations. The specific identities of the metabolites were assigned by Zeng *et al.* (1998) using a combination of ^{1}H-NMR, LC-MS/MS and HPLC retention times (ID of metabolite 5 could not be confirmed). In the current study the specific identity of metabolites were not assessed.

The elution times and order of elution for the various metabolites were slightly altered in comparison to those reported by Zeng *et al*. (1998). However, this is likely explained by the different HPLC column and mobile phase used in this study. M5 (764.4/307.4), representing a loss of the disaccharide moiety and an oxidation of the hexahydrobenzofuran moiety of the aglycone, was produced in very small quantities in the study by Zeng *et al*. (1998) and its identity could not be confirmed. In the current study this metabolite was not identified. In addition only one peak of transition 908.4/ 307.4 was identified, representing a single oxidation of the hexahydrobenzofuran moiety of the aglycone. Two metabolites with this transition with differing elution times were identified by Zeng *et al*. (1998): M3 and M9.

Transition 908.4/307.4 showed a peak at 23.4 minutes which was identified as M2. However, this transition also had a broad based double peak from 19.4-20.4min. MS/MS analysis at this time point revealed this not to be a metabolite of ivermectin and so this was ignored. There was a significant peak of transition

894.4/ 307.4 at 24.51min, the same elution time as the parent ivermectin. MS/MS analysis and assessment of the relative intensity of this peak suggested the presence of a naturally occurring isotype of ivermectin.

Initial analyses revealed low levels of metabolites in the ivermectin standard. Given the low level metabolism of ivermectin previously reported this may have hampered identification of true cytochrome P450 derived metabolites (Perez *et al.*, 2008; Zeng *et al.*, 1998; Chiu *et al.*, 1984). Ivermectin standards were purified using a prep LC method as detailed in **Section 5.2.5.2**. Analysis of the purified ivermectin using the MRM method described above revealed no metabolite impurities.

5.3.2.2 Development and validation of the HPLC-MS method for albendazole and metabolites

Albendazole is a much smaller molecule than ivermectin. An albendazole standard was found to have an intense peak using the standard phase I and II drug metabolite identification system available at Pfizer R&D. The benefits of this system included ultra performance liquid chromatography, resulting in excellent resolution of metabolite peaks, and Q-Tof (time of flight) mass spectrometry with accurate mass identification using LockSpray. This is a method by which the mass of metabolites can be accurately measured to within 0.05Da by normalising mass/ charge (m/z) data to a standard solution of a compound of known m/z, in this case leucine enkephalin. The reference compound is injected into a secondary reference ion sprayer and is analysed simultaneously with the compounds of interest.

Analysis of albendazole and metabolites used a gradient of 95:5% water: MeCN + 0.1% Formic acid (FA) to 100% MeCN + 0.1% FA over 12min. Mass spectrometry was carried out in positive ion mode, with a declustering potential of 25mV. The single protonated ion of albendazole (266.096Da) was the most abundant ion produced, showing an intense peak at 4.44min. Initial analysis of albendazole and its metabolites were carried out accurately without using a multiple reaction monitoring method.

Analysis of HLM- albendazole incubations revealed that albendazole is rapidly metabolised to albendazole sulphoxide (ABZ-SO), a pharmacologically active oxidation product of albendazole (3.18min, 282.091Da). 1μM albendazole incubated with 400pmoles human liver cytochrome P450 for 1hr at 37^{o}C had an ASOX peak 5.5 times more intense than the parent albendazole signal (**Fig. 5-6**). This is consistent with the high turnover of albendazole reported in humans and other mammals (Kitzman *et al.*, 2002; Mirfazaelian *et al.*, 2002). The pharmacologically inactive metabolite albendazole sulphoxone (ABZ-SO_2) is present in the serum of treated humans, but does not appear to be a product of human liver microsome mediated metabolism and was not present in this study (Rawden *et al.*, 2000). It is possible that this metabolite is produced extra-hepatically or by other enzymes not present in microsome preparations. In addition to ABZ-SO a less intense peak was consistently seen at 3.92min with mass 208.093Da. This has not previously been reported but is consistent with cleavage across the amino bond of albendazole and will be referred to as amino-albendazole (**Fig. 5-7**). This peak is not present in microsome minus or NADP minus controls and therefore was proposed to be a CYP P450 derived metabolite of albendazole.

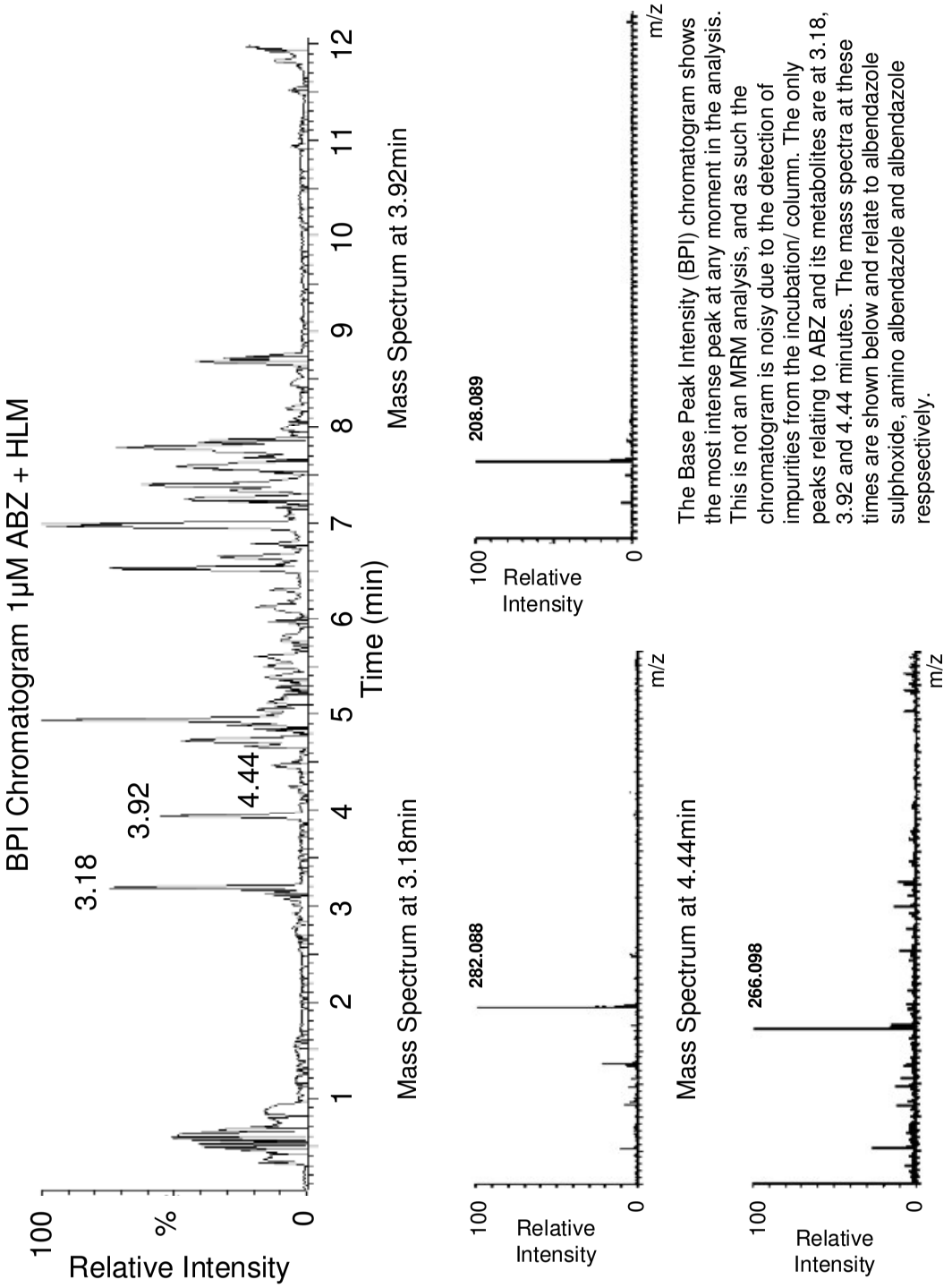


Figure 5-6: BPI chromatogram of HLM- albendazole incubation and mass spectra of significant peaks

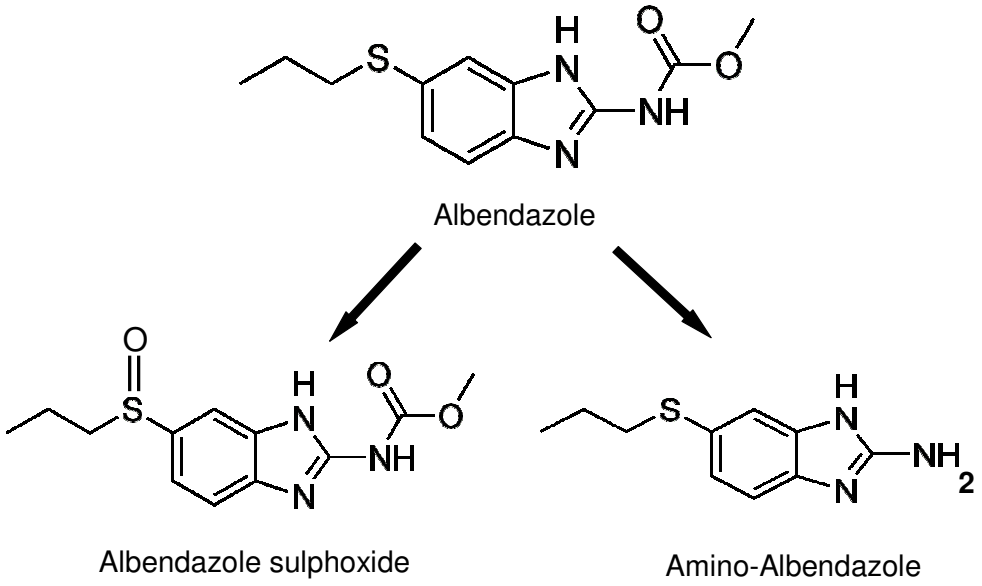


Figure 5-7: Proposed structures of albendazole and identified HLM metabolites Albendazole is rapidly metabolised to albendazole sulphoxide in mammals and high concentrations of this metabolite are seen in the blood. Amino albendazole has not previously been reported, but may be an intermediate metabolite to albendazole amino sulphoxone, which can also be found in the plasma of humans (Mirfazaelian *et al.,* 2002).

5.3.2.3 Nematode microsome preparations do not metabolise ivermectin or albendazole

Microsome preparations from *C. elegans* were incubated with both ivermectin and albendazole. Microsomal protein concentrations were varied from 0.5mg protein/ml to 2mg protein/ml. The incubations were carried out either in phosphate buffer supplemented with NADPH or in the modified incubation buffer containing FAD and FMN detailed by Kulas *et al.* (2008). Incubations were carried out at 25^{o}C for 24-72hrs. Drug concentrations were varied from 100nM to 10µM.

MRM analysis of ivermectin incubations revealed no significant metabolite peaks in any of the conditions described. Q1 scans revealed no significant metabolite peaks on the total ion chromatogram and specific searches for phase I metabolites (Holcapek *et al.*, 2008), followed by fragment ion analyses also revealed no metabolites. Similarly, no albendazole metabolites were identified.

Haemonchus contortus microsome preparations were treated in a similar manner except that incubations were carried out at 37^{o}C. Again, HPLC-MS of these reactions revealed no significant metabolite peaks.

5.3.2.4 Nematode microsome preparations do not metabolise midazolam

Midazolam is regularly used as a positive control for HLM drug incubations of new compounds. This benzodiazepine drug has extremely high turnover in the presence of HLM. Midazolam is a small molecule, accurate mass 326.781Da, whose phase I metabolites are adequately analysed using the UPLC-QTOF system described for albendazole above. Midazolam incubated with HLM at 37^{o}C for 1 hour is extensively metabolised to the 1′-hydroxy and 4′-hydroxy metabolites, accurate mass 342.772Da (Ghosal *et al.*, 1996).

C. elegans and *H. contortus* microsome preparations were incubated with midazolam at 1μM, as described for ivermectin and albendazole above. Analyses of these incubations did not reveal any significant metabolite peaks.

5.3.2.5 *C. elegans* homogenates do not metabolise ivermectin or albendazole

Previous work has cited nematode homogenates as being able to metabolise moxidectin and albendazole (Alvinerie *et al.*, 2001; Solana *et al.*, 2001). Homogenates of mixed stage *C. elegans* strain N2 grown in standard conditions for 4 days were made as per Alvinerie *et al.* (2001) and incubated with ivermectin at a total concentration of 1.5mg protein/ml for 72hrs at 25^{o}C. HPLC-MS analysis, of these incubations did not identify any significant metabolite peaks.

5.3.2.6 *C. elegans* cytosolic fractions do not metabolise ivermectin or albendazole

Microsomal fractions are thought to contain the vast majority of xenobiotic metabolising enzymes. However, several enzymes such as carboxyl esterases, epoxide hydrolases, sulphotransferase, and glutathione-s-transferases predominate in the cytosol fraction. Cytosol fractions derived from parasitic helminths have previously been reported to metabolise albendazole (Solana *et al.*, 2001). Therefore albendazole and ivermectin were incubated with 1mg cytosolic protein (the final supernatant following differential centrifugation)

prepared from *C. elegans*. HPLC-MS analysis of these incubations revealed no metabolites of either drug.

5.3.3 Inhibition of HLM reactions by nematode derived microsomal protein

It has previously been noted when preparing cytochrome P450 enzymes from *Drosophila melanogaster* that the wings and eyes of the organism contain inhibitors of cytochrome P450 reactions (*pers. comm.* Dr. D. Woods, Pfizer Animal Health). In order to identify the presence of inhibitors in nematode microsomal preparations, ivermectin was co-incubated with both HLM and those prepared from *C. elegans* at a final concentration of 1mg/ml. The presence of *C. elegans* microsomal protein resulted in an average 90.6% reduction in intensity of the HLM ivermectin metabolite peaks (lowest reduction 60%; highest reduction 100%), **Fig. 5-8**. This effect occurred in incubations at both 25^{o}C and 37^{o}C and irrespective of whether NADPH or an NADPH generating system was used as the hydrogen donor.

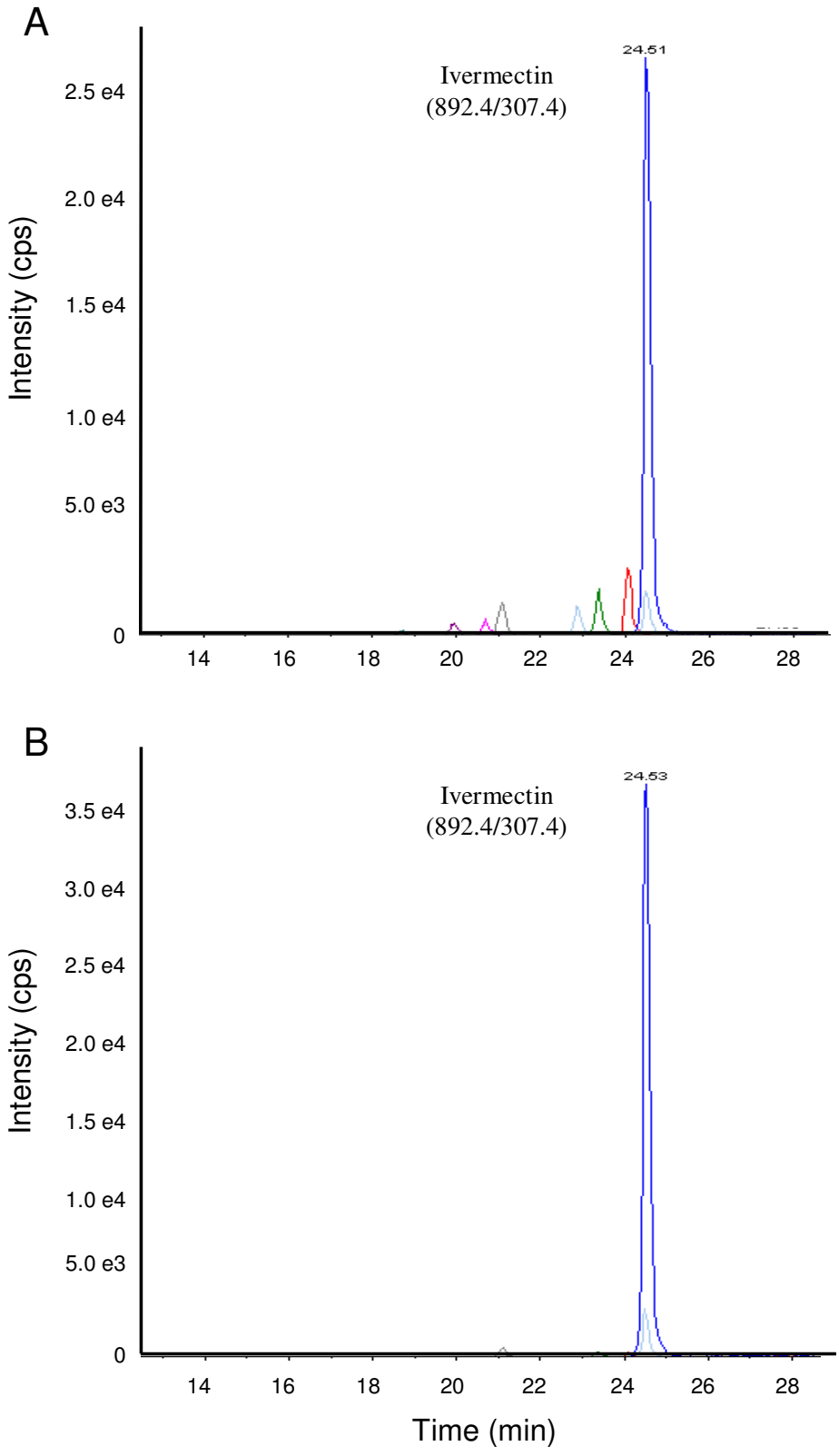


Figure 5-8: *C. elegans* microsome preparations inhibit HLM reactions
A: HLM ivermectin incubation analysis showing ivermectin peak with associated metabolite peaks. B: The same incubation with the addition of 1mg/ml *C. elegans* microsomal protein. Note the almost complete lack of metabolite production.

5.3.4 HPLC-MS analysis of ex vivo drug incubations

Due to the presence of cytochrome P450 inhibitors in the microsome preparations, a whole worm incubation protocol was adopted. Homogenates of live worms previously exposed to anthelmintic drugs would be expected to contain low levels of metabolites of the drugs. A similar technique was used by Schafer *et al*. (2009) to investigate the metabolism of PCB52.

C. elegans liquid cultures were allowed to grow at 20^{o}C for 5 days in total before the drug was added to ensure that there were many adult worms present. Worms were incubated with anthelmintic drug for 7hrs with or without prior exposure to the cytochrome P450/ UDP-glucuronosyl transferase inducer fenofibrate. Due to the presence of *E. coli* bacteria in the cultures as a food source for the nematodes, control cultures were also prepared. These cultures either had no nematodes present or *C. elegans* was added as normal but killed by heating to 50-60^{o}C for 30min prior to the addition of anthelmintic drug. Homogenates of the worms were then analysed for anthelmintic drug and metabolites.

Similar experiments were carried out using *Haemonchus contortus*. Exsheathed L3 stage larvae were exposed to albendazole or ivermectin in M9 buffer for 7hrs. Killed nematode controls (by heating as above) were also included.

5.3.4.1 Analysis of ivermectin-live worm incubations

Analyses of the homogenates of worms exposed to ivermectin for 7hrs with or without prior induction of cytochrome P450s was initially carried out using the described MRM method. Whilst an intense chromatographic peak was identified for ivermectin, there were no significant peaks for the predefined metabolite transitions. As described, the MRM transitions used were based on phase I metabolites previously identified following the incubation of ivermectin with human liver microsomes. It is possible that incubating ivermectin with whole worms could result in phase II metabolites and/or novel metabolites not produced by human liver microsomes. However, no obvious chromatographic peaks pertaining to ivermectin metabolites were noted on the total ion chromatogram. Therefore, the homogenate samples were subject to in depth

parent mass and MS/MS screening for ion masses that could correlate to either phase I or II metabolites as based on the expected mass changes described by Holcapek *et al.*, 2008. No significant metabolite peaks were found for any of the cultures.

5.3.4.2 Analysis of albendazole-live worm incubations

Homogenates of *Caenorhabditis elegans* exposed to albendazole for 7hrs were initially analysed using a total ion scan with accurate mass analysis. As well as an intense peak for albendazole at approximately 4.5 minutes there were also significant peaks for albendazole sulphoxide (mass 282.091Da, elution time 3.22min), amino-albendazole (mass 208.092Da, elution time 3.93min) and two glucose conjugates (mass 428.149Da, elution times 4.08 and 4.26min), see **Fig. 5-9** and **5-10**. Both albendazole sulphoxide and amino-albendazole were also found in the control samples. However, the glucose conjugate of albendazole was unique to the experimental samples and was also of greater intensity following prior exposure of the nematodes to fenofibrate (**Fig. 5-11**). The intensity of the albendazole-glucoside metabolite was between 0.7-2.3% of the albendazole peak in the analysed incubations. However, accurate quantitation of the metabolite was not possible as an albendazole-glucoside standard was not available.

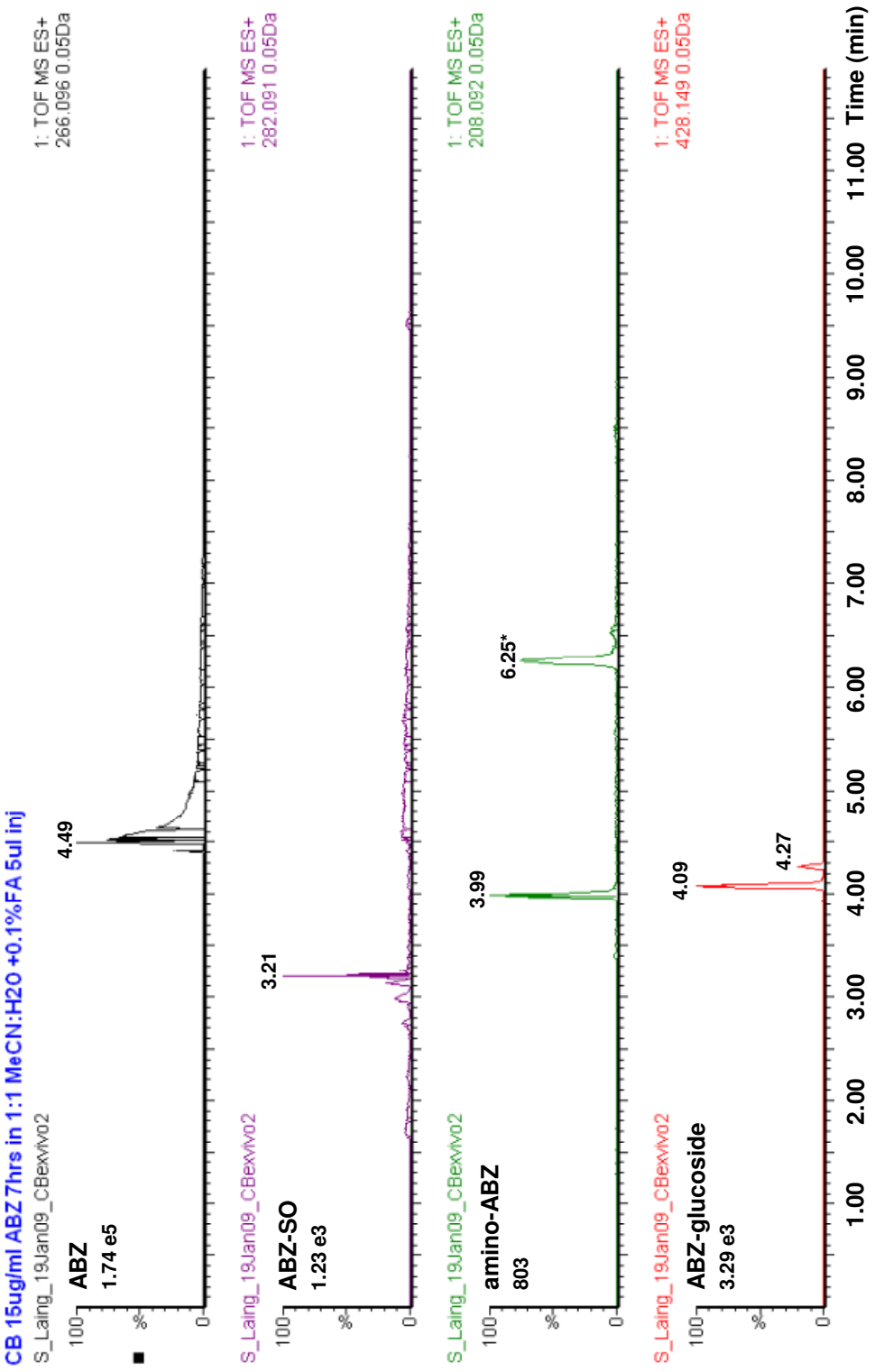


Figure 5-9: Chromatograms of albendazole and metabolites from *ex vivo C. elegans* incubation

Intensity (counts per second) of the peaks of interest is shown in top left corner of each chromatogram. The *ex vivo* incubations showed intense peaks for ABZ-SO, amino-ABZ and ABZ-glucoside. However, peak intensity for all metabolites was significantly lower than that of the parent compound (1.74 e5). * A peak at approximately 6.25 min with the same mass as amino-ABZ (208.092) was consistently present in *ex vivo* incubations. MS-MS studies showed this not to be a metabolite of albendazole.

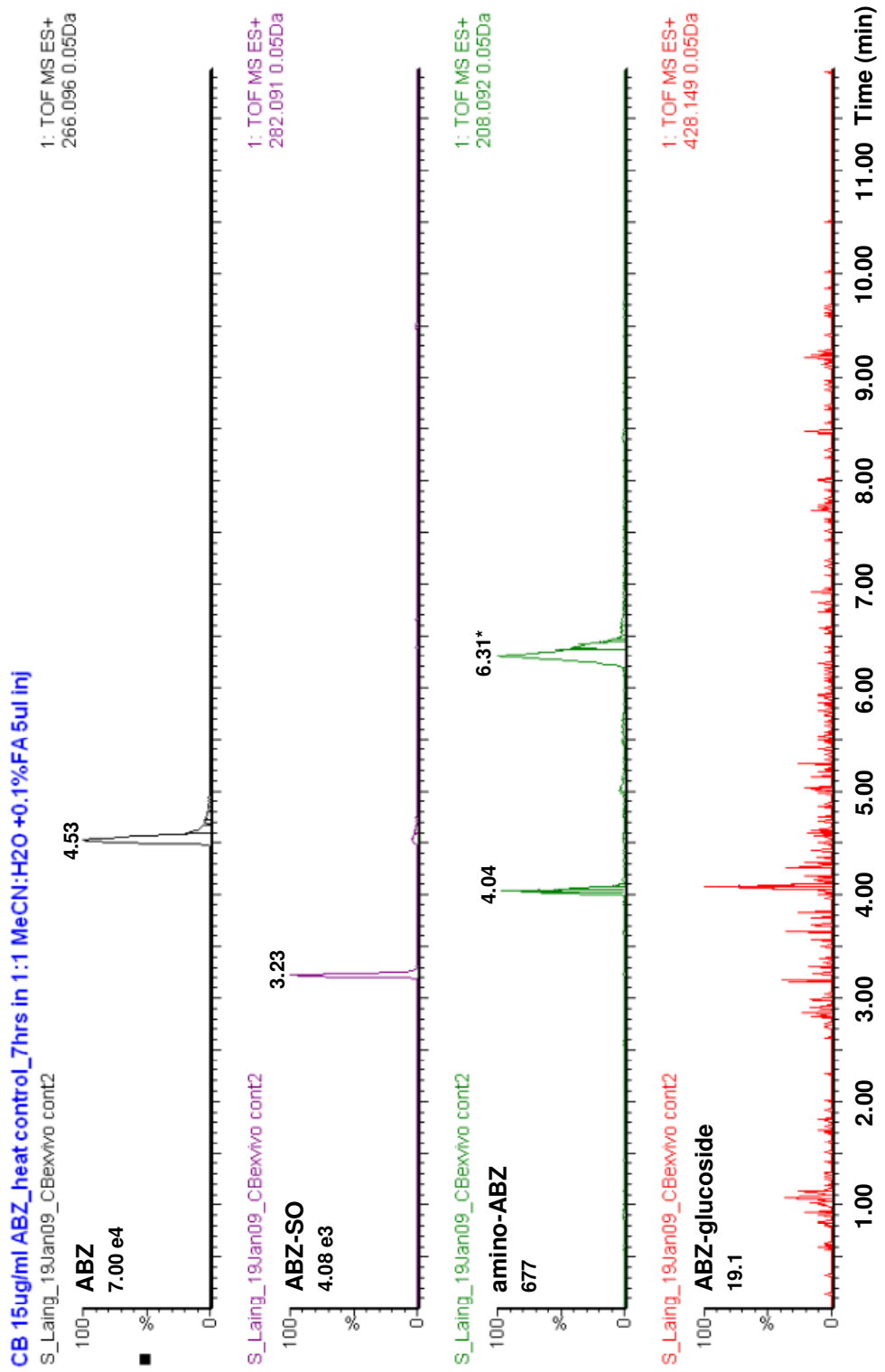


Figure 5-10: Chromatograms of albendazole and metabolites from heat killed *ex vivo C. elegans* incubation
Both ABZ-SO and amino-ABZ have clear peaks at appropriate elution times in this control sample. However, there is no clear peak for ABZ-glucoside. * see Fig. 5-9.

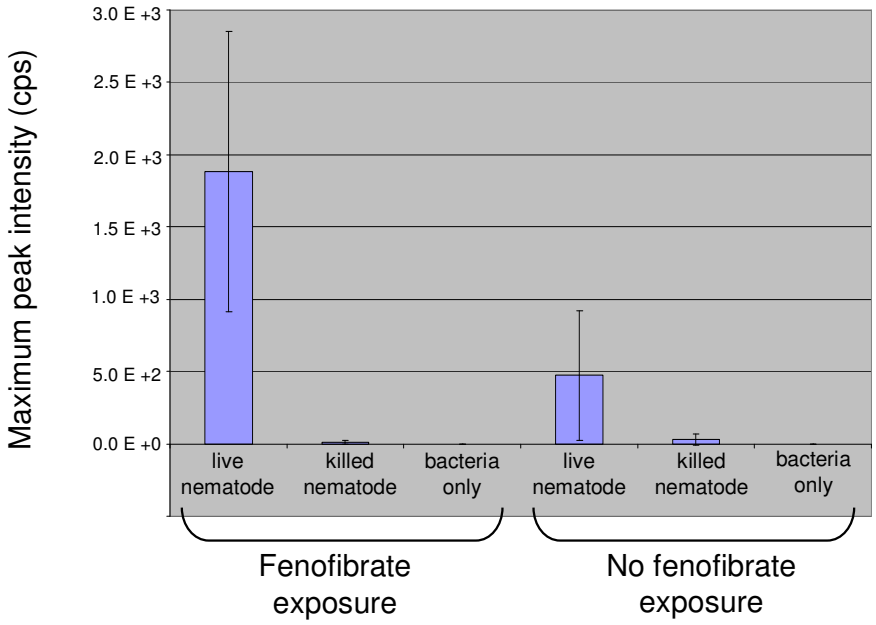


Figure 5-11: Relative intensity of albendazole glucoside metabolite (elution time 4.06) from cultures with and without pre-exposure to fenofibrate
Albendazole glucoside production is significantly greater following pre exposure of worm cultures to 20 µg/ml (55.43µM) fenofibrate. Note the lack of an albendazole-glucoside peak in the bacterial control group. Graph represents the result of three biological replicates for each condition.

In order to confirm that the peaks at 4.08 and 4.26min were indeed metabolites of albendazole, the homogenate samples were subject to MS-MS fragment analysis. The declustering potential remained at 25mV and collision energy of 25V was used to fragment albendazole and the proposed metabolites. Fragmentation of albendazole typically reveals three major fragment ions: 234.06Da, 191.00Da and 159.04Da. The proposed structure for these ions is detailed in **Fig. 5-12**. Fragmentation of the proposed glucose conjugates of albendazole identified only two major fragments. One of mass 266.09Da is proposed to be albendazole itself and the other of mass 234.06Da is the sulphur loss fragment identified in the fragment ion spectrum of the albendazole standard, see **Fig. 5-12** and **5-13**. These findings confirm the identity of the peaks of mass 428.149Da as metabolites of albendazole and the mass change is consistent with them being glucose conjugates. The lack of peaks of mass 190.997Da and 159.034Da in the fragment spectra of the metabolites is likely due to the glucose conjugate stabilising the specific bonds at the normal site of cleavage. The fragment ion spectra for each of the glucoside metabolites are identical; therefore they do not clarify the molecular position at which the glucose has been conjugated.

Analysis of *Haemonchus contortus* albendazole incubations did not reveal any significant metabolite peaks.

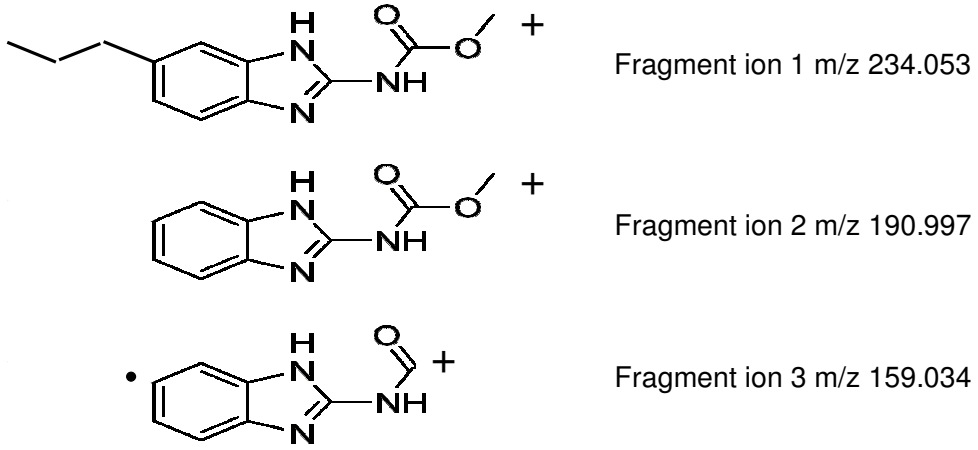


Figure 5-12: Structure of albendazole fragment ions
Fragment ion 1 is proposed to be a simple loss of the sulphur atom and reseal of the hydrocarbon chain. This is a common ion type of sulphur containing compounds. Fragment ion 2 is the result of complete loss of the C_3H_7S chain. Fragment ion 3 is a radical resulting from loss of the C_3H_7S chain with maintenance of an electron in the aromatic ring and loss of CH_3O.

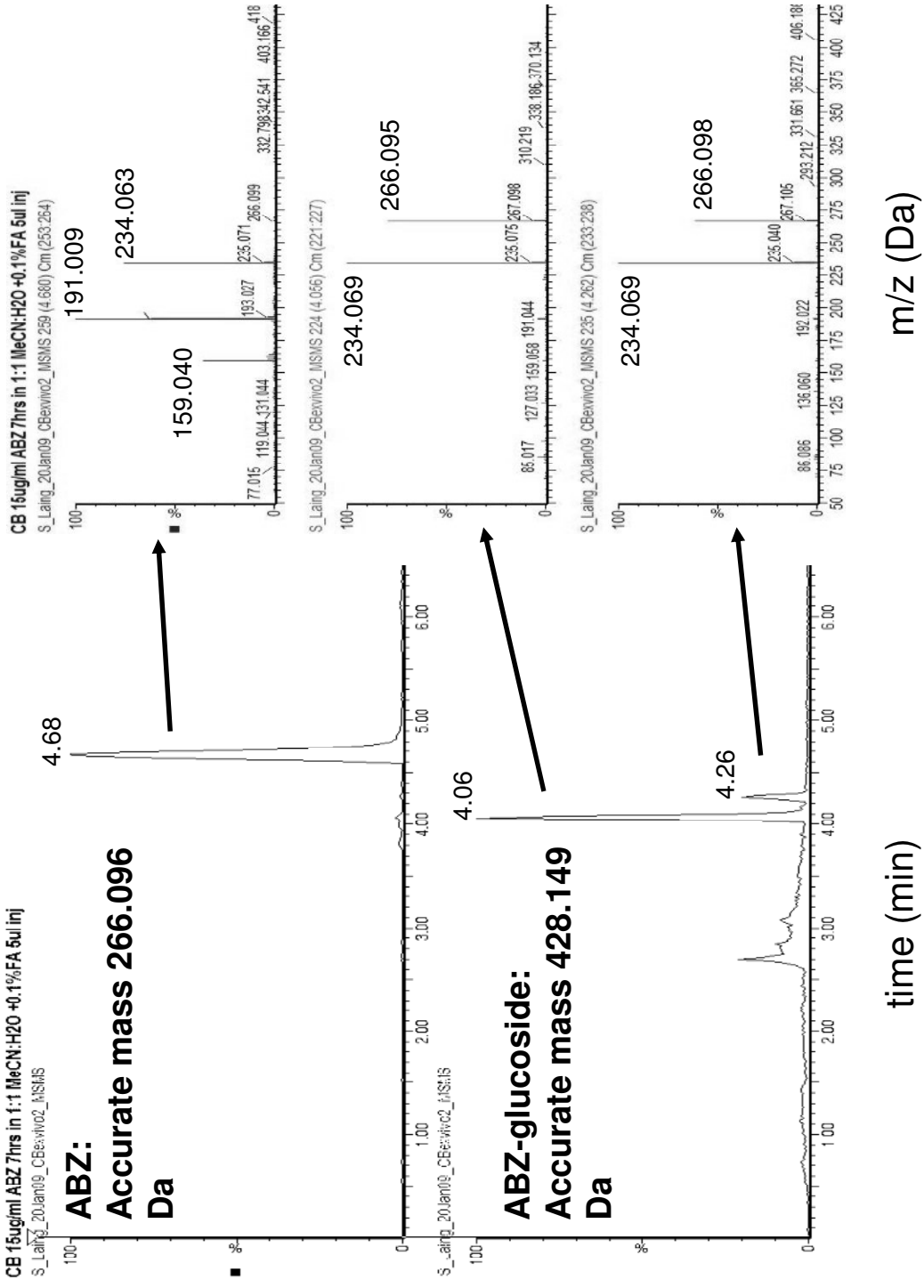


Figure 5-13: Confirmation of peaks m/z = 428.149Da as true albendazole metabolites Chromatograms reveal a single peak for albendazole at 4.68min and two more polar compounds, mass 428.149Da, with peaks at 4.06 and 4.26min. Fragment ion spectra confirm these peaks as metabolites of albendazole, likely to be glucose conjugates.

5.4 Discussion

The failure to visualise a P450 soret peak in nematode derived microsomal protein was not entirely unexpected. Only one published paper has previously reported absorbance spectra showing a 450nm peak in such proteins (Kulas *et al.*, 2008). The protocol from this paper was followed closely and personal communication with both Dr. Kulas and Dr. Menzel suggested the inclusion of fenofibrate/ delta- aminolevulinic acid and substrate within cultures to increase yields, none of which were successful. The only apparent difference in protocols was the method of homogenisation: Kulas *et al.* (2008) made use of a liquid CO_2 cooled automated homogeniser from Braun. Unfortunately, a similar system was not available at the time of this study. It may be that this method resulted in more complete homogenisation of the small nematodes or that the cooling system more effectively inhibited the denaturing of P450 than the methods described here. However, an absorption spectrum obtained from OP50 bacteria in liquid culture medium showed a peak at the same wavelength as that from microsomal preparations. As the culture medium did not undergo any potentially denaturing homogenisation steps, this would suggest that the nematodes were not producing proteins with a soret peak at 450nm, or that the intense 421nm peak was masking the presence of a small 450nm peak. *C. elegans* cannot produce haem and relies on exogenous sources (Rao *et al.*, 2005). It may be that the liquid culture system or bacterial food source used here did not produce sufficient haem, but these were standard protocols also used by Kulas *et al.*(2008).

The cytochrome P450 enzymes of mammals and insects can be inhibited by a wide range of compounds. In many cases, including midazolam metabolism by human CYP3A4, autoinhibition is a feature of the enzyme kinetics (McNulty *et al.*, 2009; Roy *et al.*, 2009; Zhu *et al.*, 2009; Baliharova *et al.*, 2005; Houston *et al.*, 2000; Ghosal *et al.*, 1996). During the preparation of microsomes from *D. melanogaster*, as part of a Pfizer R&D project, it was discovered that the eye pigment and wings of the insect contained potent inhibitors of CYP reactions (*pers. comm.*, Dr. D.J. Woods, Pfizer R&D). In order to prepare non contaminated microsomes, the heads and wings of each individual fly had to be removed. The data presented here suggests that *C. elegans* may also contain

P450 inhibitors. The nature of these inhibitors and their anatomic location within the worm remains unknown. However, the extremely small size of the nematode makes dissection of unaffected tissues, presumably the gut, for microsome preparation an unrealistic goal. Therefore, an *ex vivo* approach to further studies may be more appropriate. It is interesting to note that despite the success of Kulas *et al.* (2008) in extracting functional microsomes, more recent studies from the Menzel group have relied on an *ex vivo* approach (Schafer *et al.*, 2009)

The whole worm approach to nematode drug metabolism studies does have several draw backs. Rigorous controls and replicates are more difficult to produce as the exact concentration of enzymes cannot be defined and many metabolic pathways are assessed simultaneously. In addition, the concentration of drug within liquid culture medium is unlikely to relate to the concentration of drug within the nematode and available for biotransformation. In this study, nematodes were washed several times to remove bacteria and the liquid culture medium. The aim of this step was to reduce the confounding effect of excessively high drug concentration in the final sample and the involvement of bacterial metabolism. However, it is possible that polar metabolites of these drugs were immediately excreted into the medium and therefore were not assessed. Finally, in order to accurately quantify the production of metabolites and aid in the identification of novel metabolites, experiments using radioactively labelled drug were originally planned. Given the large volume, shaking cultures necessary to carry out *ex vivo* experiments this was not possible. The concentration of radioisotope necessary was prohibitively high and the possibility of contamination through splashing of the cultures was unacceptable. However, a whole worm approach does represent a more physiologically relevant comparison to the process in living nematodes and allows analysis of a greater spectrum of potential metabolism pathways.

Glucoside conjugates of xenobiotics are uncommon in mammals. Drugs will more commonly be glucuronidated in the liver of these species (Gessner *et al.*, 1973). However, this study clearly shows the production of *C. elegans* derived albendazole glucose conjugates. Whilst no *H. contortus* metabolites were apparent in the current study, this may be related to the stage of the nematode, mass of nematode per reaction or incomplete homogenisation of the L3 larvae.

Recently published data by Cvilink *et al.* (2008) showed that that adult *H. contortus* incubated with albendazole produced albendazole sulphoxide and two albendazole glucoside conjugates similar to those produced by *C. elegans*. These were present in both the homogenised worms and in the medium, which was not analysed in this study. The similarity of the metabolites produced by *C. elegans* and *H. contortus* is remarkable and validates the use of *C. elegans* as a model to investigate metabolism as a mechanism of anthelmintic resistance in parasitic nematodes.

The apparent increase in rate of metabolism of albendazole to glucoside conjugates following exposure to fenofibrate is extremely interesting. Fenofibrate is a peroxisome proliferator- activated receptor α (PPARα) agonist. Drugs of this group are used to treat hyperlipidaemia and hypercholesterolaemia in humans. They are known to be potent inducers of both hepatic and renal cytochrome P450s, UGTs, sulphotransferases and to a lesser extent GSTs (Runge-Morris *et al.*, 2009; Graham *et al.*, 2008; Knight *et al.*, 2008; Waxman, 1999; Kroetz *et al.*, 1998). This result would not only suggest that the glucoside conjugates are produced through these pathways but that *C. elegans* contains a functional PPARα homologue. In fact, several studies have drawn comparisons between the mammalian PPARα and nhr-49 in *C. elegans* (Atherton *et al.*, 2008; Van Gilst *et al.*, 2005a).

It is likely that *C. elegans* is able to metabolise albendazole to albendazole sulphoxide, but due to the presence of this metabolite in the bacterial culture controls this will require further investigation. Axenic culture techniques are becoming better defined and would provide an ideal platform from which to further investigate this question (Castelein *et al.*, 2008). Albendazole sulphoxide is a pharmaceutically active metabolite and therefore this pathway is unlikely to be directly involved in anthelmintic resistance. Further investigation will be necessary to evaluate the molecular identity and pharmaceutical activity of the albendazole-glucoside conjugates. Potentially nuclear magnetic resonance spectroscopy could be used to identify the conjugation site of the glucose moiety in each of the metabolites. Further to this, the compounds could be synthesised and their activity compared to albendazole and albendazole sulphoxide. The amino albendazole metabolite noted in both HLM and *Caenorhabditis elegans* incubations has not previously been described and its

relevance is unknown. However, it is possible that this is simply an intermediate metabolite in the pathway that produces albendazole amino sulphoxone. This is a pharmaceutically inactive metabolite found in the plasma of humans dosed with albendazole (Mirfazaelian *et al.*, 2002).

No nematode derived metabolites of ivermectin were noted using any protocol. Ivermectin has a low rate of metabolism in human and mammal studies and it is possible that the rate of ivermectin metabolism by nematodes is too low to measure (Gonzalez *et al.*, 2009). In the case of *C. elegans*, the lack of evidence of ivermectin metabolism in the strains used does not rule out the involvement of metabolism in naturally occurring resistant parasite isolates. It is possible that transgenic overexpression of *cyp*s in *C. elegans* would result in measurable ivermectin metabolism. It is difficult to draw conclusions from the lack of metabolites following ivermectin incubation with resistant strains of *H. contortus*. These strains did not bioconvert albendazole in this experiment either, despite the fact that *H. contortus* has previously been shown to metabolise albendazole (Cvilink *et al.*, 2008). In addition, the *C. elegans* incubations revealed that the method used in the current study was sensitive to the expected *H. contortus* derived albendazole metabolites. Further assessment of metabolism of both albendazole and ivermectin using adult *H. contortus* is warranted to assess this further.

In conclusion, this study has shown that *C. elegans* can metabolise albendazole. The metabolite appears to be produced via a pathway that is uncommon in vertebrates, but which has been reported in several other invertebrates including the parasitic nematode *H. contortus* (Cvilink *et al.*, 2008). The enzymes directly involved in this pathway are as yet undefined in both *C. elegans* and *H. contortus* and warrant further investigation. *C. elegans* knock out mutants for several *cyp* and *ugt* genes are available, and RNA inhibition of *cyp* genes has been reported in the literature (Schafer *et al.*, 2009; www.wormbase.org). In addition, *C. elegans* can easily be manipulated to over-express genes of interest. The HPLC-MS techniques described here could be used in combination with knock outs, RNAi and transgenic worms to further investigate the role of specific enzymes in the metabolism of albendazole and other anthelmintics. In addition, genes of interest from *H. contortus* may be expressed in *C. elegans* in order to assess their involvement in these pathways.

Chapter 6: General Discussion

6.1 Exposure to high dose ivermectin and albendazole elicit very different responses in *C. elegans*

Chapters 3 and 4 have outlined the very different transcriptomic responses of *C. elegans* to ivermectin and albendazole. In the case of albendazole a small group of 42 genes were up-regulated (FDR< 10%, rank products) in response to 4hrs exposure of young adults to 300µg/ml (1.13mM) ABZ, and only four genes were down-regulated. The list of up-regulated genes was enriched for those with predicted transferase activity and monooxygenase activity and was consistent with a detoxification response being mounted by the nematode. In addition, specific *cyp* genes were up-regulated, mainly the *cyp-35* family, which corroborates recent studies suggesting that this family is highly responsive to xenobiotic exposure (Menzel *et al.*, 2005; Menzel *et al.*, 2001).

In contrast, the response of *C. elegans* to 4hrs exposure to 1µg/ml (1.14µM) ivermectin was far more complex. 254 genes were up-regulated and 192 genes were down-regulated (FDR<10%, rank products). The greater number of genes with significantly changed expression level, compared to the albendazole experiments, may be explained by several factors. A greater number of biological replicates were available for microarray analysis of the ivermectin experiments, resulting in greater statistical power to identify differentially expressed genes. However, of greater importance is the fact that whilst the ABZ resistant strain (CB3474) was completely unaffected by the dose of drug used in the ABZ experiments, the ivermectin resistant strain used (DA1316) was not fully resistant to ivermectin. This resulted in gene expression changes associated with intoxication being noted, perhaps alongside a subset of genes involved in detoxification. The resultant transcriptomic response appears to be extremely complex in which many completely uncharacterised genes are involved, see **Fig. 6-1**.

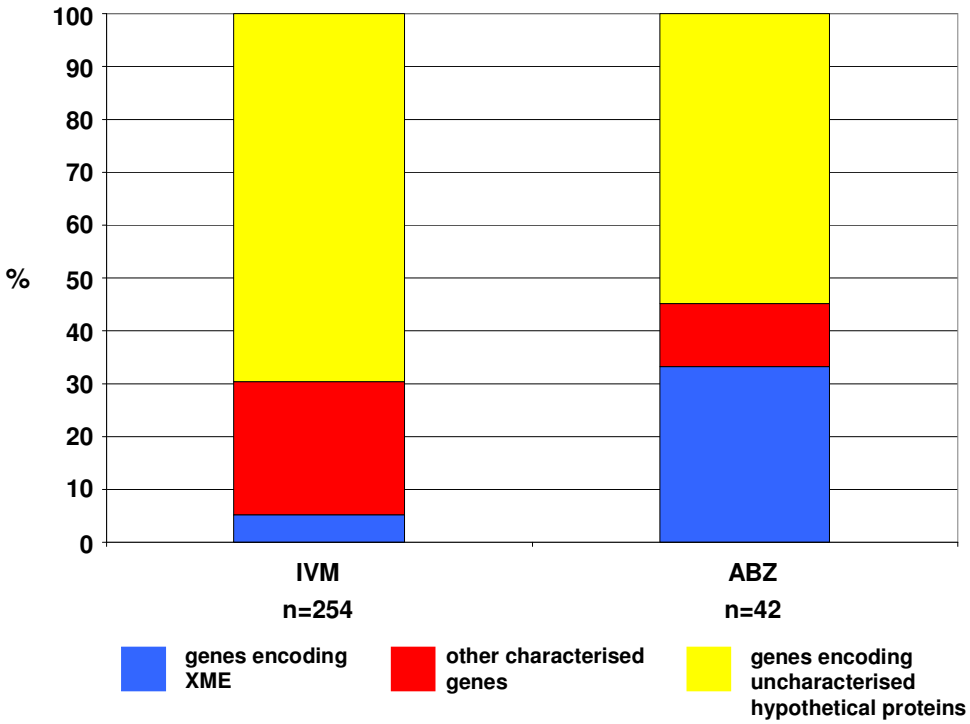


Figure 6-1: Comparative ontologies of genes up-regulated in response to ivermectin and albendazole
The response to ivermectin was characterised by up-regulation of few genes encoding classical xenobiotic metabolising enzymes (XME) and many uncharacterised genes. In comparison the response to albendazole resulted in up-regulation of a much smaller but more defined group of genes including a large number of genes encoding XMEs.

Albendazole has proven repeatedly to increase the expression and activity of several XMEs in mammalian systems, including CYPs and UGTs (Velik *et al.*, 2005; Velik *et al.*, 2004; Bapiro *et al.*, 2002; Rolin *et al.*, 1989; Souhaili-el *et al.*, 1988a). By comparison, there are only few reports in the literature regarding the inductive effect of ivermectin on XMEs, with conflicting results (Bapiro *et al.*, 2002; Skalova *et al.*, 2001). Information on the interaction of ivermectin with nuclear hormone receptors, such as CAR/PXR/PPARα, is lacking; but it may be that the structure of this drug is less conducive to the up-regulation of XMEs. This may partially explain the long plasma half-life of ivermectin in mammalian systems compared to that of albendazole (Gonzalez *et al.*, 2009; Marriner *et al.*, 1986; Prichard *et al.*, 1985).

Unpublished work using GFP reporter constructs, to investigate several anthelmintic responsive genes elucidated in the current study, has been undertaken by members of the Gilleard lab (Dr. V. Butler and Ms. S. Ravikumar). This has shown that whilst nearly all of the top 10 genes up-regulated in

response to albendazole are exclusively expressed in the gut of the nematode, those up-regulated in response to ivermectin may be expressed in many tissues (Tables 6-1 and 6-2). The intestine has been proposed to be the major organ of detoxification in *C. elegans* and nematodes as a whole (McGhee, 2007). Therefore, this work is again suggestive that albendazole exposure results in a detoxification response, whereas ivermectin exposure does not.

Gene ID	Gene Description	Type of reporter	GFP expression
C06B3.3	*cyp-35C1*	PCR-fusion (transcriptional) AND plasmid PJM-355 (transcriptional)	intestine
K07C6.5	*cyp-35A5*	PCR fusion (translational)	intestine (highly expressed)
C03G6.15	*cyp-35A2*	PCR fusion (translational)	intestine (highly expressed)
C29F7.2	Predicted small molecule kinase	PCR-fusion (transcriptional)	pharynx (highly expressed), posterior intestine (weak)
T16G1.6	Predicted small molecule kinase	PCR-fusion (transcriptional)	anterior and posterior intestine (plus head neurones at L3)
C04F5.7	*ugt-63*	PCR-fusion (transcriptional)	hypodermis
R03D7.6	*gst-5*	PCR fusion (translational)	intestine, pharynx and circum-pharynx neurones
ZC443.6	*ugt-16*	PCR-fusion (transcriptional)	intestine (highly expressed)

Table 6-1: Expression pattern of selected genes up-regulated in response to 4hrs exposure to 300µg/ml (1.13mM) ABZ

Gene ID	Gene Description	Type of reporter	GFP expression
K11G9.6	*mtl-1*	PCR-fusion (transcriptional)	intestine and terminal bulb of pharynx
F49E11.10	*scl-2*	PCR-fusion (transcriptional)	intestine
C23G10.11	uncharacterised	PCR-fusion (transcriptional)	hypodermis (dorsal and ventral)
F28G4.1	*cyp-37B1*	PCR-fusion (transcriptional) AND plasmid PJM-355 (transcriptional)	intestine
F57G8.7	uncharacterised	PCR-fusion (transcriptional)	hypodermis at L2 and older
K03D3.2	uncharacterised	PCR-fusion (transcriptional)	hypodermis and head neurones
C45G7.3	*ilys-3*	PCR-fusion (transcriptional)	anterior intestine (weak)

Table 6-2: Expression pattern of selected genes up-regulated in response to 4hrs exposure to 1µg/ml (1.14µM) IVM

6.2 Implications of the fasting response upon exposure to ivermectin

Exposure of strain DA1316 to 1µg/ml (1.14µM) IVM for 4hrs appears to result in a fasting response. This study represents the first whole genome microarray investigation of this type of response in *C. elegans*. In addition to the up- and down- regulation of several genes previously reported by van Gilst *et al.* (2005b) to be responsive to short term fasting, this study has uncovered several novel genes which had not previously been associated with fasting in *C. elegans*. These include *cyp-37B1, mtl-1* and *scl-2*, whose up-regulation in response to fasting was confirmed by real-time QPCR. Up-regulation of several similar genes in response to fasting of mammals has been noted: the *mtl-1* gene of the rat is up-regulated following short-term fasting of this species and the human homologue of CYP37B1 (CYP4V2) is thought to be a fatty acid hydroxylase (Nakano *et al.*, 2009; Sogawa *et al.*, 2003; Shinogi *et al.*, 1999). Whilst *scl-2* is largely uncharacterised, the putative protein that it encodes carries a sterol carrier-like domain, which could feasibly be involved in the transport of lipid breakdown products. This would explain its up-regulation in response to periods of fasting. Both *mtl-1* and *cyp-37B1* did not appear to be up-regulated following exposure of N2 to IVM in microarray analyses. The reason for the different response between DA1316 and N2 is unknown, but may be due to a higher level of constitutive expression in strain DA1316. Alternatively, both genes may represent an immediate response to fasting and were only significantly up-regulated in the resistant strain due to the longer length of IVM exposure required for it to succumb to pharyngeal paralysis. This will be investigated further by analysing gene expression in *C. elegans* exposed to IVM for different durations. Many of the other differentially regulated genes in this study may also be involved in the fasting response, but further analysis will be necessary to confirm or refute this, as some of the genes may well be involved in a detoxification response specific to ivermectin exposure. However, *C. elegans* may provide an interesting model to investigate fasting responses at a whole organism level.

Many of the genes up-regulated in response to 4 hrs exposure to ivermectin were also up-regulated in dauers compared to non-dauers. This is to be expected as the dauer represents a non-feeding stage that must rely on stored fat as an

energy source. The comparison made to the data published by Wang *et al*. (2003) is perhaps not ideal, as this was a comparison of dauers and dauer exit worms 12hrs after exposure to food. A more recent paper by Jeong *et al*. (2009) compared the transcriptomes of fed L1, L2 and L3 larvae, prior to entry in to the dauer-stage, to long-term dauer stage worms. A full comparison to this data was not possible. However, the top 10 up-regulated and down-regulated genes following exposure to 1µg/ml (1.14µM) ivermectin appeared to be similarly regulated in the dauer stage in the Jeong analysis, see **Tables 6-3** and **6-4**. Despite the overlap between the transcriptomes of ivermectin exposed and dauer nematodes, there are many more genes differentially expressed in the dauer stage. The dauer stage may survive, without feeding, for several months and the metabolic pathways involved in this process are likely to be very different to those that react to short-term fasting over a period of hours. It is likely that the overlapping genes noted between the current study and that of Wang *et al*. (2003) represent a subset of these genes that are involved in fatty acid metabolism and gluconeogenesis.

Interestingly, Harvey *et al*. (2009) recently carried out a study comparing the transcriptome of various *C. elegans* lines in presence of daumone or without for 8 hours from the L1 stage, i.e. prior to entry into the dauer stage. Daumone is a hormonal substance secreted by *C. elegans* which, at high enough concentrations, causes entry into the dauer stage, normally when the habitat of the worm is overpopulated. They identified a small subset of 89 genes that were consistently differentially expressed in the daumone exposed group. There was very little overlap between the genes up-regulated in response to ivermectin exposure and daumone exposure. However, eleven genes were up-regulated in both experiments. Most were uncharacterised, but *acs-7*, representing a fatty acid CoA synthetase, and *dhs-18,* representing a short chain dehydrogenase, were both up-regulated and both likely to be involved in fatty acid oxidation. The general lack of similarity would suggest that entry into the dauer-stage in response to daumone does not immediately result in a switch to metabolism of stored energy supplies. In fact, Jeong *et al*. (2009) propose there to be a period of preparatory fat storage prior to dauer entry in response to daumone.

Gene ID	Log_2 FC in response to IVM exposure	Log_2 FC in dauer vs. L3 (Jeong, 2009)
mtl-1	4.99	5.62
scl-2	3.27	-3.59
C23G10.11	3.2	2.43
cyp-37B1	3.09	2.90
F57G8.7	3.01	2.55
K03D3.2	2.83	4.46
F45D3.4	2.77	1.22
F54F3.3	2.51	-2.35
ilys-3	2.51	4.50
dod-3	2.33	-0.73

Table 6-3: Comparison of top 10 up-regulated genes following 4hrs exposure of strain DA1316 to 1µg/ml (1.14µM) IVM to dauer data (Jeong *et al.*, 2009)

Gene ID	Log_2 FC in response to IVM exposure	Log_2 FC in dauer vs. L3 (Jeong, 2009)
spp-23	-2.79	-6.85
folt-2	-2.55	-4.98
F46F2.3	-2.36	-5.48
F07H5.9	-1.88	-3.66
C35A5.3	-1.83	0.81
gst-10	-1.77	-2.13
ugt-63	-1.77	-0.42
F18E3.11	-1.72	-2.16
F58G6.9	-1.72	-2.73
F21F8.4	-1.7	-2.70

Table 6-4: Comparison of top 10 down-regulated genes following 4hrs exposure of strain DA1316 to 1µg/ml (1.14µM) IVM to dauer data (Jeong *et al.*, 2009)
In general, genes up-regulated or down-regulated in response to ivermectin exposure are regulated similarly in the dauer stage compared to L3 larvae. The most notable exception to this rule is *scl-2* which is strongly down-regulated in the dauer stage, but up-regulated in response to ivermectin.

The predominating effect of ivermectin on *C. elegans* is the paralysis of the nematode pharynx. Ivermectin has also been shown to inhibit pharyngeal pumping in the parasitic nematodes *A. galli, T. colubriformis, A. suum* and *H. contortus* (Holden-Dye *et al.*, 2006; Sheriff *et al.*, 2002; Paiement *et al.*, 1999; Kotze, 1998; Brownlee *et al.*, 1997; Adelsberger *et al.*, 1997). Therefore, it is possible that modulation of genes encoding enzymes involved in fasting responses could provide an advantage to parasites under selective pressure from ivermectin exposure.

Strain DA1316 did not contain all of the mutations reported by the CGC and Dent *et al.* (2000). *avr-15* appeared to be wild type over the locus of the proposed *ad1051* mutation. As this gene is thought to encode the glutamate-gated chloride

channel subunit which confers ivermectin sensitivity to the *C. elegans* pharynx, this could easily explain the transcriptomic response to 1µg/ml (1.14µM) ivermectin. However, personal communication with Dr. Dent has suggested that the strain should still have a null mutation of this gene and therefore behave phenotypically as an *avr-14*/*avr-15*/*glc-1* triple mutant. Sequencing of the entire *avr-15* gene is currently being undertaken. Studies with GluCl triple mutants have shown the pharynx to be unaffected by up to 2.5hrs exposure to ivermectin concentrations of 5µM (4.5µg/ml). In the current study, pharyngeal pumping rate was reduced approximately five-fold following 4hrs exposure of DA1316 to 1µg/ml (1.14µM) IVM. If this strain is truly a triple mutant then this would suggest that ivermectin was able to inhibit pharyngeal pumping in *C. elegans* via another pathway than the currently accepted AVR14/ AVR15 interaction (Dent *et al.*, 2000). An *avr-14*/*avr-15*/*glc-1* triple mutant has been requested from the Dent laboratory to allow further investigation of this issue.

6.3 Mammalian xenobiotic metabolism pathways are likely to be extremely divergent from those of nematodes

Attempts have been made throughout this study to compare the functions of particular mammalian cytochrome P450s to the most similar *C. elegans* enzymes based on amino acid sequence. As has been detailed at several points, inferences of this kind are fraught with inaccuracy and the functions of cytochrome P450s are likely to be very different in mammals and nematodes. Alignment of the *C. elegans* P450 family revealed that amino acid sequence identity is similar enough to assess phylogeny accurately only at the level of a particular family, for example the CYP35 family. Alignment of all of the CYPs of the free-living nematode reveals a remarkably divergent family of proteins. Therefore, addition of the major human CYPs involved in xenobiotic metabolism and the major *D. melanogaster* CYP involved in insecticide resistance to the alignment, only served to further increase the complexity. With these caveats in mind a best assessment of phylogeny was created, see **Fig. 6-2**.

The topology of the cladogram presented in **Fig. 6-2** is unlikely to be completely accurate. However, it has successfully separated the CYPs of *C. elegans* into the three major families (CYP2, CYP3 and CYP 4) noted by Gotoh *et al.* (1998). The CYP35 family, of which several members were up-regulated in response to exposure of *C. elegans* to ABZ, represent members of the CYP2 family. Therefore, they may be distantly related to several of the human cytochrome P450s involved in xenobiotic metabolism. The human cytochrome P450 involved in the metabolism of most of the drugs in use, including IVM and ABZ, is CYP3A4 (Guengerich *et al.,* 2006; Li *et al.,* (2003); Zeng *et al.,* 1998). Interestingly, CYP6G1, from *D. melanogaster*, appears to be in a clade with this enzyme (boot strap value 100). However, none of the CYPs up-regulated in response to exposure of *C. elegans* to IVM or ABZ are members of the CYP3 family.

Chakrapani *et al.* (2008) proposed the use of transgenic *C. elegans* expressing GFP under the control of various *cyp* promoters to investigate the possible mechanisms by which drugs intended for use in humans may be metabolised. The work presented in this study and the phylogenetic analysis of the human and

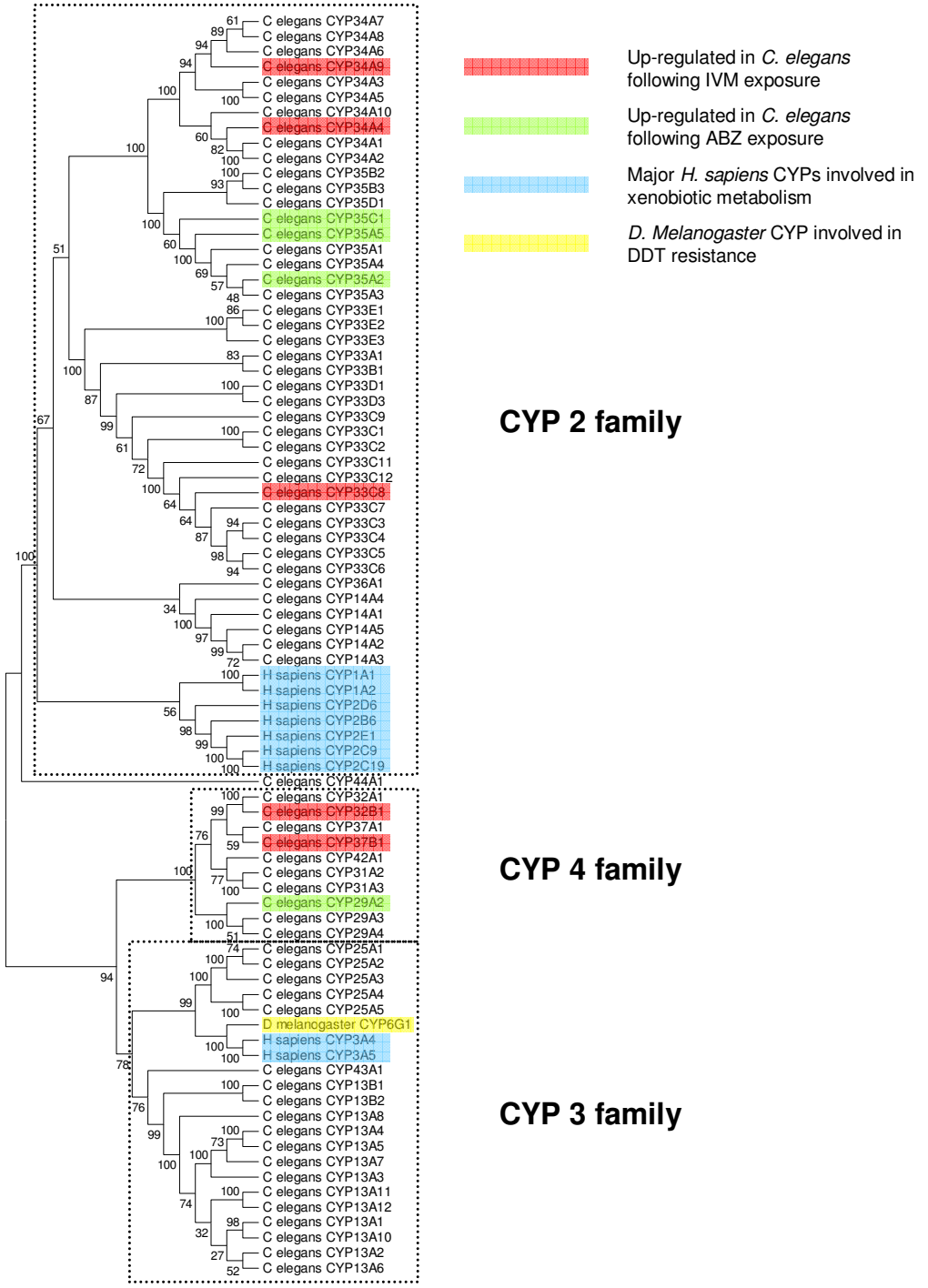


Figure 6-2: Cladogram of *C. elegans* CYPs, the major *H. sapiens* CYPs involved in xenobiotic metabolism and *D. melanogaster* CYP6G1
The evolutionary history was inferred using the Neighbour-Joining method (Saitou *et al.*, 1987). The optimal tree with the sum of branch length = 32.658 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the JTT matrix-based method (Jones *et al.*, 1992) and are in the units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 639 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

C. elegans CYP families, would suggest that this is not likely to be successful. The *C. elegans* P450s induced upon exposure of the nematode to ABZ were not the orthologues of those thought to be involved in ABZ metabolism in humans. In addition, the drug appears to be metabolised by glucosylation in nematodes, a pathway which is rarely described in mammals.

Comparisons between mammalian and nematode UGTs are likely to be equally as problematic. This family of enzymes is also highly divergent even within *C. elegans*. *ugt-63* and *ugt-16* were in the top 10 up-regulated genes following exposure of *C. elegans* to ABZ. The proteins encoded by these genes were subject to BLASTp analysis against the human proteome and the best hits were UGT1A1 and UGT2B7 respectively. The cladogram presented in **Fig. 6-3** includes all of the putative *C. elegans* UGTs and the human UGTs proposed to be most important in xenobiotic metabolism (Williams *et al.*, 2004). The UGTs that were up-regulated in response to the exposure of *C. elegans* to anthelmintic drugs do not appear to belong to any particular clade. In addition, they show no clear relationship to the human UGTs.

In summary, whilst the CYPs and UGT enzymes up-regulated in response to exposure of *C. elegans* to ABZ are not homologues of those involved in ABZ metabolism in mammalian systems, this is likely to be irrelevant. Both the CYPs and UGTs are rapidly evolving families and sequence similarities between mammals and nematodes should not be expected. The fact that a small subset of genes, including those encoding XMEs, was up-regulated in response ABZ exposure is more compelling evidence that these proteins are likely to be involved in detoxification than any phylogeny studies.

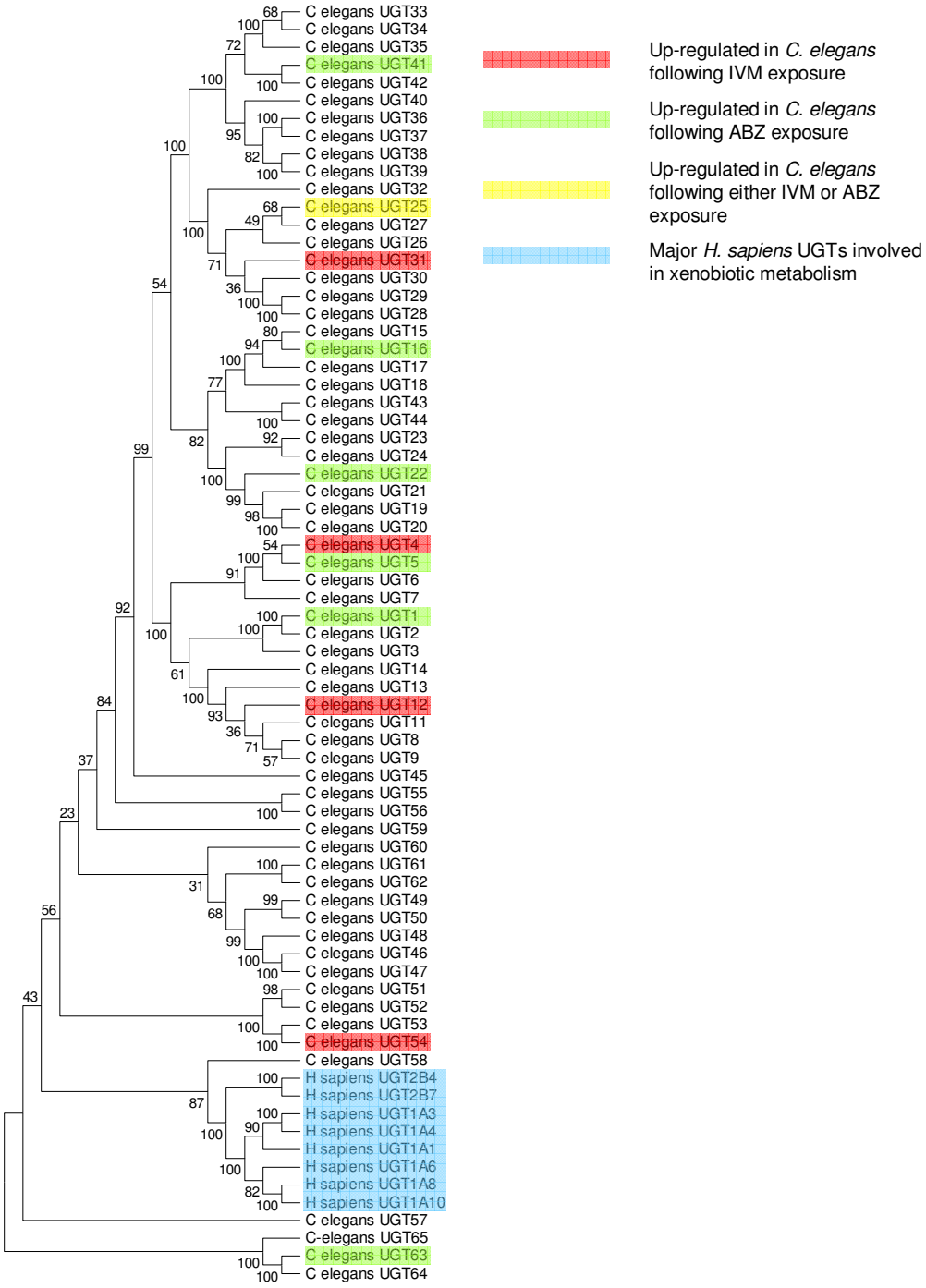


Figure 6-3: Cladogram of *C. elegans* UGTs and the major *H. sapiens* UGTs involved in xenobiotic metabolism
The evolutionary history was inferred using the Neighbour-Joining method (Saitou *et al.*, 1987). The optimal tree with the sum of branch length = 35.043 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the JTT matrix-based method (Jones *et al.*, 1992) and are in the units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 628 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

6.4 Transcriptomic changes upon exposure of *C. elegans* to albendazole are consistent with the albendazole metabolites identified by HPLC-MS

Following incubation with *C. elegans* cultures, albendazole was shown to be metabolised to albendazole sulphoxide and two albendazole-glucoside metabolites. Unfortunately, albendazole sulphoxide was also present in the control samples suggesting that the bacterial culture in which the worms are grown may also be able to metabolise albendazole. However, the unique albendazole-glucoside metabolites appear to be nematode specific. The predominating gene classes up-regulated in response to albendazole exposure were members of the UDP-glucuronosyl/glucosyl-transferase family and members of the cytochrome P450 family. The P450s, as has been discussed, are important in oxidation reactions, such as the conversion of albendazole to albendazole sulphoxide. In mammals, this step has been proposed to be carried out by a combination of CYPs and FMOs. There were no flavin monooxygenase genes up-regulated in the current albendazole study. UGTs are likely to catalyse the conjugation of glucose to xenobiotics in *C. elegans* and other invertebrates in which this pathway is common. Within mammals UGT activity has been found to be increased in the rat following exposure to albendazole and ABZ-glucuronide conjugates have been found in the bile of sheep dosed with albendazole (Hennessy *et al.*, 1989; Souhaili-el *et al.*, 1988a). In addition, prior exposure to fenofibrate, which is known to induce the activity of CYPs and UGTs, appears to result in increased production of ABZ-glucoside in ABZ-*C. elegans* incubations.

In conclusion, whilst the specific enzymes involved in the metabolism of albendazole by the nematode *C. elegans* require further investigation, the evidence presented in this study is strongly suggestive of the role of cytochrome P450 enzymes and UDP-glucosyl transferases.

6.5 *C. elegans* is a valid model for nematode metabolism of anthelmintics

In addition to the evidence presented in **Chapter 1**, the suitability of *C. elegans* as a model for nematode metabolism has been further confirmed by the current study. HPLC-MS analysis of albendazole-*C. elegans* incubations revealed the presence of three metabolites: albendazole sulphoxide and two albendazole-glucoside conjugates. Recent studies by Cvilink *et al.* (2008) also revealed albendazole sulphoxide and two glucose conjugates to be produced following incubation of the parasitic nematode *H. contortus* with albendazole. This provides strong evidence of the ability to extrapolate data derived from *C. elegans* metabolism experiments to other nematodes within the same phylogenetic clade. Whether or not this data may also be applicable to more distantly related nematodes remains to be assessed.

In the current study, *H. contortus* L3 larvae did not produce any metabolites of albendazole. L3 stage larvae were initially chosen as they were easier to attain and have been reported to have higher levels of oxidase activity compared to adult parasites (Kotze, 1997). It is possible that the ability to metabolise anthelmintics is a stage-specific phenomenon and may be due to differential expression of specific xenobiotic metabolising genes, rather than an up-regulation of general oxidase activities. However, comparison of SAGE tags in a developmental series of *C. elegans* does not reveal any of the *cyps* to be expressed at a significantly greater level in adults. UGT activity in free-living L3 larvae and adult *H. contortus* has not been compared. It is also possible that metabolism of albendazole is a slower process in *H. contortus* than in *C. elegans*. The study conducted by Cvilink *et al.* (2008) incubated *H. contortus* with albendazole for 24hrs prior to analysis by HPLC-MS, compared to only 7hrs incubation time in the current study. Further HPLC-MS studies will be necessary to clarify the differences between *C. elegans* and *H. contortus* metabolism of albendazole.

6.6 The role of drug metabolism in anthelmintic resistance requires further investigation

The data presented within the current study provides solid evidence that the genome of the free-living nematode *C. elegans* encodes genes that are transcriptionally responsive to the presence of albendazole and that the nematode is able to metabolise the drug. Studies by other groups suggest that these pathways are also present in certain parasitic nematodes including *H. contortus* and *A. suum* (Cvilink *et al.*, 2008; Solana *et al.*, 2001). However, in order to assess whether these pathways are involved in resistance to anthelmintics will require further studies. Whether or not the drug metabolites produced by *C. elegans* and parasitic nematodes are pharmacologically active or not must be assessed. In many cases within mammals the activity of drugs is actually increased following interaction with XMEs. For example albendazole sulphoxide is an active metabolite and production of this metabolite by nematodes will not provide any protection from the drug. Analysis of the albendazole-glucoside metabolites produced by *C. elegans* could be carried out using nuclear magnetic resonance spectroscopy in an attempt to identify the molecular position of the glucose conjugate. Following these studies, synthesis of albendazole glucoside and assessment of its pharmacological activity could be carried out in both *C. elegans* and parasitic nematodes.

Resistance to anthelmintics in parasites in the field is unlikely to be conferred by an alteration in the rate of induction of XMEs following drug exposure, especially in the case of rapidly acting drugs such as ivermectin. A far more likely scenario is that of a mutation in an enzyme resulting in increased activity against an anthelmintic(s), or in regulatory regions or regulators resulting in a constitutively overexpressed enzyme. However, studies investigating the induction of XMEs following exposure to drug are relevant to discovering enzymes potentially involved in resistance. Giraudo *et al.* (2009) reported that eight of the twelve CYPs known to be involved in insecticide resistance in *D. melanogaster* have also been shown to be inducible by xenobiotic exposure. Differences in the expression level of XME encoding genes between anthelmintic resistant and susceptible populations of *H. contortus* are currently being investigated in the Gilleard lab (*pers. comm.*, R. Laing and J.S. Gilleard). Should

these studies show an association between gene overexpression and resistance, then functional studies can be carried out to prove a definite causal relationship. Studies in *C. elegans* will be fundamental in carrying out these experiments. RNAi experiments, similar to those carried out by Schafer *et al.* (2009), will allow the elucidation of the specific identity of the enzymes involved in anthelmintic metabolism. This will guide the analysis of expression data from *H. contortus* and potentially other parasites. More importantly, functional studies will require the use of RNAi, specific knock-out mutants and nematodes over-expressing genes of interest. Given the difficulty of carrying out these types of experiments in a parasitic nematode, with a limited arsenal of genomic tools available, it is likely that any such experiments must be carried out in a heterologous system. Thus far *C. elegans* provides the best platform upon which to carry out such studies and the methods presented in the current study will require little modification for this purpose. The most important alteration required will be the use of an axenic culture system to rule out bacterially derived metabolites of the drugs.

Knowledge of the metabolism of anthelmintics by nematodes and modification of the HPLC-MS techniques may also have applications in the design of novel therapeutics. Currently potential drug candidates are screened for their rate of metabolism in mammalian systems early in the drug discovery process. Similar screens investigating target organism metabolism may also be of use in screening out compounds that are likely to be easily deactivated by nematode metabolising enzymes. Many cytochrome P450 genes are closely positioned in the *C. elegans* genome. We hoped to investigate whether transgenic expression of whole fosmids, containing several of these genes, could result in significant up-regulation of the genes of interest and be of use as a screening mechanism for nematode metabolism. Several transgenic lines were created containing the fosmid WRM0616dG11 (Geneservice), which contains *cyp-37B1*. Initial studies using RT-QPCR analysis of the transgenic nematodes showed a 40-fold up-regulation of *cyp-37B1* in the transgenic line compared to wild-type worms. However, this experiment has thus far only been carried out once and further studies will be necessary to assess whether or not the result is repeatable. An alternative to this process would be to express parasite XME encoding genes in bacteria.

The current study was unable to define XME encoding genes that are transcriptionally responsive to ivermectin exposure or any nematode derived metabolites of ivermectin. This may suggest that wild-type nematodes are not able to metabolise the drug or that they do so at an extremely low level. However, the complication of the overwhelming fasting response in ivermectin exposure experiments may have masked the presence of important drug metabolism pathways. In addition, overexpression of a XME in a resistant isolate may increase the rate of ivermectin metabolism so that it results in a physiologically significant decrease in ivermectin concentration at the active site. Further studies with XME over-expressing mutants may help to investigate this further.

Finally, one of the main aims in investigating mechanisms of anthelmintic resistance is the development of a sensitive diagnostic test for resistance emergence in the field. Overexpression of a gene is assessed using RNA or protein quantitation, but these assays will be of little use in the field due to the unstable nature of both RNA and proteins. Therefore, the genetic mechanism by which overexpression of XMEs may occur must be elucidated. Daborn *et al*. (2002) have demonstrated that overexpression of a single CYP isoform in *D. melanogaster,* resulting population wide multidrug resistance, was due to the upstream insertion of an *Accord* transposon. However, as has been seen with other examples of insecticide resistance, gene duplication events may also result in the functional overexpression of XMEs (Li *et al.*, 2007). Investigation of these mechanisms should allow the development of a DNA based assay for the presence of these mutations, which is far more likely to be of use to farmers and clinicians.

Appendices

7.1 RT-QPCR primers and typical reaction efficiencies

Gene ID	Primers	Efficiency (%)
acs-2	F: 5′-GGA GAT ACC GCC ACG ATG AA-3′	103.9
	R: 5′-ATG TTC TCT CCG TAC CTG TCA T-3′	
ama-1	F: 5′-AAG CGG CTC ACA ATG ATC TAC GA-3′	96.3
	R: 5′-ACA CGG CGG TAT GAT GGT TGA-3′	
C06B3.1	F: 5′-GGC TAC CAC ATT GTC CGA GTT-3′	102.8
	R: 5′-GTA GTT TCG GTA GAT TCG GCT T-3′	
C23G10.11	F: 5′-ATT CTA GCC GTC CTA CTC ATC TT-3′	92.5
	R: 5′-GCT TGC ATT CCA CCA GTG GTT-3′	
C29F7.2	F: 5′-CGG AGT TAG GGT ACA TGT CAA-3′	102
	R: 5′-CAA CAT TAG CAG AGT GGT CAG TT-3′	
C35C5.8	F: 5′-GGA GTG TAA CAC TCT TGG TCA T-3′	100.6
	R: 5′-AGC TGC ATT TCA TAC TTC TCA CAA-3′	
C45G7.1	F: 5′-GTC GTT GGT TTT TCT GAC TAT TG-3′	84.9
	R: 5′-CTT CCA CGC GGC TTC AGT T-3′	
clec-174	F: 5′-CGT TTG CCC AGT CGG TAA TGA-3 ′	90.1
	R: 5′-ACC GGA CGA TAA TGG CAA GAA T-3′	

col-19	F: 5′- GTT CCA GGA TGG TAT GGT TGA ATT AGA GCT-3′ R: 5′-GGT CCG CAG TTA CAT TGC TCG AAT CC-3′	96.8
cyp-35A2	F: 5′-ATG ACT GCA CCC GTT TGG TTT-3′ R: 5′-ACG CGT CAG TGT AAT CTT GCA-3′	98.8
cyp-35A5	F: 5′-AAA AGG TTA TCC CAT TCG GAG TT-3′ R: 5′-AAC GCT CTC TTT GCA ATA CTG TA-3′	98.1
cyp-35C1	F: 5′-GAG ATT TGA TGG AGG AGA AGA TT-3′ R: 5′-CAT CAA ATC GAA ATC CTA AGA GCA-3′	90.1
cyp-37B1	F: 5′-AAG AAC GGT GGA GCA GGA TGT-3′ R: 5′-TTC GGG GTC CAG CAC TAA TG-3′	100.1
dod-3	F: 5′-GAT TGT TAC GCC ACC ACC GTA T-3′ R: 5′-TGG GCG GGC CAC ATG AAC A-3′	95.6
F09F7.6	F: 5′-TTA GAA TCC ACG ACG CGC CAA AT-3′ R: 5′-TCG GCG GCT TCC AGA TCA TCA-3′	97.3
F21C10.10	F: 5′-TGC TGA AAC TGT CGT CGG TCT T-3′ R: 5′-CTT GTC AGC GAG TTT TTG TTG TTG-3′	96.8
F43C11.7	F: 5′-CTG ACC AGT GAG GAG GAC A-3′ R: 5′-GGT TTT CAA TTC CAT TGG TGG TTT-3′	85.9

F45D3.4	F: 5′-AAC CAA CTA CAA CAA CTA CTG AAA-3′	77.7
	R: 5′-TTC AGT AAC AAA AGA TCC AGT GA-3′	
F53A9.8	F: 5′-GAA CAC GGA CAC GGA GAT GGT-3′	99.9
	R: 5′-GTG ATG TTG CTC GTG GTG TTC T-3′	
F54F3.3	F: 5′-CCA GCA TAC GAC TTC ACT ACT-3′	85.6
	R: 5′-CAC GGA GTC CCC AGA TGA A-3′	
F57G8.7	F: 5′-TCG TGG GGC CAA ATA AGG GAA-3′	95.5
	R: 5′-TCA ACA TGA ACA CCT GGT GGA A-3′	
gei-7	F: 5′-GAT TCG GTG GAG CCC TGA AT-3′	96.2
	R: 5′-CGC AGA CAT CAG CAG CCA AA-3′	
gst-1	F: 5′-CCG GAG ACG AGG AGA TTG TTC AA-3′	92.8
	R: 5′-GCC TTG CCG TCT TCG TAG TTT CT-3′	
gst-5	F: 5′-CCG GAC AAC AAT ACG AGG AT-3′	105.3
	R: 5′-GCG GTT TTT CCG TTG AGC TT-3′	
hsf-1	F: 5′-CGT TGG ATG ATG ATG AAG AAG GAT-3′	95.1
	R: 5′-AGC CGG TGA ATG TGG GAA GAT-3′	
ilys-3	F: 5′-GTT GTA ACA TGG ACG TCG GAT-3′	99.3
	R: 5′-CAC ATT GAC TCT TGT AGC GGT T-3′	

K03D3.2	F: 5′-GCC TGG AAG ATG ACG ATG ATA A-3′	108.3
	R: 5′-CAG GAC GAC ATT CTT GCC CTT-3′	
K12G11.3	F: 5′-ACG AAG GAG CTG GAA GTG TTG TT-3′	101.7
	R: 5′-CTC CTG GAA AGT TCC AGA ACG AT-3′	
lea-1	F: 5′-CGC AGA TTC CTT CAA AGC CCA-3′	104.1
	R: 5′-CCC AAG CAT CAC CAG CCT TAT-3′	
mtl-1	F: 5′-CTT GCA AGT GTG ACT GCA AAA AC-3′	92.7
	R: 5′-CTT GCA GTC TCC CTT ACA TCC-3′	
pgp-1	F: 5′-GGA GCC GCG TCT GGT ATC TAT-3′	96.7
	R: 5′-GAC CTG CAT TTA CAC GGA GAT TCA-3′	
scl-2	F: 5′-ACT CAA ATG GCG TGG GCG AA-3′	82.7
	R: 5′-GAC GCA GAG CCC TGT GGA-3′	
sip-1	F: 5′-AGC CGG AAG AGT TGA AGG TCA AT-3′	89.7
	R: 5′-TGG AGC CGG TCT TTG GAG CA-3′	
T12D8.5	F: 5′-CCG TAT GTA GCC TCG GAG A-3′	93.1
	R: 5′-CGG TCG ATC TCC TGT TTC AA-3′	
T16G1.6	F: 5′-GGG AAT GGA ATA TGT CGA TGA T-3′	86.6
	R: 5′- CTT TTA GAC CAT CGT CGT TGA A-3′	

T22F3.11	F: 5′-ATC CCA GCC GAG AAC AAG TAT T-3′	85.6
	R: 5′-GGT GGT GAC GAA GAG AGC AA-3′	
tts-1	F: 5′-TTT GAT GTA GGT GGA AAT TGG CA-3′	91
	R: 5′-GTT GAG CCG GTC AAG TTT TCT-3′	
ugt-16	F: 5′-TGC ATC AAT GCC GGA AAC TAC TT-3′	103.6
	R: 5′-TTC CAA GCC CTC CGT GAG TT-3′	
ugt-25	F: 5′-GTA CTA GAC GAA CGA CCA CAT AA-3′	100
	R: 5′-AAG AGC TGT TTG AGG AAC CCA TT-3′	
ugt-63	F: 5′-CGC CAG GAC ATT GAT TTT GGA A-3′	103.3
	R: 5′-ACG GTG CTT CAG GAT GTT GTT-3′	

7.2 GFP fusion construct primers

7.2.1 cyp-35C1

35C1FuPrA	5′-ATC CTA CGA GCG ACC CAG TT-3′
35C1FuPrB	5′-CCT TTG GGT CCT TTG GCC AAT CCC TGT TTT GCA ATA GAA ATG AAC AA-3′
35C1FuPrA*	5′-CTA CGA GCG ACC CAG TTT TC-3′

7.2.2 cyp-37B1

37B1FuPrA	5′-TCA CTG TTG TAC TCG AAT CTG-3′
37B1FuPrB	5′-CCT TTG GGT CCT TTG GCC AAT TTT TTA ATT TCA ATT TCA AAA ACT AG-3′
37B1FuPrA*	5′-ACT CGA ATC TGT TAA AAA CG-3′

7.2.3 mtl-1

mtl-1FuPrA	5′-CTT CCC GTT GTC TGT CTA TAG A-3′
mtl-1FuPrB	5′-CCT TTG GGT CCT TTG GCC AAT CCC GAT TTC TTA ATT TCA GCA GTC-3′
mtl-1FuPrA*	5′-CCG TTG TCT GTC TAT AGA GTT TTT-3′

7.2.4 scl-2

scl-2FuPrA	5′-AGC CGT TCG TGA TAC TTG TA-3′
scl-2FuPrB	5′-CCT TTG GGT CCT TTG GCC AAT CCC AAT TGG AGA AAA AAG TGC AAG TC-3′
scl-2FuPrA*	5′-GTG ATA CTT GTA AAC GTC TGA ATA-3′

7.2.5 GFP (pPD95.67 template)

95.67FuC	5′-GGG ATT GGC CAA AGG ACC CAA AGG-3′
95.67FuD	5′-AAG GGC CCG TAC GGC CGA CTA GTA GG-3′
95.67FuD*	5′-GGA AAC AGT TAT GTT TGG TAT ATT GGG-3′
GFP_R	5′-GAG CAT GTA GGG ATG TTG AAG AG-3′

7.3 DA1316 sequencing primers

7.3.1 avr-14 (ad1302)

ad1302F 5′-ACT TTG CTG AAT CGG CAG GTT-3′

ad1302R 5′-CTG AAT GTG AAT TGA GCA CTG TA-3′

ad1302F[+] 5′-AAT CGG CAG GTT CAG GAG TT-3′

ad1302R[+] 5′-GTG AAT TGA GCA CTG TAT TCC AT-3′

7.3.2 avr-15(ad1051)

ad1051F 5′-ACC AGG AGA GGA TGG AAC AA-3′

ad1051R 5′-GGA AGA ACG AGT CGG GCA T-3′

ad1051F[+] 5′-GAG AGG ATG GAA CAA TAC AT-3′

ad1051R[+] 5′-AGA ACG AGT CGG GCA TCC AA-3′

7.3.3 glc-1(pk54)

DKV1.3 5′-TAA TGG AGG ACC AGT TGT GG-3′

Tcl_R1 5′-GCT GAT CGA CTC GAT GCC ACG TCG-3′

[+]Nested primers used for direct sequencing of PCR fragments

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