Transcriptional responses in *Parascaris univalens* after *in vitro* exposure to ivermectin, pyrantel citrate and thiabendazole

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Abstract

Background: *Parascaris univalens* is a pathogenic parasite of foals and yearlings worldwide. In recent years *Parascaris* spp. worms have developed resistance to several of the commonly used anthelmintics, though currently the mechanisms behind this development is unknown. The aim of this study was to investigate the transcriptional responses in adult *P. univalens* worms after *in vitro* exposure to different concentrations of three anthelmintic drugs, focusing on drug targets and drug metabolising pathways.

Methods: Adult worms were collected from the intestines of two foals at slaughter. The foals were naturally infected and had never been treated with anthelmintics. Worms were incubated in cell culture media containing different concentrations of either ivermectin (10^{-9} M, 10^{-11} M, 10^{-13} M), pyrantel citrate (10^{-6} M, 10^{-8} M, 10^{-10} M), thiabendazole (10^{-5} M, 10^{-7} M, 10^{-9} M) or without anthelmintics (control) at 37 °C for 24 h. After incubation the viability of the worms was assessed and RNA extracted from the anterior end of 36 worms and sequenced on an Illumina NovaSeq 6000 system.

Results: All worms were alive at the end of the incubation but showed varying degrees of viability depending on the drug and concentration used. Differential expression ($p < 0.05$ and fold change ≥ 2) analysis showed similarities and differences in the transcriptional response after exposure to the different drug classes. Candidate genes up- or downregulated in drug exposed worms include members of the phase I metabolic pathway short-chain dehydrogenase/reductase superfamily (SDR), flavin containing monooxygenase superfamily (FMO) and cytochrome P450-family (CYP) as well as members of the membrane transporters major facilitator superfamily (MFS) and solute carrier superfamily (SLC). Generally, different targets of the anthelmintics used were found to be up- and downregulated in an unspecific pattern after drug exposure, apart from the GABA receptor subunit *lgc-37*, which was upregulated only in worms exposed to 10^{-9} M of ivermectin.

Conclusions: This is the first time the expression of these genes has been described in *P. univalens* and future work should be focused on characterising these candidate genes further to explore their potential involvement in drug metabolism and anthelmintic resistance.

Background

Nematodes within the genus Parascaris are pathogenic parasites of foals and yearlings worldwide. Traditionally the parasite has been referred to as Parascaris equorum but recent cytological studies have established that the major species currently infecting horses in the USA and Europe is P. univalens (1–3). Parascaris spp. infection causes nasal discharge, coughing and impaired growth, while large burdens can be lethal due to obstruction and perforation of the small intestine (4, 5). To avoid parasite-related disease most foals are usually treated with anthelmintics from the drug classes macrocyclic lactones, benzimidazoles or tetrahydropyrimidines several times during the first year (6). Macrocyclic lactones act by binding to parasite specific glutamate- and γ-aminobutyric acid (GABA) - gated ion channels in nerve and muscle cells, increasing the cells permeability to Cl^- ions and leading to hyperpolarization which

results in paralysis of the parasite (7). Benzimidazoles bind parasite β-tubulin molecules, thereby disrupting the polymerisation of microtubules, causing starvation and death of the worm (8). Tetrahydropyrimidines act as an agonist to the L-type nicotine acetylcholine gated ion channels, allowing Cl^- to flow through, leading to depolarisation of muscle cells and spastic paralysis of the parasite (9).

Overuse of anthelmintic drugs has contributed to the development of resistance in several parasites of veterinary importance (10). The first reported case of anthelmintic resistance in Parascaris spp. was to the macrocyclic lactone ivermectin in 2002 (11) and since then ivermectin resistance has been reported from around the world and is now considered wide spread (12). Resistance to the tetrahydropyrimidine pyrantel was first discovered in the USA in 2008 (13) and has also been found in Australia (14) and, more recently, in Europe (3, 15). The benzimidazole fenbendazole is generally effective against Parascaris spp. in Europe (3, 15), but sporadic cases of treatment failure have been reported from Australia (14) and Saudi Arabia (16). Considering the risk of lethal complications in foals infected with Parascaris spp. and the lack of new anthelmintic drugs for the equine market the development of resistance to all available drug classes in Parascaris spp. is a major threat to equine health and the equine industry.

Despite the increasing problem of anthelmintic resistance, the underlying causes of the development of resistance in parasitic nematodes are still poorly understood, particularly in ascarids. Advances in molecular techniques and genetics, such as the recent publication of a high quality reference genome of P. univalens (17), have provided new possibilities to investigate responses in potential drug targets and pathways that may lead to resistance. There are several suggested mechanisms of anthelmintic resistance, including conformational changes or altered expression of the target molecule of the drug. Examples of conformational changes in the drug target are the three single nucleotide polymorphisms (SNPs) in the β-tubulin gene of strongyle nematodes. These mutations lead to amino acid substitutions and have been associated with the loss of action of benzimidazoles, particularly in strongyle nematodes of veterinary importance (18–20). Decreased sensitivity to macrocyclic lactones in Haemoncus contortus was suggested to be connected to SNPs in the GABA receptor subunit gene lgc-37 (21, 22), although this was challenged (23). Additionally, reduced expression of the glutamate gated chloride channel avr-14 was described in ivermectin resistant isolates of Cooperia oncophora and Ostertagia ostertagi (24).

Other proposed mechanisms of anthelmintic resistance include changes in drug metabolising enzymes and alterations in efflux pumps leading to inactivation or removal of the drugs (25). Drug metabolism is usually divided into two phases; in phase I oxidation, reduction or hydrolysis convert the drug to a more reactive compound that can be conjugated with an endogenous molecule such as glutathione or glucose in phase II. This results in a soluble, inactive drug that can be removed from the cell (26). The phase I enzymes of the cytochrome P450-family (CYP) have been shown to be involved in drug resistance in insects (27), but the involvement of phase I and II enzymes in anthelmintic resistance of parasitic nematodes has not been thoroughly investigated, particularly not in ascarids. However, constitutively higher expression of a CYP34/35 family member (28) as well as the phase II enzyme uridine 5'-diphospho-glucuronosyltransferase-glucosyltransferase (UGT) (29) have been found in BZ resistant strains of H. contortus. Several studies have also shown evidence for upregulation of genes encoding

drug efflux pumps, such as ATP-binding cassette (ABC) transporters, in macrocyclic lactone resistant strongyle nematodes (30–32). Taken together, these results indicate that changes in drug metabolism and efflux play a role in anthelmintic resistance.

Despite anthelmintic resistance in P. univalens being a growing threat to equine health little is known about the molecular mechanisms behind the development of resistance. To our knowledge only two reports have studied the expression of genes after in vitro drug exposure of adult Parascaris spp. (33, 34).

The aim of our study was to identify genes that respond to drug treatment, focusing on drug targets, metabolising enzymes and transporters in adult P. univalens. We have used a whole transcriptome approach to compare the gene expression after in vitro exposure to sub-lethal doses of ivermectin, pyrantel citrate and thiabendazole.

Methods

No ethical permissions were necessary for this study as the parasites were collected from horses slaughtered for meat production.

Parasite material and karyotype

Adult P. univalens were collected from the intestines of two Icelandic foals, approximately six-months old at an abattoir in Selfoss, Iceland. The horses originated from the same farm in southern Iceland and had never been treated with anthelmintic drugs. After removal from the intestine, worms were rinsed with 37 °C PBS (Life Technologies) and transported to the laboratory (Institute for Experimental Pathology at Keldur, University of Iceland, Reykjavík, Iceland) in an insulated box. A faecal sample was taken from one of the foals for karyotyping of Parascaris spp. eggs as described in Martin et al. (3).

In vitro incubation experiment

To investigate the effect of in vitro incubation on viability and gene expression two groups of worms were used. One group of nine worms was divided in to three containers containing cell culture media (RPMI-1640 with the addition of 10% foetal bovine serum, 1% Penicillin/Streptomycin and 1% L-glutamine (Life Technologies)) and then incubated for 24 h at 37 °C (control 24 h^{-DMSO}). The other group of worms was immediately killed by decapitation upon arrival to the laboratory to serve as controls for the in vitro incubation (control 0 h).

In vitro drug exposure experiment

Worms were divided into three groups, one for each drug class. The worms were exposed to three different concentrations of ivermectin (IVM) (Sigma), pyrantel citrate (PYR) (Santa Cruz Biotechnology) or thiabendazole (TBZ) (Sigma), according to Table 1. The drug concentrations used were based on previous studies by Janssen et al. (34) for ivermectin exposure and Zhao et al. (35) for pyrantel citrate and thiabendazole exposure. All anthelmintic drugs were dissolved in DMSO (Swedish Veterinary

Institute) and serially diluted before addition to the media. For each concentration three biological replicates, each consisting of three worms, were divided into different containers containing media and drug (final concentration of DMSO 0.1%) and incubated at 37 °C for 24 h (Table 1). An additional group of three worms, serving as control was incubated in media with the addition of 0.1% DMSO (control 24 h^{+DMSO}).

Table 1
Samples included in the in vitro exposure experiment. Mean viability score of adult Parascaris univalens, number of differentially expressed genes ($p < 0.05$ and fold change ≥ 2) and proportion of unknown differentially expressed genes after exposure to the anthelmintic drugs ivermectin (IVM), pyrantel citrate (PYR) and thiabendazole TBZ) at different concentrations for 24 h. The gene expression of drug exposed worms were normalized to control 24 h^{+DMSO}.

Drug and concentration	Viability	Differentially expressed genes	Unknown genes
IVM 10^{-9} M	3.5	256	44%
IVM 10^{-11} M	4.5	119	44%
IVM 10^{-13} M	6.0	177	46%
PYR 10^{-6} M	3.0	38	50%
PYR 10^{-8} M	4.5	191	45%
PYR 10^{-10} M	6.0	84	50%
TBZ 10^{-5} M	4.0	154	46%
TBZ 10^{-7} M	5.0	46	43%
TBZ 10^{-9} M	5.0	161	51%

Viability scoring and dissection

After 24 h incubation, worm viability was assessed according to the scoring system developed by Scare et al. (36) to evaluate the effects of the different drug concentrations on viability. The worms in each container were observed and viability scores between 2 (movement only when stimulated with forceps) and 6 (seven or more whole body movements without stimulation), were given for each container. One worm from each container (n = 3) was dissected and the anterior end, containing the pharynx and a small part of the anterior intestine, was placed in individual tubes containing RNAlater (Ambion). Three worms were dissected directly upon arrival at the laboratory, serving as controls for the in vitro incubation experiment (control 0 h). The samples were then transported to Sweden (Swedish University of

Agricultural Sciences, Department of Biomedical Sciences and Veterinary Public Health, Division of Parasitology) for the molecular analysis.

RNA extraction and sequencing

The samples were removed from RNAlater and cut into smaller pieces. 1 mL of Trizol (Invitrogen) was added to each sample and the tissue was homogenized in a glass tissue grinder. After addition of 0.2 mL of chloroform and centrifugation of the sample 100 µL of the upper aqueous phase was mixed with 350 µL lysis buffer from the NucleoSpin® RNA Plus kit (Macherey Nagel). RNA was then isolated as described in the user manual of the kit. rDNase treatment and subsequent clean-up (NucleoSpin RNA Clean-up, Macherey Nagel) was performed according to the manufacturer´s protocol to ensure RNA purity.

Sample preparation and sequencing were performed at SciLifeLab Uppsala, SNP&SEQ Technology platform. RNA concentration and integrity were checked by Fragment Analyzer (Agilent) before sequencing libraries were prepared from 500 ng total RNA using the TruSeq stranded mRNA library preparation kit including polyA selection (Illumina Inc.) resulting in insert sizes of approximately 140 bp. Three biological replicates per condition were sequenced using Illumina NovaSeq S1 flow cells and 100 bp paired end v1 sequencing chemistry, resulting in 36 transcriptomes.

RNA-seq analysis

Read quality assessment, adaptor removal, filtering and removal of duplicates was performed using fastp (37) (for pipeline see supplementary info). The resulting reads were mapped against the P. univalens reference transcriptome (parascaris_univalens.PRJNA386823.WBPS11.mRNA_transcripts.fa) available in WormBase ParaSite https://parasite.wormbase.org/Parascaris_univalens_prjna386823/Info/Index/ and transcripts were quantified using salmon version 0.11.3 (38). Transcript-level abundance, estimated counts and lengths from salmon were summarised together with gene-ids from the P. univalens transcriptome into matrices by the tximport R package (39) for downstream differential expression analysis. Genes with five or more read counts were included in the differential expression analysis performed by the R package DESeq2, version 1.22.2 (40). Resulting p-values were adjusted for multiple testing using the Benjamini–Hochberg procedure (41) as applied by the R base p.adjust function. For principal component analysis (PCA), gene counts were transformed into log2 scale by applying regularized logarithm approach using the rlog function provided in DESeq2 package and plotted using the plotPCA function. For all the above analysis R version 3.5.2 was used (42). Only genes with a fold change of two or more and an adjusted P-value < 0.05 were considered differentially expressed, both up- and downregulated. Orthologues of differentially expressed genes were identified by searching the protein sequences against the Swiss-Prot database (43) using BlastP (e-value $\leq 10^{-5}$). Candidate genes for drug metabolism and drug targets significantly differentially expressed after exposure to one or more drugs were identified by their orthologue name. To identify the number of genes common among the concentrations of a particular drug, gene IDs were used to construct comparative Venn diagrams using the venn.diagram function of the VennDiagram package in R (44), version 3.6.1. Gene IDs corresponding

to individual Venn diagram partitions were retrieved using the get.venn.partition function. Superfamilies of genes shared between concentrations were identified by BlastP searches (e-value $\leq 10^{-6}$) in NCBI (https://www.ncbi.nlm.nih.gov/).

Results

Karyotype

P. univalens was identified in the karyotype as DAPI stained eggs from the first mitotic division showed one pair of chromosomes (Fig. 1).

Viability after in vitro incubation

After 24 h incubation, all worms were alive irrespective of drug and concentration used for exposure. Control worms were highly viable (score 6), whereas worms incubated in the highest drug concentrations were visibly less viable (score 3–4) than those incubated in lower concentrations (score 5–6) (Table 1).

Sequencing

RNA with RIN values between 6.6 and 8.7 were sequenced on Illumina Nova SEq. The number of reads per sample varied between 63 million and 132 million after quality assessment and filtering by fastp. Sequences have been deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB37010 (https://www.ebi.ac.uk/ena).

Analysis of differentially expressed genes

The number of differentially expressed genes for each drug concentration, and the proportion of uncharacterised genes are shown in Table 1. The PCA plot illustrates the differences in gene expression between individual worms (Fig. 2). It should be noted that the largest difference in gene expression was seen between the non-incubated worms (control 0 h) and both control and drug-exposed worms that were incubated in vitro for 24 h. Though care was taken to choose the largest worms (i.e. females) for the experiment it was discovered during the dissection that two individuals (IVM 10^{-11} M individual 2 and TBZ 10^{-5} M individual 1) lacked egg containing uterus. These two individuals did not show up as outliers in the PCA plot (Fig. 2) and were therefore included in the analysis.

Transcriptional response in control worms after in vitro incubation

The largest number of differentially expressed genes was seen in the in vitro incubation experiment, where unexposed worms incubated in media (control 24 h^{-DMSO}) were compared to non-incubated worms (control 0 h). Between these two groups 1061 genes were differentially expressed and interestingly,

among these were a number of candidate genes putatively involved in drug metabolism, such as CYPs, flavin containing monooxygenase (FMO), short chain dehydrogenase/reductase (SDR), glutathione S-transferase (GST) and UGTs as well as ABC-transporters (Supplementary table 1). Forty percent of the differentially expressed genes were uncharacterised.

Differential expression of putative drug targets

Putative drug targets were identified by their annotation and orthologue. Transcripts of ten drug targets were differentially expressed in worms exposed to IVM, PYR or TBZ compared to control 24 $h^{+ DMSO}$ (Fig. 3a). An orthologue to the GABA-receptor lgc-37 in H. contortus (GenBank accession number X73584), transcript PgR047_g061, was upregulated after exposure to the highest concentration of IVM (10^{-9} M). Three transcripts orthologous to acetylcholine receptor subunits (AChRs); PgB02X_g213, PgR034_g017 and PgR034_g018, were upregulated, whereas five AChRs; PgR043_g026, PgR075_g041, PgR005X_g207, PgR006_g054 and PgR045_g040 were downregulated, all in an unspecific pattern regardless of drug used (Fig. 3a). The transcript PgE153_g002, identical to the previously described P. equorum β-tubulin isotype 2 (GenBank accession number KC713798), was downregulated after exposure to the highest dose of TBZ (10^{-5} M) but also after exposure to IVM (10^{-11} M) and PYR (10^{-8} M and 10^{-10} M) (Fig. 3a).

Comparison of the transcriptional response after exposure to different concentrations of IVM, PYR or TBZ

Differentially expressed genes shared among the three concentrations of each drug are visualized in Fig. 4 and Table 2. After IVM exposure 14 transcripts showed overlapping differential expression for all three concentrations (Fig. 4a). Of these, nine transcripts were upregulated whereas five were downregulated. The differentially expressed genes included four transcripts belonging to the SDR superfamily, putatively involved in phase I metabolism, two transcripts of the major facilitator superfamily (MFS), known to be involved in drug efflux and resistance in bacteria and yeast, and one transcript of the solute carrier superfamily (SCS), involved in transportation. Of the remaining seven differentially expressed transcripts three do not have any known connection to drug metabolism or transport, while four transcripts are uncharacterised (Table 2).

Table 2

Differentially expressed genes (fold change ≥ 2 or and adjusted P-value < 0.05) and their corresponding superfamilies shared among Parascaris univalens worms exposed to three different concentrations of the anthelmintic drugs ivermectin (IVM), pyrantel citrate (PYR) or thiabendazole (TBZ) for 24 h. The gene expression of drug exposed worms were normalized to control 24 h^{+DMSO}.

Gene-id	Superfamily	Log2FC		
Ivermectin	Concentration:	10^{-9} M	10^{-11} M	10^{-13} M
PgB01_g106	Short-chain dehydrogenases/reductases (SDR) cl25409	− 1.05	− 1.57	− 1.50
PgR004_g112	Short-chain dehydrogenases/reductases (SDR) cl25409	1.94	1.94	1.27
PgR007_g080	Short-chain dehydrogenases/reductases (SDR) cl25409	1.92	1.75	1.80
PgR127_g021	Short-chain dehydrogenases/reductases (SDR) cl25409	1.54	1.50	1.36
PgR006_g137	The major facilitator superfamily (MFS) cl28910	-1.12	-1.39	-1.92
PgR015_g078	The major facilitator superfamily (MFS) cl28910	2.32	2.11	2.44
PgR011_g039	Solute carrier families 5 and 6-like superfamily (SLC) cl00456	1.15	1.29	1.47
PgR135_g007	C-type lectin (CTL) cl02432	1.06	1.29	1.28
PgR422_g001	RNA recognition motif (RRM) superfamily cl17169	1.62	1.63	1.99
PgR037_g063	Clc-like superfamily cl06205	1.72	1.84	1.39
PgB06_g058	No conserved domains detected	1.69	1.77	2.51
PgR061_g018	No conserved domains detected	-1.55	-1.84	-2.14
PgR142_g012	No conserved domains detected	-2.15	-1.26	-1.53
PgR011_g030	No conserved domains detected	-1.04	-1.40	-1.79
Pyrantel	Concentration:	10^{-6} M	10^{-8} M	10^{-10} M
PgR006_g137	Major facilitator superfamily cl28910	-1.97	-1.88	-1.70

Supplementary table 1.xls Parascaris univalens in vitro incubation experiment. Differentially expressed genes (fold change ≥ 2 or and adjusted P-value < 0.05) and their corresponding orthologues according to Swiss-Prot database after 24 h in vitro incubation in media (control 24 h^{-DMSO}). The gene expression was normalized to non-incubated worms (control 0 h).

Gene-id	Superfamily		Log2FC	
PgB08_g086	alpha/beta hydrolases cl21494	-3.89	-4.04	-2.64
PgB27X_g004	C-type lectin (CTL) cl02432	-3.11	-4.46	-2.05
PgR050X_g009	Olfactomedin-like domain cl02549	-3.20	-3.62	-1.62
PgB08_g034	CUB domain cl00049	-5.65	-4.90	-1.13
PgE206_g001	No conserved domains detected	-2.88	-3.92	-3.08
PgR022X_g030	No conserved domains detected	-5.72	-5.15	-2.92
PgR022X_g031	No conserved domains detected	-8.43	-4.43	-2.23
PgR093_g009	No conserved domains detected	-4.87	-4.88	-2.64
PgR186_g008	No conserved domains detected	1.05	1.82	1.51
Thiabendazole	Concentration:	10^{-5} M	10^{-7} M	10^{-9} M
PgB08_g034	CUB domain cl00049	-2.82	-3.52	-5.09
PgR004_g040	Carbonic anhydrase alpha (vertebrate-like) group cl00012	-1.70	-3.65	-1.91
PgE192_g001	No conserved domains detected	1.30	3.48	2.17
PgR004_g250	No conserved domains detected	-1.50	-1.73	-1.81
PgR022X_g030	No conserved domains detected	-4.64	-5.65	-6.83
PgR049_g007	No conserved domains detected	-6.11	-9.59	-8.19

Supplementary table 1.xls Parascaris univalens in vitro incubation experiment. Differentially expressed genes (fold change ≥ 2 or and adjusted P-value < 0.05) and their corresponding orthologues according to Swiss-Prot database after 24 h in vitro incubation in media (control 24 h^{-} ^{DMSO}). The gene expression was normalized to non-incubated worms (control 0 h).

After exposure to PYR ten transcripts were commonly differentially expressed (Fig. 4b) and all but one were downregulated. As in the IVM exposed worms the transcript PgR006_g137, a member of MFS, was downregulated (Table 2). Of the remaining differentially expressed transcripts four belong to superfamilies not connected to drug metabolism or transportation, whereas five transcripts are uncharacterised (Table 2).

Six transcripts were commonly differentially expressed after exposure to all three concentrations of TBZ (Fig. 4c) where all but one were downregulated. Two transcripts are involved in processes not connected to drug metabolism or transport while four are uncharacterised or not similar to any known superfamilies (Table 2).

Differential expression of other putative candidate genes

Putative candidate genes involved in drug metabolism were identified by their annotation and orthologue. Transcripts of 12 metabolising genes involved in phase I and phase II drug metabolism were identified in worms exposed to all drugs (Fig. 3b).

Four phase I transcripts were differentially expressed after drug exposure. One transcript belonging to the CYP family (PgR020_g037) was downregulated after exposure to the medium concentration of IVM (10^{-11} M). Three transcripts belonging to the flavin containing monooxygenase (FMO) superfamily were differentially expressed in an inconsistent pattern after drug exposure. The transcript PgR003_g196 was downregulated after exposure to IVM (10^{-9} M), TBZ (10^{-5} M) and PYR (10^{-10} M). The transcript PgR016_g066 was upregulated after exposure to IVM (10^{-9} M) only, whereas the transcript PgR161_g008 was upregulated after exposure to PYR (10^{-8} M) and TBZ (10^{-9} M) (Fig. 3b).

Eight genes belonging to two super families of the phase II metabolizing pathway were also differentially expressed in an inconsistent pattern irrespective of drug and concentration used (Fig. 3b). One transcript, PgR024_g121, of the glutatione S-transferase superfamily (GST) was down regulated, but only after exposure to the lowest concentration of IVM (10^{-13} M). Seven transcripts of the uridine 5'-diphospho-glucuronosyltransferase superfamily (UGT) were downregulated in an unspecific pattern after exposure to all drugs (Fig. 3b).

Discussion

The equine roundworm Parascaris spp. is resistant to several classes of anthelmintic drugs, but regardless of this few studies focusing on the mechanisms involved have been published (33, 34, 45). In this study the transcriptomes of 36 individual P. univalens worms, confirmed by karyotyping, were analysed after in vitro exposure to different concentrations of IVM, PYR and TBZ. We found, for the first time in ascarid worms, that the expression of phase I gene families SDR and FMO were affected by exposure to anthelmintic drugs. We also found a 250-fold upregulation of a GABA receptor subunit, orthologous to the H. contortus gene lgc-37 (PgR047_g061), after exposure to the highest IVM concentration (10^{-9} M) (Fig. 3). GABA receptors are targets for IVM in nematodes, but generally show lower affinity to the drug than the glutamate receptors (22). Even so, mutations in lgc-37 have been found to decrease the sensitivity of ML drugs in H. contortus (21) suggesting a role in resistance. Transcriptional regulation of lgc-37 (PgR047_g061) in association with IVM response has not been reported previously, but taken together these results indicate that this gene could be an interesting candidate for further research in P. univalens. In contrast, the putative drug targets for PYR and TBZ were un-specifically differentially expressed after drug exposure. Eight transcripts orthologous to AChR subunits showed no clear pattern after exposure to IVM, PYR and TBZ. Since the L-subtypes of AChRs have not yet been characterized in Parascaris spp. we cannot conclude whether these transcripts are the drug targets for pyrantel. Previous studies have shown that truncated forms of AChRs as well as reduced

expression of AChR subunits have been associated with decreased sensitivity to PYR in strongyle nematodes (46, 47). Furthermore, β-tubulin isotype 2 was downregulated after exposure to all three drug classes, however to a higher degree after TBZ exposure than after exposure to IVM or PYR. So far only two β-tubulin isotypes (isotype 1 and 2) have been described in Parascaris spp. (48), though more might be present in the genome since six putative β-tubulin genes were found in the closely related parasite Ascaridia galli (49). It has also been mentioned that the Ascaris suum genome contains at least nine β-tubulin genes (50). It should be noted that the isoforms of ascarid β-tubulins should not be directly compared to the strongyle nematodes as phylogenetic studies have shown that they are evolutionary separated (48, 49). In a study by Martis et al. (49) where the transcriptomes of A. galli were compared before and after in vivo exposure to flubendazole, no differential expression of β-tubulins was found, whereas a study by Tyden et al. (51) showed an upregulation of β-tubulin isotype 1 in Parascaris spp. eggs exposed to thiabendazole during embryogenesis. However, downregulation of specific isotypes of β-tubulins has been seen in human cancer cells resistant to microtubule destabilising drugs (52, 53), indicating that expressional regulation of β-tubulin could be a cellular response to certain drugs. Comparing the differential expression after exposure to three different drugs gave us valuable insights on how expression of putative drug targets are similarly affected regardless of drug used. This unspecific expressional response of drug targets is an important finding to consider in the analysis of expression data and to our knowledge this is the first time this has been shown in P. univalens.

Several members of the phase I superfamily SDR were found to be differentially expressed after drug exposure in our study. The SDR family is a large superfamily present in all life forms (54) containing enzymes with conserved structure that metabolise endogenous and xenobiotic compounds by phase I reduction (55, 56). Upregulation of SDR enzymes is believed to contribute to resistance towards chemotherapeutic agents in cancer treatment in human medicine (57) and has also been seen in Caenorhabditis elegans after in vitro exposure to benzimidazole drugs (58). The involvement of SDR in drug metabolism in parasitic nematodes is largely unknown. It has been shown that reduction is the main metabolic pathway for flubendazole metabolism in H. contortus, though it has not been established if the enzymes involved belonged to the SDR family or other related superfamilies (59). In summary, members of the SDR family may participate in the xenobiotic metabolism in helminths, but their possible involvement in anthelmintic resistance has not been studied in parasitic nematodes. Members of the FMO family are phase I enzymes known to be involved in the biotransformation of xenobiotic compounds in many phyla (60). FMO enzymes have been shown to play a role in albendazole metabolism in the common liver fluke Fasciola hepatica, and to be involved in the resistance to this drug (61, 62). In contrast, Vokral et al. (63) did not see any activity of FMO enzymes in H. contortus in response to albendazole. In our study two transcripts of the FMO family were upregulated after exposure to all drugs, indicating that they may play a universal role in the drug metabolism in P. univalens. Only one member of the CYP family (PgR020_g037) was differentially expressed in this study, with a downregulation after exposure to IVM (10^{-11} M). This finding is in contrast to the upregulation of the CYP family member PgR071_g005 in P. univalens in vitro exposed to IVM and oxibendazole (33). Upregulation of CYP genes has also been seen in susceptible strains of H. contortus after in vitro exposure to IVM (26).

In summary, we found that several phase I enzymes were differentially expressed in P. univalens after drug exposure. To our knowledge, SDR and FMO enzymes have not yet been characterized in parasitic helminths and therefore their roles in drug metabolism or anthelmintic resistance have not been investigated. Although no consistent pattern was observed regardless of drug and concentration used, we found downregulation of several members of the phase II enzymes of the UGT family. In contrast, several studies have shown an increased expression and activity of UGTs in benzimidazole resistant strains of H. contortus (29, 63). Though the expression and function of phase I and phase II enzymes have been investigated in parasitic helminths, few studies have focused on ascarid worms and thus further investigation is required.

The upregulation of genes encoding transporters and drug efflux pumps have been suggested as a mechanism behind multidrug resistance in parasitic worms (30–32). Focus has so far mainly been on certain ABC-transporters genes, but in agreement with Janssen et al. (34) we did not see any changes in gene expression of pgp-11 and pgp-16 after drug exposure. On the other hand, we found that transcripts of the transportation superfamilies MFS and SCS were differentially expressed after exposure to anthelmintic drugs. These are large superfamilies of membrane proteins present in both pro- and eukaryotes, transporting endogenous substances and drugs across cell membranes (64, 65). The role of MFS proteins in drug resistance in parasitic helminths has so far not been investigated. However, their involvement in resistance to specific drugs as well as multidrug resistance in bacteria and yeast are well studied (64), indicating that these could be interesting candidates to investigate further in regards to anthelmintic resistance.

In a recently published study, Scare et al. (33) studied the transcriptomes of adult P. univalens after in vitro exposure to ivermectin and oxibendazole. Genes identified as potentially involved in drug detoxification and regulatory mechanisms differed from our results with only one similarity between the two studies, a member of the CYP family. These varying results are most likely due to the differences in study design between the two experiments, such as different drug concentrations and drug exposure times. Scare et al. (33) also allowed a 24 h acclimatization period for the worms in tissue culture media before drug exposure, whereas we started the drug exposure at 0 h. These two studies show that despite similar aims experimental design can affect differential expression considerably and thus also highlight the potential for future studies to identify important genes involved in drug interactions not found in either study.

In our experiment unexposed control worms (control 24 h^{-DMSO}) were not visibly affected by incubation at 37 °C for 24 h. Even so, we saw a large number (1061) of differentially expressed genes in these worms when compared to non-incubated worms (control 0 h). Among these genes were several candidates putatively involved in drug metabolism and efflux. This implies that even though not visibly affected by the in vitro incubation, the worms become stressed from the change in environment and nutritional supply when removed from the host intestine to incubation in media. This is an important point to consider when performing in vitro experiments. But since whole live animal experiments in foals

are both expensive and pose ethical dilemmas, further development of in vitro models are crucial for research in parasitic nematodes.

Conclusion

We have explored the changes in gene expression across the complete transcriptome of adult P. univalens in response to in vitro exposure of the anthelmintic drugs ivermectin, pyrantel citrate and thiabendazole. We found a 250-fold upregulation of a possible target of ivermectin, an orthologue to the GABA receptor subunit lgc-37, which is an interesting candidate gene to investigate further. Surprisingly few other candidate genes found in other worms, such as P-gps, were differentially expressed in our experiment. However, our data revealed differential expression of several novel gene candidates belonging to the phase I superfamilies SDR and FMO as well as transporters belonging to the MFS and SLC families. This is the first time the expression of these genes has been described in P. univalens and future work should be focused on characterising these candidate genes further to explore their potential involvement in drug metabolism and anthelmintic resistance.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Nucleotide sequence data reported in this paper are available in the European Nucleotide Archive (ENA) under the accession number PRJEB37010 (https://www.ebi.ac.uk/ena).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

FM, ET, OKL, JH and TB designed the study. FM, ET and ME collected parasite material. FM and ET performed the experiments. FM, OKL, FD and ET analysed the data. FM and ET wrote the manuscript. JH and FD reviewed the manuscript. All authors read and approved the final manuscript.

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Supplementary information

The pipeline used for quality assessment, mapping and quantification is available (with nextflow and docker support) at https://github.com/SLUBioinformaticsInfrastructure/RNAseq_nf.

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Figures

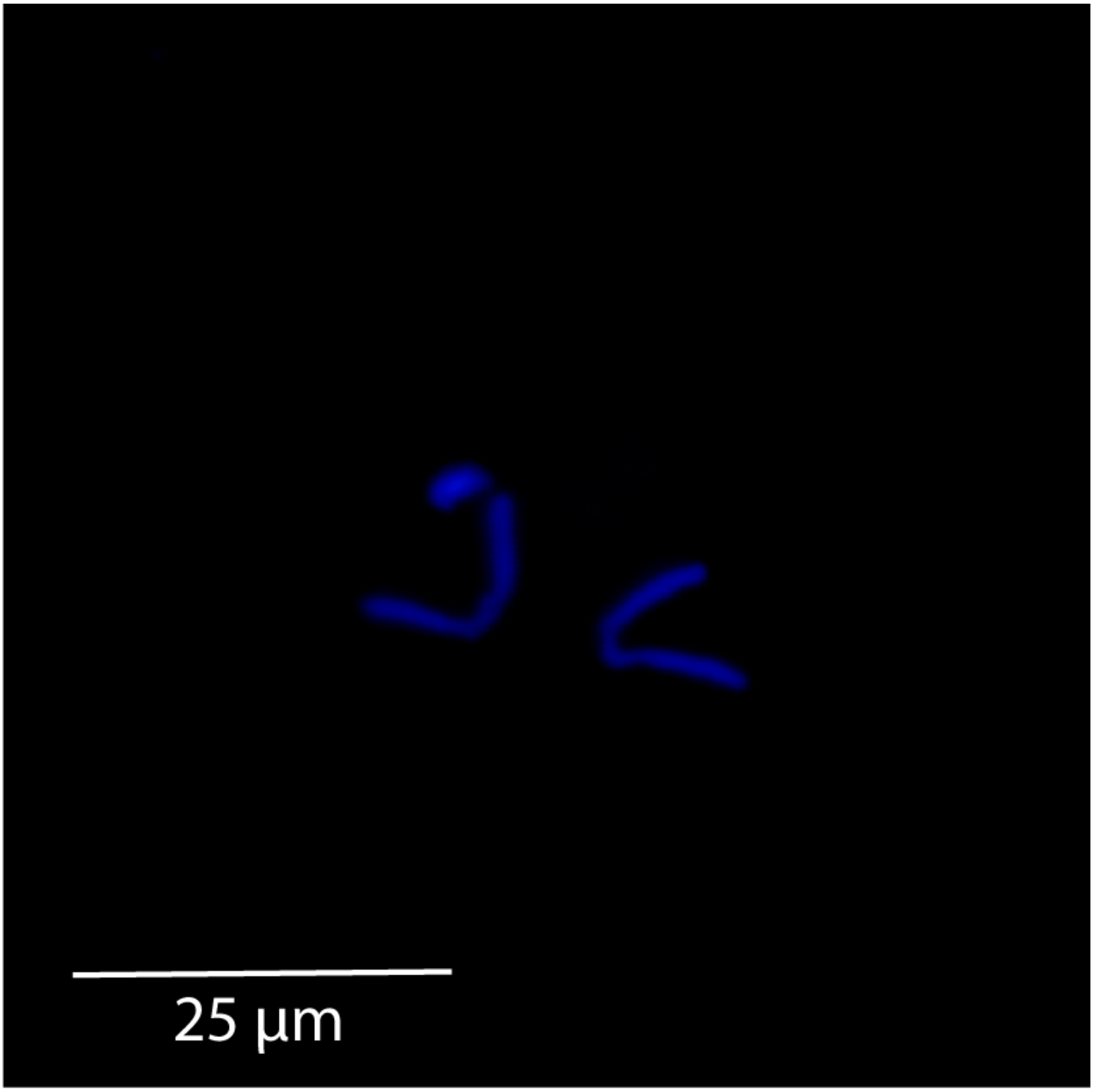

Figure 1

DAPI stained Parascaris univalens egg at first mitotic division showing one pair of chromosomes. Eggs collected from the faeces a 6 month old Icelandic foal at slaughter. 400X magnification.

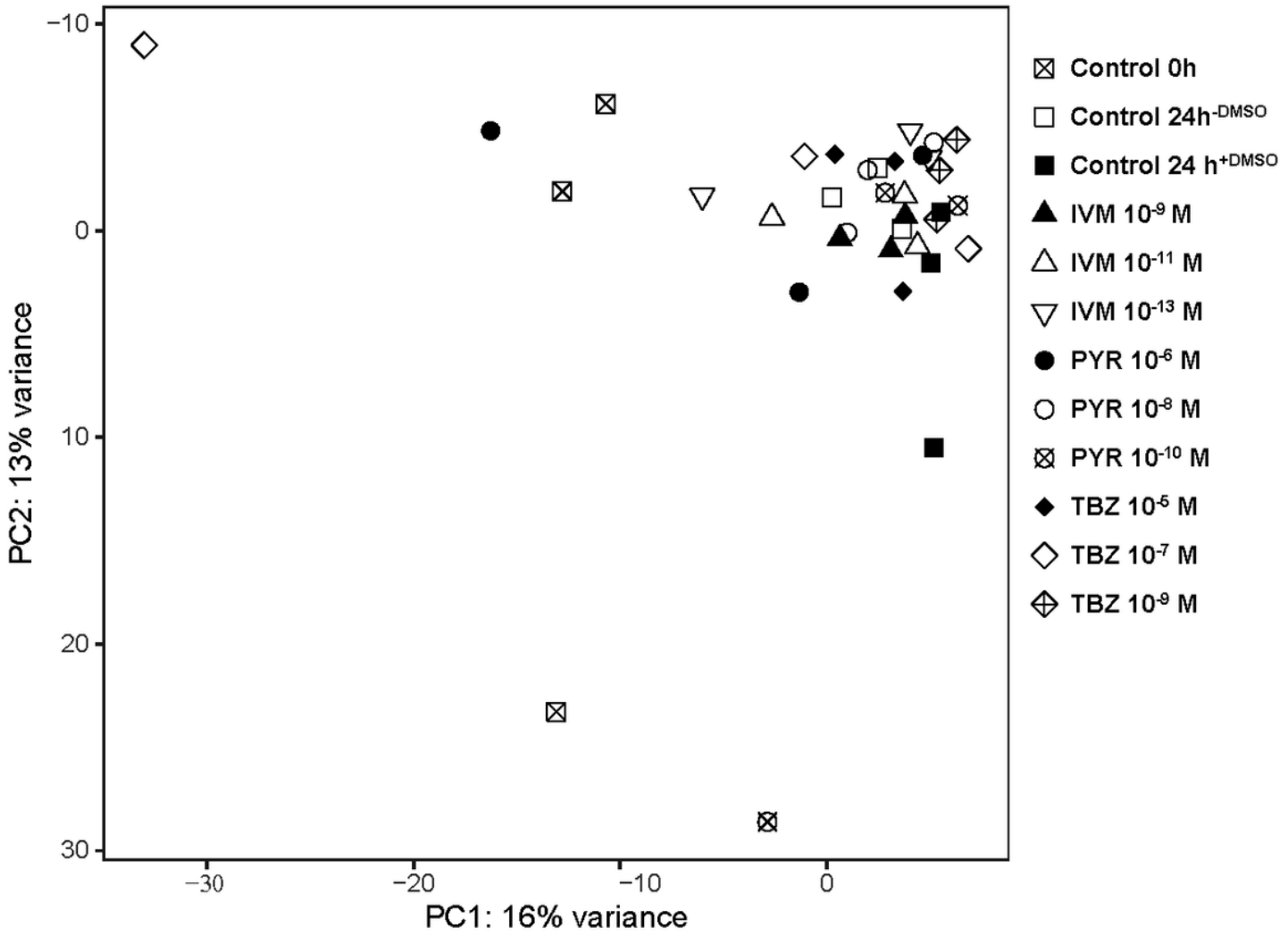

Figure 2

Principal component analysis plot showing the clustering of adult Parascaris univalens incubated in the presence of the anthelmintic drugs ivermectin (IVM), pyrantel citrate (PYR) and thiabendazole (TBZ) at different concentrations as well as control worms. The largest variation in gene expression is observed between the non-incubated worms (control 0h) and all worms incubated in vitro. Each symbol corresponds to an individual worm.

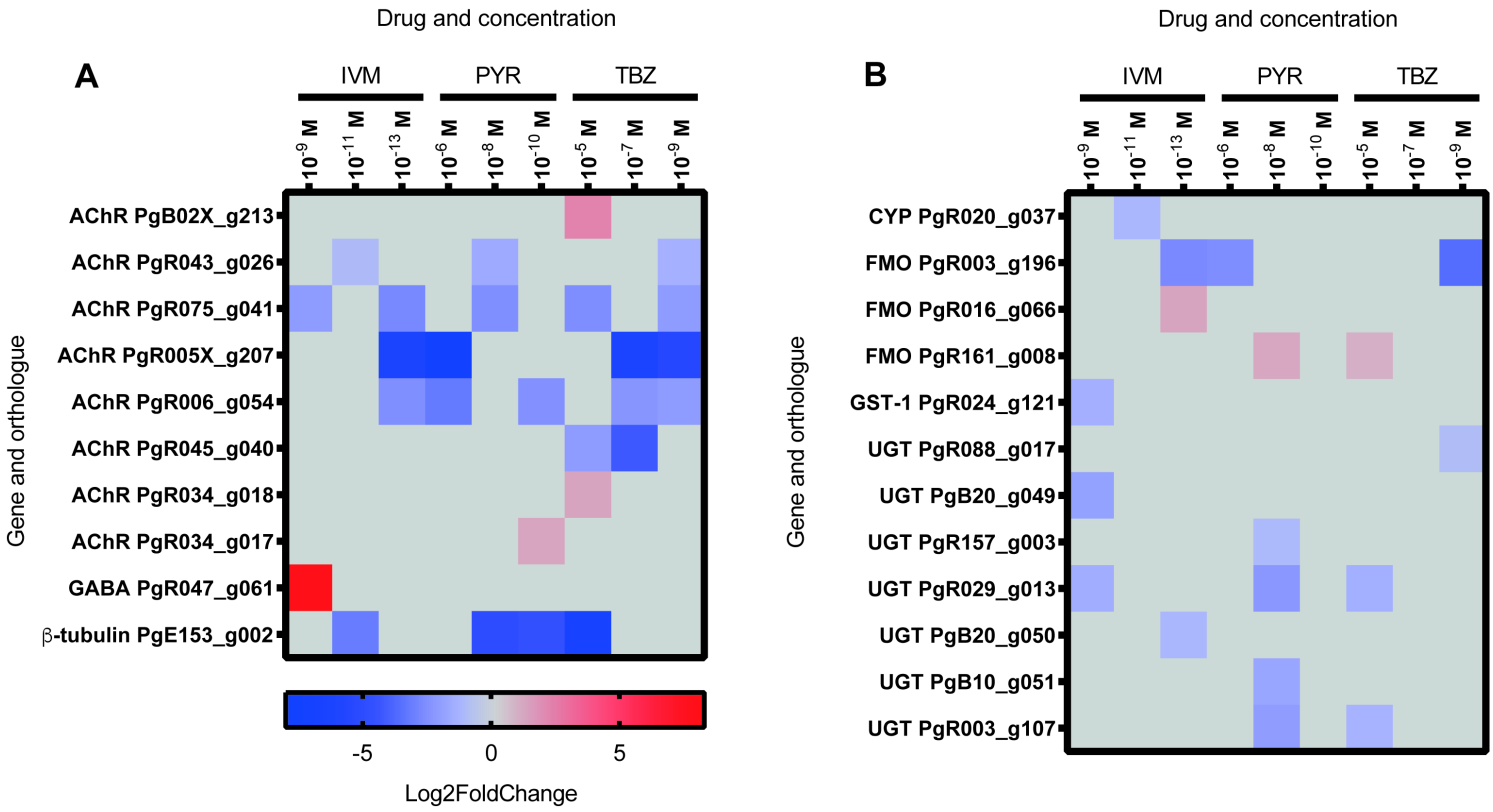

Figure 3

Heat map showing the differential expression of (A) genes coding for putative drug targets and (B) genes coding for putative phase I and phase II metabolizing enzymes after in vitro exposure of Parascaris univalens to the anthelmintic drugs ivermectin (IVM), pyrantel citrate (PYR) and thiabendazole (TBZ) at different concentrations. Genes with a fold change of 2 or more and an adjusted P-value < 0.05 were considered differentially expressed.

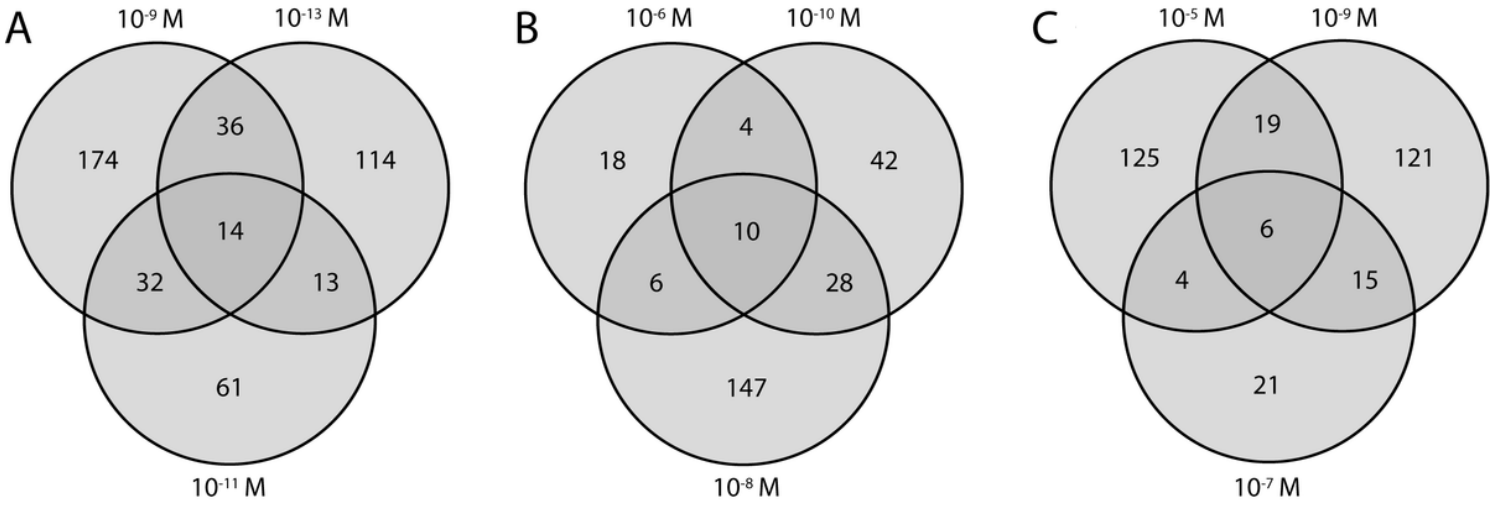

Figure 4

Venn diagrams showing the number of differentially expressed genes unique to and shared among Parascaris univalens worms exposed to three different concentrations of to the anthelmintic drugs (A)

ivermectin, (B) pyrantel citrate and (C) thiabendazole. Genes with a fold change of 2 or more and an adjusted P-value < 0.05 were considered differentially expressed.

Supplementary Files

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- Graphicalabstract.tif
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