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 are 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 region 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 (Padj < 0.05 and log2 fold change ≥ 1 or ≤ -1) analysis showed similarities and differences in the transcriptional response after exposure to the different drug classes. Candidate genes upregulated 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 upregulated 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: To our knowledge, this is the first time the expression of *lgc-37* and members of the FMO, SDR, MFS and SLC superfamilies have been described in *P. univalens* and future work should be focused on characterising these candidate genes to further explore their potential involvement in drug metabolism and anthelmintic resistance.

Keywords: Transcriptome, Anthelmintic resistance, RNA sequencing, Differential expression, *lgc-37*
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 *Parascaris 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 widespread [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 draft 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 *Haemonchus 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 CYP3a35 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, no in vivo treatment experiments have been performed in this parasite to date and only two studies have reported 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
eggs as described in Martin et al. [3].

citrate and thiabendazole.

exposure to sub-lethal doses of ivermectin, pyrantel

h

in media with the addition of 0.1% DMSO (control 24

group of three worms, serving as control, was incubated

0.1%) and incubated at 37 °C for 24 h. An additional

tainers containing media and drug (final concentration of DMSO

three worms, were divided into different containers con-

centration, three biological replicates, each consisting of

worms were rinsed with 37 °C PBS (Life Technologies,

Carlsbad, USA) and serially

way Institute, Uppsala, Sweden) and transported to the labo-

ratory (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

pecies identification by 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 into three con-

ainers containing cell culture media (RPMI-1640 with

the addition of 10% foetal bovine serum, 1% penicillin/

streptomycin and 1% L-glutamine (Life Technologies,

Carlsbad, USA) and then incubated for 24 h at 37 °C

(control 24 h⁻¹DMSO). The other group of worms (n=3)

was immediately killed by removal of the anterior region

upon arrival to the laboratory to serve as controls for the

in vitro incubation (control 0 h) (Fig. 1).

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-Aldrich,

Saint Louis, USA), pyrantel citrate (PYR) (Santa Cruz

Biotechnology, Dallas, USA) or thiabendazole (TBZ)
(Sigma-Aldrich), according to Table 1. The drug concen-

trations used were based on previous studies by Jans-

sen et al. [34] for ivermectin exposure and Zhao et al.
[35] for pyrantel citrate and thiabendazole exposure.

All anthelmintic drugs were dissolved in DMSO (Swed-

ish Veterinary Institute, Uppsala, Sweden) and serially

diluted before addition to the media. For each concen-

tration, three biological replicates, each consisting of

three worms, were divided into different containers con-

taining media and drug (final concentration of DMSO

0.1%) and incubated at 37 °C for 24 h. An additional

group of three worms, serving as control, was incubated

in media with the addition of 0.1% DMSO (control 24

h⁻¹DMSO) (Table 1, Fig. 1).

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 concen-

trations 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 region, contain-

ing the pharynx and a small part of the anterior intesti-

ne, 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 Sci-

ces and Veterinary Public Health, Division of Parasitol-

ogy) for the molecular analysis.

RNA extraction and sequencing

The samples were removed from RNAlater and cut into

smaller pieces. One ml of Trizol (Invitrogen, Carls-

bad, USA) 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 (Mach-

erey Nagel, Düren, Germany). RNA was then isolated

according to the manufacturer’s instructions as described

in the user manual of the kit. rDNase treatment and sub-

sequent 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 Plat-

form. RNA concentration and integrity were checked by

Fragment Analyzer (Agilent, Santa Clara, USA) before

sequencing libraries were prepared from 500 ng total

RNA using the TruSeq Stranded mRNA Library Prepa-

ration Kit including polyA selection (Illumina Inc., San

Diego, USA) 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] (Pipeline with nextflow and docker support avail-

able at https://github.com/SLUBioinformaticsInfrastruc-
ture/RNAseq_nf). The resulting reads were mapped
against the predicted transcriptome of *P. univalens* (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 v.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 R package tximport [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, v.1.22.2 [40]. *P*-values resulting from the Wald test incorporated into DESeq2 were adjusted for multiple testing using the Benjamini-Hochberg procedure [41] as applied by the R base p.adjust function. Genes with a log2 fold change of ≥ 1 or ≤ –1 and an adjusted *P*-value < 0.05 were considered differentially expressed.

### Table 1  Samples included in the in vitro exposure experiment

| Drug and concentration Viability | Differentially expressed genes | Unknown genes (%) |
|---------------------------------|-------------------------------|-------------------|
| IVM 10⁻¹⁰ M                    | 3.5                           | 256               | 44                |
| IVM 10⁻¹¹ M                    | 4.5                           | 119               | 44                |
| IVM 10⁻¹² 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                |
| Control 24 h + DMSO            | 6.0                           | –                 | –                 |

**Notes:** Mean viability score of adult *Parascaris univalens*, number of differentially expressed genes (adjusted *P* < 0.05 and log2 fold change of ≥ 1 or ≤ –1) 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 24h. The gene expression of drug-exposed worms was normalized to control 24 h + DMSO.
For principal components analysis (PCA), gene counts were transformed into log2 scale by applying regularized logarithm approach using the rlog function provided in the DESeq2 package and plotted using the plotPCA function. For all the above analyses, R v.3.5.2 was used [42]. Functional annotations of differentially expressed genes were identified by searching the protein sequences against the Swiss-Prot database [43] using BlastP (e-value ≤ 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 v.3.6.1 [44]. Gene IDs corresponding to individual Venn diagram partitions were retrieved using the get.venn.partition function. Superfamilies of genes shared between partitions were retrieved using the get.venn.partition function of the VennDiagram package in R v.3.6.1 [44].

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 ≤ 10\(^{-5}\)) in NCBI (https://www.ncbi.nlm.nih.gov/).

**Results**

**Karyotype**

*Parascaris univalens* was identified in the karyotype as DAPI stained eggs from the first mitotic division showed one pair of chromosomes.

**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, quality control and mapping**

RNA with RIN between 6.6–8.7 were sequenced on Illumina Nova Seq. The number of reads per sample varied between 63–132 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). Mapping against the transcriptome resulted in an average mapping rate of 80–90% indicating high similarity between the transcriptomic data and the reference transcriptome.

**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 observed between the non-incubated worms (control 0 h) and both control and drug-exposed worms that were incubated in vitro for 24 h. Although 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. Log2 fold change and adjusted P-values for the differentially expressed genes discussed in this article are listed in Additional file 1: Table S1.

**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 (Additional file 2: Table S2).

**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 subunit lgc-37 in *H. contortus* (GenBank: 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, PgR055X_g207, PgR006_g054 and PgR045_g040, were downregulated according to Fig. 3a. The transcript PgE153_g002, identical to the previously described *P. equorum* β-tubulin isotype 2 (GenBank: 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

The ten most upregulated and downregulated genes after exposure to each drug are shown in Table 2. Of the most upregulated genes across all drugs, 57% are uncharacterized and only one transcript, the GABA-receptor subunit PgR047_g061 (lgc-37), has previously been described as a drug target to IVM and to be involved in anthelmintic resistance. Of the most downregulated genes after exposure to the three different drugs, 53% are uncharacterized and two transcripts, the acetylcholine receptor PgR005X_g207 and the B-tubulin isotype 2 gene PgE153_g002, are previously described as drug targets of PYR and TBZ. No genes previously described to be involved in drug metabolism were found in this list.

Differentially expressed genes shared among the three concentrations of each drug are visualized in Fig. 4 and Table 3. 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 3).

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 3). Of the remaining differentially expressed transcripts, four belong to superfamilies not connected to drug metabolism or transportation, whereas five transcripts are uncharacterised (Table 3).

Six transcripts were commonly differentially expressed after exposure to all three concentrations of TBZ (Fig. 4c), five downregulated and one upregulated. Of these, two transcripts are involved in processes not connected to drug metabolism or transport, while four are uncharacterised or not similar to any known superfamily (Table 3).
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 monoxygenase (FMO) superfamily were differentially expressed in an inconsistent pattern after drug exposure. The transcript PgR003_g196 was downregulated after exposure to IVM (10^{-7} 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 superfamilies 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 glutathione 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, to our knowledge 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.

The GABA receptor subunit PgR047_g061, orthologous to the *H. contortus* gene *lgc-37*, was upregulated after exposure to the highest IVM concentration (10^{-9} M) (Fig. 3). GABA receptors are targets for IVM in...
Table 2: The 10 most up- and downregulated genes (adjusted P-value < 0.05) in *Parascaris univalens* worms exposed to three different concentrations of the anthelmintic drugs ivermectin (IVM), pyrantel citrate (PYR) and thiabendazole (TBZ) for 24 h

| Gene ID         | Log2FC | Orthologue                              | Condition          |
|-----------------|--------|-----------------------------------------|--------------------|
| PgR001X_g190    | 23.45  | Uncharacterised                         | IVM 10^{-5} M     |
| PgR013_g129     | 8.93   | RPE Ribulose-phosphate 3-epimerase      | IVM 10^{-13} M    |
| PgR047_g061     | 8.23   | GABRA6 Gamma-aminobutyric acid receptor subunit alpha-6 | IVM 10^{-5} M    |
| PgB04_g079      | 6.77   | RPL3 60S ribosomal protein L3           | IVM 10^{-11} M    |
| PgR010_g071     | 5.77   | Uncharacterised                         | IVM 10^{-9} M     |
| PgR152_g010     | 5.36   | COL9A1 Collagen alpha-1(IX) chain       | IVM 10^{-11} M    |
| PgR045_g001     | 5.32   | Uncharacterised                         | IVM 10^{-9} M     |
| PgR004_g064     | 4.85   | Uncharacterised                         | IVM 10^{-11} M    |
| PgR001X_g079    | 4.35   | Uncharacterised                         | IVM 10^{-9} M     |
| PgB05_g040      | 4.30   | Uncharacterised                         | IVM 10^{-11} M    |
| PgB04_g079      | 9.10/5.51 | RPL3 60S ribosomal protein L3               | PYR 10^{-6} M/PYR 10^{-10} M |
| PgE230_g001     | 8.25   | Uncharacterised                         | PYR 10^{-6} M     |
| PgR020_g019     | 6.13   | Uncharacterised                         | PYR 10^{-10} M    |
| PgR004_g204     | 5.88   | Uncharacterised                         | PYR 10^{-8} M     |
| PgR152_g010     | 5.67   | COL9A1 Collagen alpha-1(IX) chain       | PYR 10^{-6} M     |
| PgR001X_g079    | 5.01   | Uncharacterised                         | PYR 10^{-8} M     |
| PgR099_g042     | 4.80   | hpo-4 Putative GPI-anchor transamidase  | PYR 10^{-8} M     |
| PgE192_g001     | 3.71   | Uncharacterised                         | PYR 10^{-8} M     |
| PgR002_g035     | 3.63   | ani-2 Anillin-like protein 2             | PYR 10^{-6} M     |
| PgR123_g005     | 3.48   | Pym Partner of Y14 and mago              | PYR 10^{-8} M     |
| PgR307_g002     | 12.41/11.44 | EDF1 Endothelial differentiation-related factor 1 homolog | TBZ 10^{-5} M/TBZ 10^{-5} M |
| PgR256X_g001    | 8.82   | Uncharacterised                         | TBZ 10^{-9} M     |
| PgR010_g071     | 8.22   | Uncharacterised                         | TBZ 10^{-9} M     |
| PgR190X_g006    | 8.03   | Uncharacterised                         | TBZ 10^{-9} M     |
| PgR001X_g019    | 7.85   | Uncharacterised                         | TBZ 10^{-7} M     |
| PgR152_g010     | 7.15   | COL9A1 Collagen alpha-1(IX) chain       | TBZ 10^{-5} M     |
| PgB04_g079      | 6.49   | RPL3 60S ribosomal protein L3           | TBZ 10^{-5} M     |
| PgR034_g082     | 6.33   | Uncharacterised                         | TBZ 10^{-5} M     |
| PgR099_g042     | 5.01   | hpo-4 Putative GPI-anchor transamidase  | TBZ 10^{-7} M     |
| PgR001X_g079    | 4.76   | Uncharacterised                         | TBZ 10^{-9} M     |
| PgR166_g010     | −7.80/−5.92 | agmo-1 Alkylglycerol monoxygenase         | IVM 10^{-11} M/IVM 10^{-9} M |
| PgR049_g007     | −7.50  | Uncharacterised                         | IVM 10^{-11} M    |
| PgE193_g002     | −6.88  | cyb-3 G2/mitotic-specific cyclin-B3      | IVM 10^{-9} M     |
| PgR260_g001     | −6.74  | Eif4a3 Eukaryotic initiation factor 4A-III | IVM 10^{-9} M |
| PgR005X_g207    | −6.28  | cup-4 Acetylcholine receptor-like protein cup-4 | IVM 10^{-11} M |
| PgE182_g003     | −6.11  | ZFP36 mRNA decay activator protein      | IVM 10^{-9} M     |
| PgR002_g265     | −6.02  | Uncharacterised                         | IVM 10^{-9} M     |
| PgR022X_g031    | −5.72  | Uncharacterised                         | IVM 10^{-11} M    |
| PgB32_g014      | −5.71  | Uncharacterised                         | IVM 10^{-9} M     |
| PgE068_g001     | −5.70  | Uncharacterised                         | IVM 10^{-9} M     |
| PgR022X_g031    | −5.43  | Uncharacterised                         | IVM 10^{-9} M     |
| PgB08_g035      | −8.13  | ZC116.3 Probable cubilin                | IVM 10^{-8} M     |
| PgR389_g001     | −7.93  | Uncharacterised                         | IVM 10^{-8} M     |
| PgR005X_g207    | −7.90  | cup-4 Acetylcholine receptor-like protein cup-4 | IVM 10^{-8} M |
| PgR049_g007     | −7.84  | Uncharacterised                         | IVM 10^{-10} M    |
| PgB22_g038      | −6.34  | F01G4.6 Phosphate carrier protein, mitochondrial | IVM 10^{-10} M |
| PgR022X_g030    | −5.72  | Uncharacterised                         | IVM 10^{-6} M     |
| PgB08_g034      | −5.65  | ZC116.3 Probable cubilin                | IVM 10^{-6} M     |
| PgR166_g010     | −5.57  | agmo-1 Alkylglycerol monoxygenase       | IVM 10^{-6} M     |
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 macrocyclic lactone 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.

Eight transcripts orthologous to AChR subunits, the putative drug target for PYR, were differentially expressed, but with 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 (iso-type 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 observed 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.

Table 3 Differentially expressed genes (log2 fold change ≥ 1 or ≤ −1 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) and thiabendazole (TBZ) for 24 h

| Gene ID          | Superfamily                              | Fold Change | Ivermectin Concentration | Pyrantel Concentration | Thiabendazole 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                   |
| PgR37_g063       | C-type lectin (CTL) 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                  |
| PgR006_g137      | Major facilitator superfamily cl28910    | −1.97       | −1.88                    | −1.70                  |
| 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                   |
| PgR004_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                  |

Note: The gene expression of drug-exposed worms was normalized to control 24 h DMSO.

Comparing the differential expression after exposure to three different drugs provided valuable insights on how expression of putative drug targets are similarly affected regardless of the drug used. Another point to consider is the inconsistent expression of putative drug targets and metabolising enzymes across the different concentrations of a drug. This could be explained by individual variation in gene expression between the biological replicates or can be an effect of changes in gene expression from drug metabolism to severe stress or apoptosis in response to
high doses of anthelmintics. These are important findings 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, Vokřál et al. [63] did not observe 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 observed in susceptible strains of *H. contortus*. The SDR family is a large superfamily present in both prokaryotes and eukaryotes, transport 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.

Among the ten most upregulated and downregulated genes after exposure to each of the three drugs, there were no previously described genes encoding xenobiotic enzymes or transporters. Even though the function of more than 50% of these genes is unknown, this list might contain novel candidates for drug metabolism and needs to be further investigated.

In a recently published study, Scare et al. [33] studied the transcriptomes of adult *P. univalens* *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 and the use of different tissues for RNA extraction. 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 observed a large number (n = 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 although 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. Although we cannot be sure that the in vitro response to drug treatment reflects what would happen in a similar in vivo situation, it is a way to obtain more knowledge about the parasite response to drug exposure. Since whole live animal experiments in foals are both expensive and pose ethical dilemmas, further development of in vitro models is crucial for research in parasitic nematodes.

Conclusions
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 parasitic 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. To our knowledge, 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.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-020-04212-0.

Additional file 1: Table S1. Log2 fold change and adjusted P-values for the differentially expressed genes discussed in this article.

Additional file 2: Table S2. *Parascaris univalens* in vitro incubation experiment. Differentially expressed genes (fold change ≥ 2 or and adjusted P-value (Walds test and Benjamini-Hochberg procedure) < 0.05) and their corresponding orthologues according to Swiss-Prot database after 24 h in vitro incubation in media (control 24 h−DMSO). Gene expression was normalized to non-incubated worms (control 0 h).

Abbreviations
SDR: short-chain dehydrogenase/reductase superfamily; FMO: monoxygenase superfamily; CYP: cytochrome P450 family; MFS: major facilitator superfamily; SLC: solute carrier superfamily; GABA: γ-aminobutyric acid; SNP: single nucleotide polymorphisms; PBS: phosphate-buffered saline; UGT: uridine 5′-diphospho-glucuronosyltransferase-glucosyltransferase; ABC: ATP-binding cassette; IVM: ivermectin; PYR: pyrantel; TBZ: thiabendazole; DMSO: dimethyl sulfoxide; PCA: principal components analysis; DAPI: 4',6-diamidino-2-phenylindole; RIN: RNA integrity number; GST: glutathione S-transferase; AChRs: acetylcholine receptor subunits.

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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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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).

Ethics approval and consent to participate
No ethical permissions were necessary for this study as the parasites were collected from horses slaughtered for meat production.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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