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Constitutive and differential expression of transport protein genes in *Parascaris univalens* larvae and adult tissues after *in vitro* exposure to anthelmintic drugs

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ABSTRACT

The equine roundworm *Parascaris univalens* has developed resistance to the three anthelmintic substances most commonly used in horses. The mechanisms responsible for resistance are believed to be multi-genic, and transport proteins such as the P-glycoprotein (Pgp) family have been suggested to be involved in resistance in several parasites including *P. univlaens*. To facilitate further research into the mechanisms behind drug metabolism and resistance development in *P. univalens* we aimed to develop an *in vitro* model based on larvae. We developed a fast and easy protocol for hatching *P. univalens* larvae for *in vitro* studies, resulting in a hatching rate of 92 %. The expression of transport protein genes *pgp-2*, *pgp-9*, *pgp-11.1*, *pgp-16.1* and major facilitator superfamily (MFS) genes PgR006_g137 and PgR015_g078 were studied in hatched larvae exposed to the anthelmintic drugs ivermecin (IVM) 10^{-9} M, pyrantel citrate (PYR) 10^{-6} M and thiabendazole (TBZ) 10^{-5} M for 24 h. In comparison, the expression of these transport protein genes was studied in the anterior end and intestinal tissues of adult worms *in vitro* exposed to IVM, TBZ and PYR, at the same concentrations as larvae, for 3 h, 10 h and 24 h. Larval exposure to sub-lethal doses of IVM for 24 h did not affect the expression levels of any of the investigated genes, however larvae exposed to PYR and TBZ for 24 h showed significantly increased expression of *pgp-9*. *In vitro* drug exposure of adult worms did not result in any significant increases in expression of transport protein genes. Comparisons of constitutive expression between larvae and adult worm tissues showed that *pgp-9*, *pgp-11.1*, *pgp-16.1* and MFS gene PgR015_g078 were expressed at lower levels in larvae than in adult tissues, while *pgp-2* and MFS gene PgR006_g137 had similar expression levels in larvae and adult worms. All investigated transport protein genes were expressed at higher rates in the intestine than in the anterior end of adult worms, except *pgp-11.1* where the expression was similar between the two tissues. This high constitutive expression in the intestine suggests that this is an important site for xenobiotic efflux in *P. univalens*. Despite the fact that the results of this study show differences in expression of transport protein genes between larvae and adult tissues, we believe that the larval assay system described here will be an important tool for further research into the molecular mechanisms behind anthelmintic resistance development and for other *in vitro* studies.

1. Introduction

Equine parasitic roundworms in the genus *Parascaris* have developed resistance to the three drug classes most commonly used for treatment: benzimidazoles (BZs), such as fenbendazole (FBZ) and thiabendazole (TBZ); macrocyclic lactones (MLs), such as ivermectin (IVM); tetrahydropyrimidines (TPs), such as pyrantel. Macrocyclic lactone resistance was first reported in the Netherlands in 2002 (Boersema et al., 2002) and is now found worldwide, as reviewed by Peregrine et al. (2014). Resistance to the TPs has also been reported in several parts of the world, such as the USA (Craig et al., 2007), Australia (Armstrong et al., 2014) and Europe (Martin et al., 2018) while few cases of resistance to BZs have so far been found only in Australia (Armstrong et al., 2014) and Saudi Arabia (Alanazi et al., 2017). This development is alarming since severe infections can be lethal to foals and control of the parasite usually relies on the use of efficient anthelmintic drugs (Cribb et al., 2006).

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Despite more than 50 years since the first reported case of drug resistance in parasitic nematodes, the knowledge about drug metabolism and resistance development mechanisms is scarce, especially in ascarid worms. One reason for this is the lack of *in vitro* models, complicated by the complex life cycle of these parasites, which require a host to be completed. In several previous studies of *P. univalens* (Janssen et al., 2013; Martin et al., 2020; Scare et al., 2020), live adult worms have been collected from horses at slaughter or from euthanized research herd foals. A problem with this approach is that slaughter of foals is uncommon and the number of research herds where foals are necropsied are limited. In addition, there are no isolated laboratory strains available for *P. univalens*, making comparisons between studies difficult due to genetically different populations and unknown resistance phenotypes. An *in vitro* model based on *P. univalens* larvae could be an option as large numbers of parasite eggs can easily be extracted from the faeces of naturally infected foals. However, certain procedures are required as ascarid eggs, unlike those of strongyle nematodes, do not hatch spontaneously outside their host (Urban et al., 1981; Ponce-Macotela et al., 2011). Previously, only a few studies of *in vitro* hatching of *Parascaris* spp. eggs have been made with techniques using glass beads resulting in hatching ratios ranging from 48 % - 98 % (Burk et al., 2014; Rakhshandehroo et al., 2017)

The mechanisms of anthelmintic resistance have been suggested to be multi-genic, including mutations leading to conformational changes in drug targets or changes in expression of target molecule genes. Altered metabolism by drug metabolizing enzymes and elevated expression of drug efflux pumps have also been suggested (James et al., 2009; Whittaker et al., 2017). Increased efflux of drugs by transport proteins such as the P-glycoprotein (Pgp) family and other ATP-binding cassette (ABC) transporters has been suggested to be involved in resistance to several classes of anthelmintic drugs, mainly MLs (Xu et al., 1998; Dicker et al., 2011; Raza et al., 2016b) but also BZs (Blackhall et al., 2008). P-glycoproteins are membrane bound transport proteins involved in protecting cells against xenobiotic substances (Schinkel and Jonker, 2003). Nematodes express numerous Pgps and several of these have been shown to be up-regulated either constitutively in resistant strains or in response to drug exposure, however results between different studies are ambiguous (Williamson and Wolstenholme, 2012; Gerhard et al., 2020). In multidrug-resistant adult and larval *Teladorsagia circumcincta pgp-9* was constitutionally up-regulated compared to drug sensitive worms (Dicker et al., 2011), while expression of *pgp-2* and *pgp-9* were higher in multidrug-resistant *Haemonchus contortus* larva than in susceptible larvae (Williamson et al., 2011). In *Cooperia oncophora pgp-11* was up-regulated in adult ivermectin (IVM) susceptible worms after *in vivo* exposure to IVM, and in IVM-resistant larvae after *in vitro* IVM exposure (De Graef et al., 2013). In addition, *pgp-16* was highly up-regulated after *in vivo* IVM selection of susceptible adult *C. oncophora* (Tyden et al., 2014).

The Pgp family has been investigated for its involvement in IVM resistance in *Parascaris* spp. in several studies. Janssen et al. (2013) found that the constitutive expression of *pgp-11.1* was increased in adult worms with reduced sensitivity to IVM compared to a random worm population. In addition, transgenic expression of *Parascaris pgp-11.1* in *Caenorhabditis elegans* led to reduced susceptibility to IVM, strengthening the theory that this efflux pump is involved in IVM resistance (Janssen et al., 2015). Gerhard et al. (2020) did a thorough investigation of the complete Pgp family in *P. univalens* and found that it contains ten genes, and that *pgp-2* and *pgp-9* interacts with IVM.

Despite the fact that Pgps and other members of the ABC transporter family have been investigated in connection with anthelmintic resistance in parasitic nematodes, additional transporters have been shown to be involved in other organisms. The major facilitator superfamily (MFS) is a large superfamily of membrane transporters present in both prokaryotes and eukaryotes, shown to be involved in drug resistance in bacteria and yeast (Paulsen et al., 1996). In a previous study, the two MFS genes PgR006_g137 and PgR015_g078 were differentially expressed in adult *P. univalens* after *in vitro* exposure to IVM and pyrantel citrate (PYR), indicating that they might respond to anthelminthic drugs in nematodes (Martin et al., 2020).

Since *Parascaris* spp. have developed resistance to the three anthelmintic substances most commonly used in horses there is an urgent need to understand the mechanism of resistance development. Here we developed a protocol for hatching and use of *P. univalens* larvae for *in vitro* experiments. The constitutive gene expression of transport proteins *pgp-2*, *pgp-9*, *pgp-11.1*, *pgp-16.1* and MFS-genes PgR006_g137 and PgR015_g078 was compared in *P. univalens* larvae and different tissues of adult worms. In addition, the expression of the transport protein genes was investigated after *in vitro* drug exposure to sub-lethal doses of IVM, PYR and TBZ.

2. Materials and methods

2.1. Parasite collection

Adult *P. univalens* were collected from the intestines of nine foals, approximately six months old, at an abattoir in Selfoss, Iceland. The horses originated from three farms in the south of Iceland and had never been treated with anthelmintic drugs. The resistance phenotypes on the farms were not investigated in this study. The worms were rinsed and transported to the laboratory (Institute for Experimental Pathology at Keldur, University of Iceland, Reykjavík, Iceland) in 37 °C PBS (Life Technologies, Carlsbad, USA). Eggs were isolated from the uterus of *P. univalens* collected in Selfoss and also from pooled faecal material collected in connection with the FECRT in the north and west of Iceland (Martin et al., 2021).

2.2. Constitutive gene expression in larvae and adult worm tissues

Adult worms were dissected after arrival to the laboratory (Institute for Experimental Pathology at Keldur). Anterior end and intestinal tissue were placed in RNAlater (Invitrogen, Carlsbad, USA) in individual tubes and transported to Sweden (Swedish University of Agricultural Sciences (SLU), Department of Biomedical Sciences and Veterinary Public Health, Section for Parasitology). Eggs collected during the FECRT were cleaned and decorticated by washing in 2 % sodium hypochlorite in 16.5 % sodium chloride (Swedish Veterinary Institute (SVA), Uppsala, Sweden) and thereafter rinsed six times with cold tap water and resuspended in water. The final egg concentration was determined by counting the number of eggs in 3 × 10 μL of water using a microscope. The eggs were incubated at 25 °C for 21 days to allow larvae to develop, then divided into three tubes containing approximately 12,000 larvated eggs each, pelleted by centrifugation for 5 min at 11,000 × g and flash frozen in liquid nitrogen.

2.3. Hatching, culture and drug-exposure of larvae

Eggs from the uterus of adult worms collected at Selfoss were cleaned and incubated for *in ovo* larval development as above. The larvated eggs were rinsed in PBS containing 0.05 % Tween 20 (VWR, Radnor, USA), final volume 5 mL, transferred to a Kimble® Kontes® 15 mL glass homogenizer (DWK Life Sciences, Mainz, Germany) and hatched by six slow strokes with pestle B (leaving 0.16 mm clearance). Hatched larvae were centrifuged 5 min at 1,600 × g and resuspended in tissue culture media, RPMI-1640 with the addition of 10 % foetal bovine serum, 1 % penicillin/streptomycin and 1 % L-glutamine (Life Technologies). Hatching ratio was determined by counting 100 larvae/eggs at five different hatching experiments and calculated by the formulae:

$$Hatching\ ratio = hatched\ larvae/(hatched\ larvae + unhatched\ larvae)\ x\ 100$$

The anthelmintic drugs IVM (Sigma-Aldrich, Saint Louis, USA) PYR (Santa Cruz Biotechnology, Dallas, USA) and TBZ (Sigma-Aldrich) were

dissolved in Dimethyl Sulfoxide (DMSO) (SVA) and serially diluted. The number of larvae in 3 × 10 µL tissue culture media were counted to calculate the larval concentration, then approximately 4,000 larvae in 1 mL tissue culture media were dispensed in each well of a 24 well tissue culture plate (TTP, Trasadingen, Switzerland) and acclimatised for 24 h at 37 °C, 5 % CO_2 before drug exposure. Larvae (n = 4,000) in triplicate wells were then exposed to either IVM 10^{-9} M, PYR 10^{-6} M, TBZ 10^{-5} M or without the addition of an anthelmintic drug (control) for 24 h at 37 °C, 5 % CO_2. The final concentration of DMSO was 0.1 % in all wells. Concentrations of anthelmintic drugs used were the same as the highest doses used in a previous study where adult worms were found to be affected but not killed by 24 h *in vitro* exposure (Martin et al., 2020). After exposure, larvae were collected and centrifuged 5 min at 11,000 × g. The larval pellets were then flash frozen in liquid nitrogen.

2.4. Drug exposure of adult worms

At the Institute for Experimental Pathology at Keldur, worms were incubated at 37 °C in tissue culture media and exposed to anthelmintic drugs of the same concentrations as in the larval experiment, IVM 10^{-9} M (n = 9), PYR 10^{-6} M (n = 9), TBZ 10^{-5} M (n = 9) or without the addition of an anthelmintic drug (control, n = 9). Anthelmintic drugs were dissolved and diluted in DMSO as above. To investigate the expressional response at different time points three worms were removed from each group and dissected after 3 h, 10 h and 24 h exposure. The anterior end and the intestine were placed in individual tubes containing RNAlater. The samples were then transported to Sweden (SLU, Department of Biomedical Sciences and Veterinary Public Health, Section for Parasitology).

2.5. RNA extraction and quality control

Adult worm samples were removed from RNAlater, cut into pieces, mixed with 1 mL Trizol (Invitrogen) and homogenized in a glass tissue grinder. 200 µL chloroform (Sigma-Aldrich) was added, the sample was mixed and then centrifuged at 13,000 × g for 15 min, 4 °C. A 100 µL aliquot of the upper aqueous phase was mixed with 350 µL lysis buffer from the NucleoSpin® RNA Plus kit (Macherey Nagel, Düren, Germany) and RNA was isolated as described in the user manual.

Frozen larval pellets were ground with a pestle after addition of 100 µL Trizol. After homogenization, the final volume of Trizol was made up to 900 µL and mixed with 200 µL chloroform. Each sample was centrifuged at 13,000 × g for 15 min, 4 °C, and the aqueous phase was split into two microfuge tubes. To each tube 200 µL lysis buffer from the NucleoSpin® RNA XS kit (Macherey Nagel) was added and RNA was isolated as described in the user manual of the kit, including a DNase step.

RNA concentration and integrity were checked by TapeStation (Agilent, Santa Clara, USA). Then cDNA was synthesized by SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturers instruction. To investigate the constitutive expression in larvae and adult tissues 18 ng of RNA (RIN 5.9–8.6) was used for each 20 µL cDNA reaction. For drug exposed larvae 10 ng of RNA (RIN 7.8–9.2) was used for each 20 µL cDNA reaction. For drug-exposed adult worms 75 ng of RNA (RIN 8.2–9.4) was treated with DNase I (Invitrogen) according to the manufacturer and then used for each 20 µL cDNA reaction.

2.6. Primer design and validation

The *P. univalens* genome (PRJNA386823) in WormBase Parasite (Howe et al., 2017) was used to identify genes of interest and reference genes (Table 1). Primer sites were picked to flank intron sequences, designed using Primer3web (Untergasser et al., 2012) and ordered from Eurofins Genomics (Galten, Denmark). PCR reactions containing: 12.5 µL Accustart II PCR ToughMix (Quanta bio, Beverly, USA), 1 µL each of F and R primer (10 µ M each), 9.5 µL H_2O and 1 µL template cDNA (unexposed adult anterior end) were performed on an Applied Biosystems (Foster City, USA) 2721 Thermal Cycler at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 90 s and a final extension at 72 °C for 9 min. PCR products were confirmed by gel electrophoresis, Sanger sequencing (Macrogen Europe, Amsterdam, Netherlands) and nucleotide searches in WormBase Parasite (Howe et al., 2017). All primers were optimized for qPCR regarding annealing temperature and concentration, then validated to a 92.9–99.9 % efficiency (Table 1). Melt curve data confirmed single products for each primer pair.

2.7. Expression of transport protein genes

qPCR was performed using QuantiTect SYBR® Green PCR kit (Qiagen, Hilden, Germany). The cDNA template was diluted 1:2 for constitutive expression in larvae and adult worm tissues. For the transcriptional response to *in vitro* drug exposure in larvae and adult worms cDNA template was diluted 1:2 and 1:3 respectively. Dilutions were made in DEPC water (Qiagen). Reactions containing 12.5 µL SYBR mix, 2 µL diluted template, primer concentration according to Table 1 and DEPC water up to 25 µL, were run on BioRad (Hercules, USA) CFX Opus 96. Each sample was run as technical duplicates (constitutive expression in larvae and adult tissue and *in-vitro* exposed larvae) or triplicates (*in-vitro* exposed adult worm tissues) and non-template controls were included for each gene. The PCR was initiated by a 15 min denaturation step at 95 °C followed by 40 cycles of 95 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s with collection of fluorescence data. Melt curve data was collected at 0.5 °C steps between 65 °C and 96 °C. Data was analysed using BioRad CFX Maestro.

Table 1
Primers used for qPCR.

Putative gene name/superfamily	qPCR primer Sequence (5'→3')	WormBase ParaSite ID	Product size (bp)	Annealing temp (°C)	Efficiency (%)	Conc (nM)
actin [a]	F: TCGTTTTTAGGGGAGGGATG R: AAACACCGAGCAAAATGGAG	PgR070_g023	137	59	99.5	500
gpd-1	F: ACAGTGGAGAGATGGACGTG R: GCCATGCCAGTCAGTTTACC	PgB20_g009	116	59	97.5	400
pgp-2	F: TTGATTGCACGATCGAAGAG R: GGTACCTTTCTCGCCAACAC	PgR011_gPgp-2	154	59	92.9	400
pgp-9	F: AACGAGGATGTCACTGAGGG R: TGTGTACCTCGATCACCCAC	PgR025_gPgp-9	118	59	97.1	400
pgp-11.1	F: TCCTGCTTGCACTGCTATGT R: GACCCCAATAACCGCGAAC	PgB04_gPgp-11.1	160	59	98.6	500
pgp-16.1	F: GTTGGATTCGGATTGGGCAT R: TGATGAATTTGTCGCTCGCA	PgR011_gPgp-16.1	142	59	98.5	500
MFS	F: AGACGTTGGGGCGATATTC R: GCTTGTTGCTATTCGCTTCA	PgR006_g137	158	59	97.4	400
MFS	F: GGAGGCTGATGTTTGCATTT R: TCCACTGCTCTTCACCATTG	PgR015_g078	158	59	99.9	400

[a] Sequence from Janssen et al. (2013).

2.8. Calculation of relative expression and statistical analysis

Relative expression was calculated in Microsoft Excel 2016 using the ΔΔCt-method (Livak and Schmittgen, 2001). Statistical analysis was performed using GraphPad Prism 9.1.0.

2.8.1. Constitutive expression of transport protein genes in larvae and adult worm tissues

The Ct values were normalized to the reference gene *gpd-1* since *actin* was not stably expressed in adult intestinal tissue. Expression levels of *pgp-2*, *pgp-9*, *pgp-11.1*, MFS genes PgR006_g137 and PgR015_g078 were calculated relative to the mean expression in larvae. A one-way ANOVA with Tukey's multiple comparisons test was performed to identify differences in expression between tissues and life stages. The expression of *pgp-16.1* was calculated relative to the mean expression in the anterior end. An unpaired *t*-test was performed to identify differences in expression between the intestine and anterior end.

2.8.2. Transcriptional response of transport protein genes to in vitro drug exposure in larvae

Relative expression compared to the control was calculated with the reference genes *actin* and *gpd-1*. Significant differences between exposed larvae and controls were identified by one-way ANOVA with Tukey's multiple comparisons test.

2.8.3. Transcriptional response of transport protein genes to in vitro drug exposure in adult tissues

Relative expression compared to the control for each time point was calculated with the reference gene *gpd-1* for intestinal tissue and reference genes *actin* and *gpd-1* for anterior end tissue. Two way ANOVAs with Tukey's multiple comparisons test were conducted to determine differences in gene expression between treated and control worms and between the different time points used for exposure.

3. Results

3.1. Constitutive expression of transport protein genes in larvae and adult tissues

The constitutive expression levels of transport protein genes varied in adult worm tissues and larvae as shown in Fig. 1. Expression of *pgp-11.1*, and MFS gene PgR015_g078 was significantly higher in both anterior end and intestinal tissues of adult worms than in larvae, while

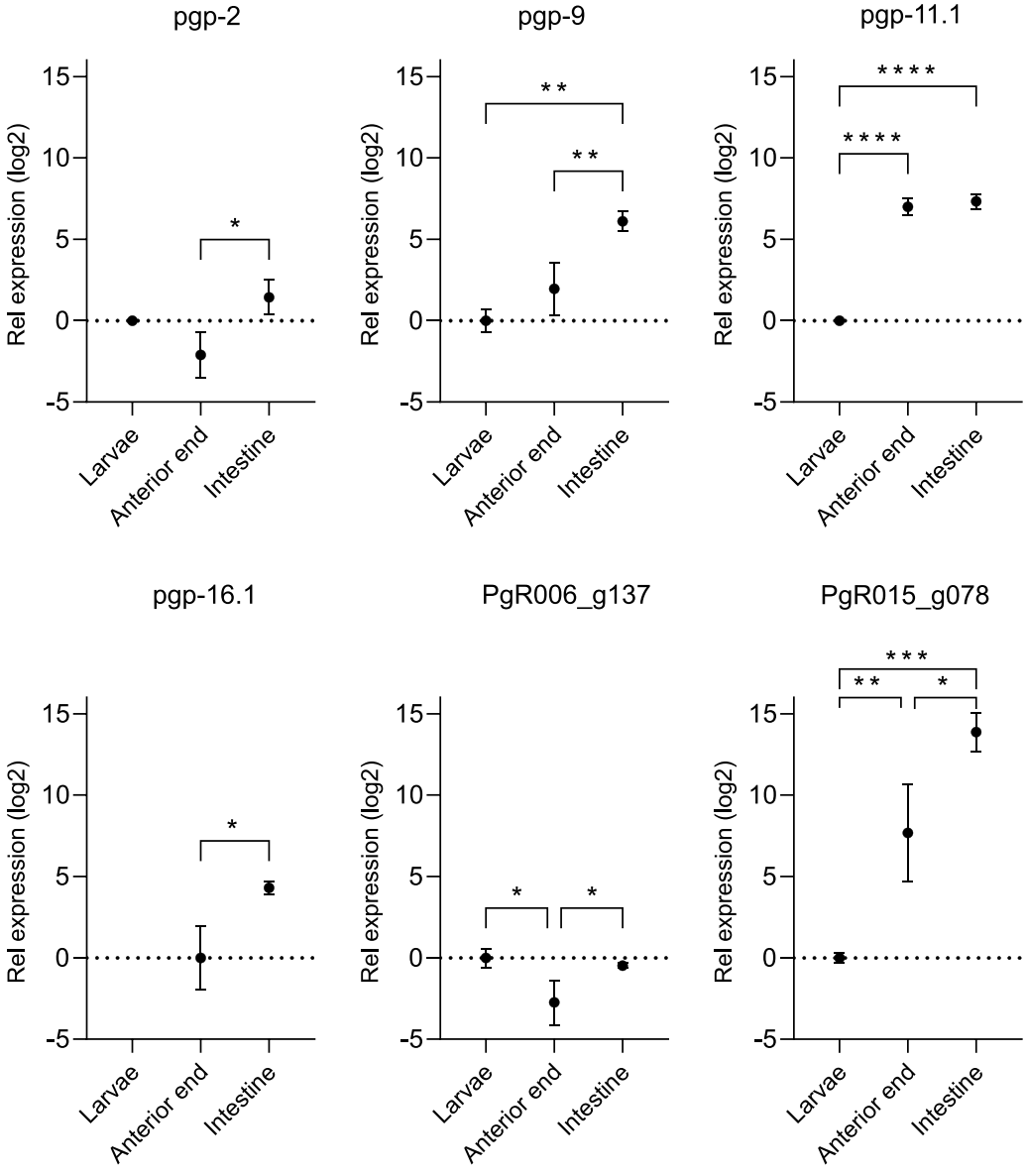

Fig. 1. Expression levels of transport protein genes in tissues from the anterior end and intestine of individual adult *Parascaris univalens* (n = 3) and pools of 12,000 larvae (n = 3). Gene expression was normalized to the reference gene *gpd-1* and expression levels of *pgp-2*, *pgp-9*, *pgp-11.1* and MFS genes PgR006_g137 and PgR015_g078 are shown relative to the mean expression in larvae. A one-way ANOVA with Tukey's multiple comparisons test was performed to identify differences in expression between tissues. Expression of *pgp-16* in larvae was too low to be detected, therefore gene expression in adult tissues is shown relative to the mean expression in the anterior end. An unpaired *t*-test was performed to identify differences in expression between anterior end and intestine. Significant differences are marked with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or **** ($p > 0.0001$).

pgp-9 expression was only significantly higher in the intestine compared to larvae. In contrast, the MFS gene PgR006_g137 was expressed significantly lower in the anterior end tissue compared to larvae whereas *pgp-2* showed no significant difference in expression levels between adult tissues and larvae. The expression of *pgp-16.1* was too low to be detected in larvae.

In adult worms, constitutive expression of all transport protein genes was significantly higher in the intestine compared to the anterior end tissue, except *pgp-11.1* that showed no difference in expression between the two tissues (Fig. 1).

3.2. Hatching of P. univalens eggs and transcriptional response of transport protein genes to in vitro drug exposure in larvae

The protocol for hatching *P. univalens* eggs resulted in a hatching ratio of 92 % (SD = 7.4 %) with fully viable larvae for the duration of the exposure experiment.

In vitro exposure to IVM did not significantly affect expression of any of the investigated transport protein genes (Fig. 2). However, the expression of *pgp-9* was significantly increased after exposure to PYR and TBZ, while the opposite was observed for *pgp-11.1* and MFS gene PgR006_g137, where the expression was significantly reduced after exposure to PYR and TBZ. Expression of MFS gene PgR015_g078 was significantly reduced only after TBZ exposure (Fig. 2).

3.3. Transcriptional response of transport protein genes to in vitro drug exposure in adult tissues

All adult worms were viable after drug exposure. The expression showed rather high variation between the biological replicates (n = 3) and no specific pattern was observed after drug exposure (Figs. 3 and 4).

Interestingly, no transcriptional responses were observed in the anterior end after *in vitro* exposure except for *pgp-9*, where expression was significantly reduced after 24 h of exposure to TBZ compared to the 24 h control. However, differences in expression between the different time points were observed for several genes. Expression of *pgp-9* and *pgp-11.1* increased significantly between the 3 h and 24 h as well as between 10 h and 24 h time points of IVM exposure, while *pgp-16.1* increased significantly between 10 h and 24 h time points of IVM exposure. In addition, *pgp-11.1* expression increased significantly between 3 h and 24 h time points of PYR exposure, while *pgp-9* decreased significantly between 3 h and 24 h time points of TBZ exposure (Fig. 3).

The intestinal tissue showed no transcriptional responses after *in vitro* exposure compared to control worms. Expression of the MFS gene PgR006_g137 was however increased significantly between 3 h and 24 h time points of TBZ exposure (Fig. 4).

4. Discussion

Anthelmintic resistance in the equine roundworm *P. univalens* is an increasing problem around the world. Novel *in vitro* models would facilitate further research to better understand how various genes in parasitic nematodes respond to anthelmintic drugs and are involved in anthelmintic resistance. We have developed a hatching protocol and explored an *in vitro* method to assess how the expression of transporter genes in larvae are affected by exposure to anthelmintic drugs. In comparison to the hatching protocol for *Parascaris* spp. presented by Burk et al. (2014) the hatching protocol presented here is easy to perform and do not require any specialised equipment or incubation during the procedure. It results in high hatching ratios (92 %) of larvae that are viable in tissue culture media and can be used for *in vitro* experiments.

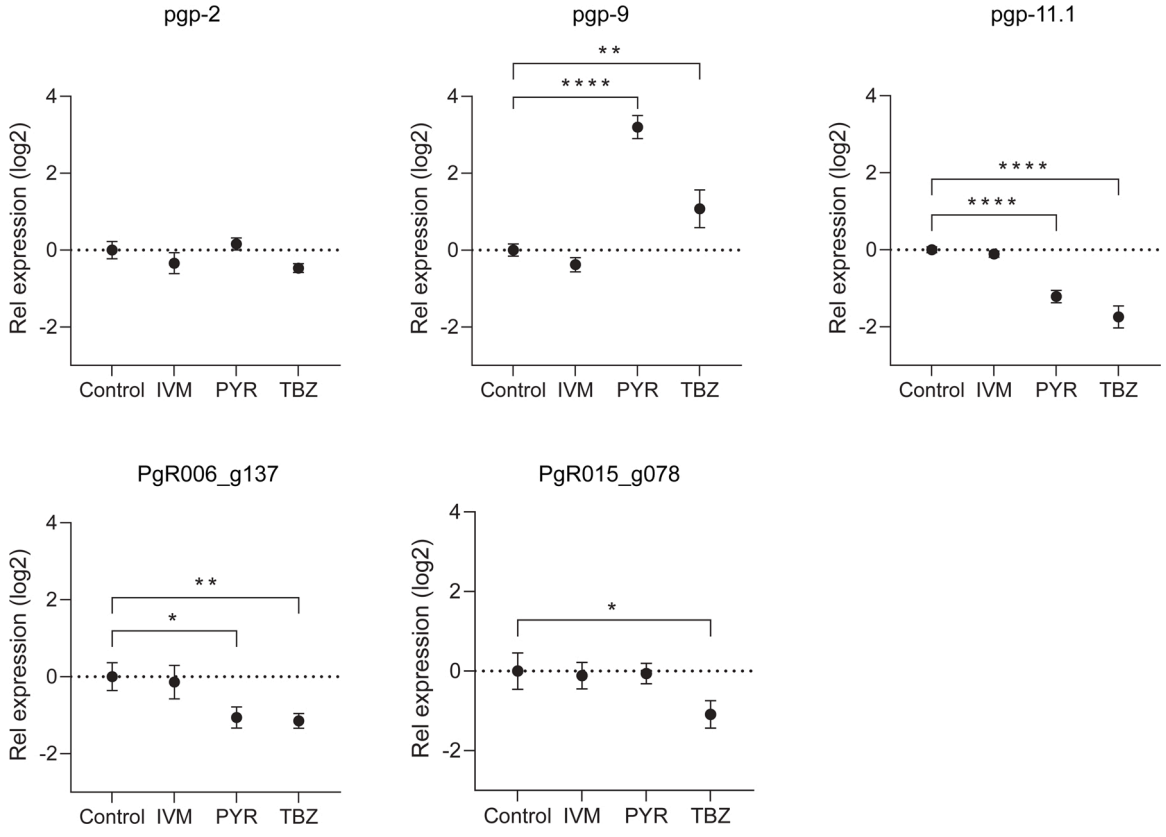

Fig. 2. Expressional responses of transport protein genes in pools of 4,000 *Parascaris univalens* larvae (n = 3) *in vitro* exposed to IVM 10^{9} M, PYR 10^{-6} M, TBZ 10^{-5} M in media with 0.1 % DMSO or media containing 0.1 % DMSO only (control) for 24 h. Gene expression was normalized to reference genes *gpd-1* and *actin* and related to the mean of the control. Significant differences between exposed larvae and controls were identified by one-way ANOVA with Tukey's multiple comparisons test and marked with * ($p < 0.05$), ** ($p < 0.01$) or **** ($p > 0.0001$).

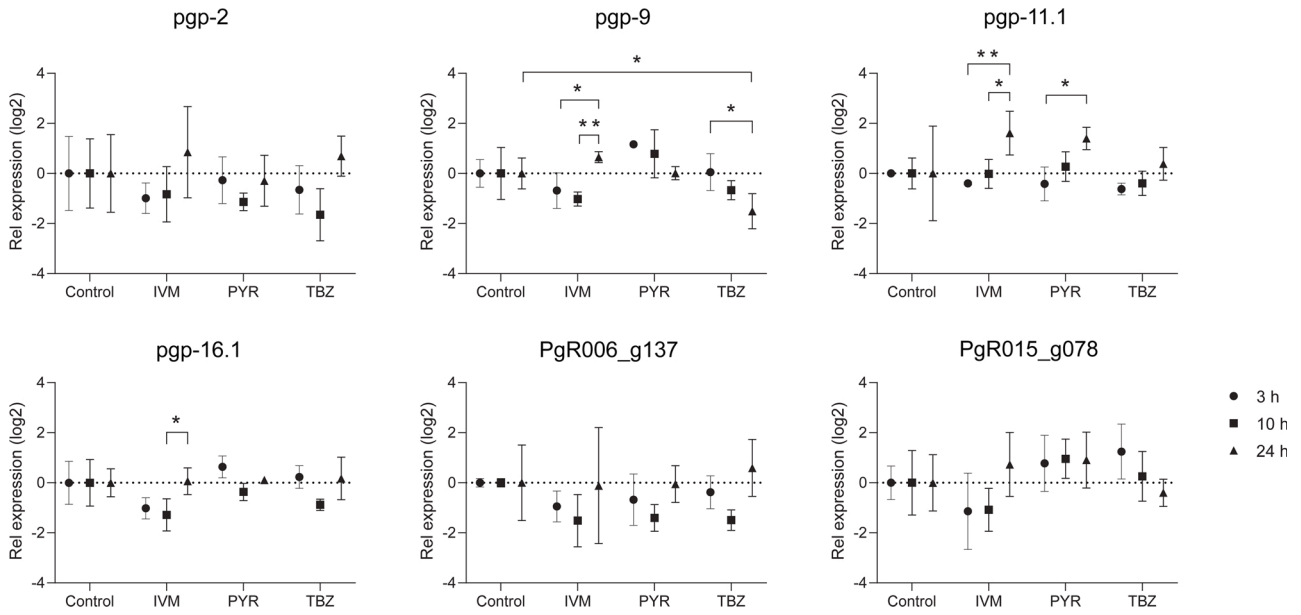

Fig. 3. Expressional responses of transport protein genes in anterior end tissue of adult *Parascaris univalens* (n = 3) *in vitro* exposed to IVM 10^{-9} M, PYR 10^{-6} M, TBZ 10^{-5} M in media with 0.1 % DMSO or media containing 0.1 % DMSO only (control) for 3 h, 10 h and 24 h. Gene expression was normalized to reference genes *gpd-1* and *actin* and related to the mean of the control for each time point. Significant differences between drug exposed worms and controls as well as between different time points within each treatment group were identified by two-way ANOVA with Tukey's multiple comparisons test and marked with * ($p < 0.05$) or ** ($p < 0.01$).

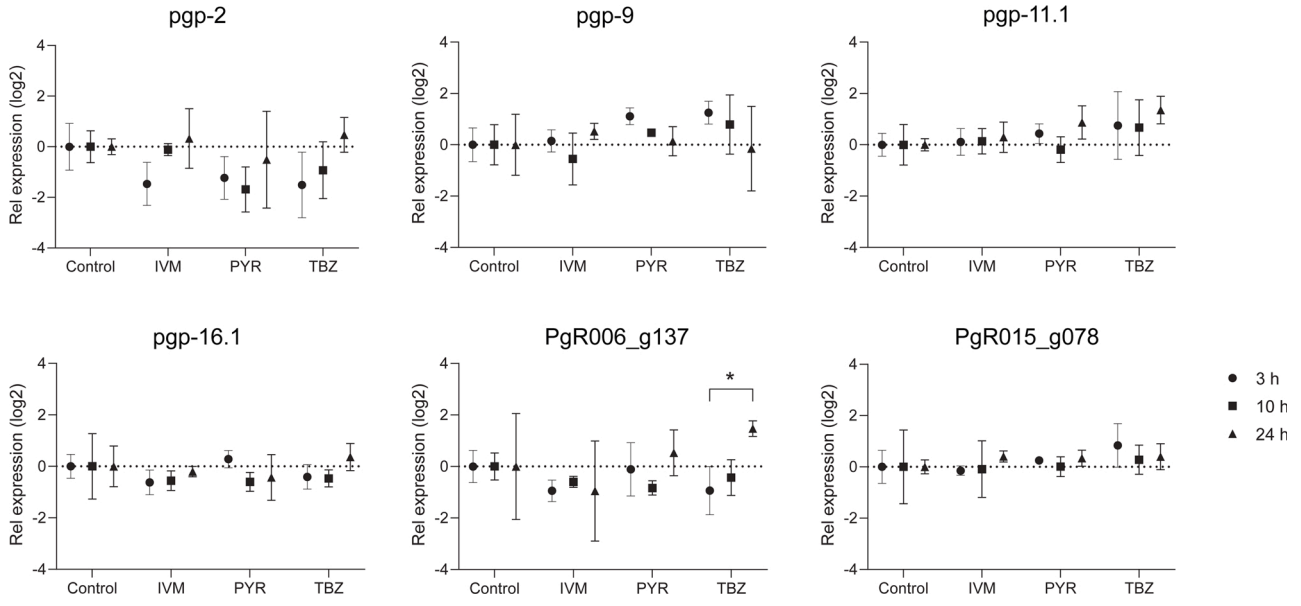

Fig. 4. Expressional responses of transport protein genes in intestinal tissue of adult *Parascaris univalens* (n = 3) *in vitro* exposed to IVM 10^{-9} M, PYR 10^{-6} M, TBZ 10^{-5} M in media with 0.1 % DMSO or media containing 0.1 % DMSO only (control) for 3 h, 10 h and 24 h. Gene expression was normalized to reference gene *gpd-1* and related to the mean of the control for each time point. Significant differences between drug exposed worms and controls as well as between different time points within each treatment group were identified by two-way ANOVA with Tukey's multiple comparisons test and marked with * ($p < 0.05$).

The mechanisms of anthelmintic resistance in parasitic nematodes are suggested to be multi-genic and to partly include increased drug efflux by transport proteins (James et al., 2009; Whittaker et al., 2017). We investigated the difference in constitutive gene expression of various Pgp and MFS genes between larvae and adult tissues. Results showed that *pgp-9*, *pgp-11.1*, *pgp-16.1* and the MFS gene PgR015_g078 were expressed at lower levels in larvae than in adult tissues, whereas *pgp-2* and MFS gene PgR006_g137 had similar expression levels in the different life stages. It is known from previous studies that gene expression may differ between larvae and adult stages of parasitic worms, as reviewed by Jex et al. (2019). However, to our knowledge, there have been no previous studies comparing expression levels of transport protein genes between larvae and adult *Parascaris* spp. In agreement with our results, comparisons between *P. univalens* adult worms and eggs as well as between adult *H. contortus* and larvae showed minor differences in expression of *pgp-2*, while expression of *pgp-9*, *pgp-11.1* and *pgp-16.1/16* was higher in adult tissues of both parasites than in eggs and larvae (Issouf et al., 2014; Gerhard et al., 2020). Thus,

differences in gene expression between larvae and adult worms needs to be considered when interpreting and comparing results from experiments performed in different life stages.

In addition to life stage specific differences, expression levels may also differ between tissues of adult *P. univalens*. We found that the constitutive expression of *pgp-2*, *pgp-9* and *pgp-16.1* were higher in the intestine than in the anterior end. In accordance, the same genes were also expressed at higher levels in the intestine than the carcass in a tissue specific transcriptome from two male and two female anthelmintic naïve worms (Gerhard et al., 2020). However, Janssen et al. (2013) saw a lower expression of *pgp-16.1* in the intestine than the body wall of male worms, while there was no differences between the tissues in female worms. The constitutive expression of *pgp-11.1* was similar in intestinal and anterior end tissue in our study, which is in contrast to Gerhard et al. (2020) where *pgp-11.1* was expressed at higher levels in the intestine than in the carcass. Similarly, Janssen et al. (2013) found significantly higher expression of *pgp-11.1* in the intestine than the body wall in male worms, while female worms had no difference in expression between the tissues, which is in agreement with our study. The variation between the studies could depend on the differences of the compared tissues (carcass, body wall and anterior end *vs* intestine), differences in experimental design and genetic differences between individual worms and populations. Both MFS genes, PgR006_g137 and PgR015_g078, were also expressed at higher levels in the intestine than in the anterior end. The high constitutive expression of transport proteins in the intestine support the theory that this is a major site for xenobiotic efflux in *P. univalens* (Janssen et al., 2013; Gerhard et al., 2020). In addition, the fact that expression *of P. univalens pgp-9* in the intestine of *C. elegans* led to reduced susceptibility of IVM (Gerhard et al., 2021), further indicates that Pgp-expression in the intestine may be involved in anthelmintic resistance.

P-glycoprotein expression has been upregulated in response to anthelmintic drug exposure in parasitic nematodes in several studies as reviewed by Lespine et al. (2012). Ivermectin is a well-known substrate for Pgp-transport and BZs have also been shown to interact with Pgps (Nare et al., 1994; Beugnet et al., 1997; Xu et al., 1998), but to our knowledge, there are no studies investigating the effects of PYR on Pgp-expression or confirming Pgp-transport of PYR so far. It has however been shown that Pgp-expression can be altered by *in vitro* exposure to both monepantel and levamisole in multidrug resistant strains of *H. contortus*, confirming that several different drugs may affect Pgp-expression in nematode larvae (Raza et al., 2016a; Raza et al., 2016b). P-glycoprotein transport of drug metabolites out of the cell has also been suggested as a contributing factor to reduced efficacy of drugs, and a metabolite of BZs has been shown to increase expression of *pgp-9* in susceptible *H. contortus* (James et al., 2009; Kellerova et al., 2020). Since *P. univalens* has developed resistance to the three commonly used anthelmintic drugs for horses, we examined the transcriptional response of transport proteins after *in vitro* exposure to IVM, PYR and TBZ. We found no significant increases in expression of transport protein genes in adult tissues after 3 h, 10 h or 24 h exposure to any of these drugs. These results are in accordance with similar studies where no significant up-regulation of Pgps was seen after 24 h of exposure to IVM, PYR or TBZ (Martin et al., 2020), 3 h of IVM exposure or 24 h exposure to the BZ oxibendazole (Scare et al., 2020) as well as 12 h of IVM exposure (Janssen et al., 2013; Gerhard et al., 2020). However, *pgp-11.1* expression in the anterior end tissue was increasing slightly during exposure to IVM and PYR, with the highest expression levels after 24 h of exposure. This suggests that these drugs may have an effect on *pgp-11.1* expression, but with a delayed response. In a study where mouse liver cells were exposed to IVM, a peak of Pgp-expression was observed after 48 h of exposure (Menez et al., 2012). In contrast, Raza et al. (2016b) found that expression of several Pgps increased in a multi-resistant strain of *H. contortus* larvae after 3 h of exposure to IVM, but then returned to basal levels after 6 h. Hence, the timing of transcriptional response seems to vary between experiments. Interestingly, there were no significant differences in Pgp-expression in intestinal tissue after *in vitro* drug exposure, supporting that the higher constitutive expression in intestinal tissue discussed above rather than up-regulation in response to drug exposure, may serve as a general defence against anthelmintic drugs as well as other xenobiotic substances.

Exposure of larvae to sub-lethal doses of PYR and TBZ for 24 h resulted in a significant increase in *pgp-9* expression, but exposure to IVM for 24 h did not affect the expression levels of any of the transport protein genes studied. A possible reason for the lack of response to IVM exposure might be the concentration of the drug. Expression of Pgps were found to be dose-dependent both in murine liver cells exposed to IVM and in *H. contortus* larvae exposed to monepantel (Menez et al., 2012; Raza et al., 2016a). Concentrations of anthelmintic drugs used in our study (IVM 10^{9} M, PYR 10^{-6} M, TBZ 10^{-5} M) were the same as the highest concentrations used in a previous study where adult worms were found to be affected but not killed by 24 h *in vitro* exposure (Martin et al., 2020). To be able to compare the expressional response between larvae and adult worms the same drug concentrations were used in both experiments. To our knowledge, there are no previous studies of *in vitro* exposed *Parascaris* spp. larvae, but in a similar experiment *C. oncophora* larvae of an IVM resistant strain showed an increase of *pgp-11* and *pgp-16* expression after 24 h *in vitro* IVM exposure (De Graef et al., 2013). In addition, another study found increases in expression of *pgp-2*, *pgp-9.1* and *pgp-11* after 3 h of IVM exposure in multi-resistant *H. contortus* larvae (Raza et al., 2016b). In conclusion, the transcriptional response of Pgps to anthelmintic drug exposure differs in larvae and adult worms. Despite these differences, we believe that the model will prove useful to compare gene expression levels between larval populations.

Though the MFS family of multidrug resistance transporters plays a major part in antibiotic resistance in bacteria (Fluman and Bibi, 2009), little is known about its possible role in anthelmintic metabolism and development of resistance in parasitic worms. In a previous study MFS gene PgR015_g078 was up-regulated in adult *P. univalens* after 24 h *in vitro* exposure to IVM, while another MFS gene, PgR006_g137 was down-regulated after exposure to IVM and PYR (Martin et al., 2020). In the present study, the expression of the MFS genes PgR006_g137 and PgR015_g078 were reduced in larvae after 24 h exposure to TBZ while PgR006_g137 was also reduced after exposure to PYR. However, there were no transcriptional response of MFS genes in adult tissues after *in vitro* exposure. Since up-regulation of several MFS family members are known to cause multidrug resistance in bacteria (Fluman and Bibi, 2009) we propose that this family of transporter needs to be further investigated regarding their possible role in anthelmintic resistance in parasites.

5. Conclusions

Similar to other studies of transcriptional responses in *P. univalens*, we did not observe any significant increases in expression of Pgp genes in adult worms (Janssen et al., 2013; Gerhard et al., 2020; Martin et al., 2020; Scare et al., 2020). Interestingly, expression of *pgp-9* was increased in larvae after exposure to PYR and TBZ, while there was no transcriptional responses after IVM exposure. All the investigated transport protein genes were expressed at higher rates in the intestine than in the anterior end in adult worms, except *pgp-11.1* where the expression was similar between the two tissues. Whether Pgps are connected to anthelmintic resistance in *P. univalens* remains to be elucidated, but the high constitutive expression in the intestine support that this is an important site for xenobiotic efflux in *P. univalens*.

We developed a fast and simple protocol for hatching of *P. univalens* larvae for use in *in vitro* studies. Though the results of this study show differences in expression of transport proteins between larvae and adult

tissues, the model could serve as an important tool for further research into the molecular mechanisms involved in drug metabolism and anthelmintic resistance development. The use of larvae opens up for refined study designs with larger and replicated study groups, which is difficult to achieve with adult *P. univalens* worms. The model can be used to compare expression of candidate genes between populations with different resistance phenotypes and for other types of *in vitro* studies such as trials of novel anthelmintic drugs.

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CRediT authorship contribution statement

Frida Martin: Conceptualization, Methodology, Investigation, Writing - original draft. **Matthías Eydal:** Resources, Project administration. **Johan Höglund:** Conceptualization, Writing - review & editing. **Eva Tydén:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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