



## Effects of *in vitro* exposure to ivermectin and levamisole on the expression patterns of ABC transporters in *Haemonchus contortus* larvae



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### ABSTRACT

This study investigated the interaction of ATP binding cassette (ABC) transport proteins with ivermectin (IVM) and levamisole (LEV) in larvae of susceptible and resistant isolates of *Haemonchus contortus* *in vitro* by measuring transcription patterns following exposure to these anthelmintics. Furthermore, we studied the consequences of drug exposure by measuring the sensitivity of L<sub>3</sub> to subsequent exposure to higher drug concentrations using larval migration assays. The most highly transcribed transporter genes in both susceptible and resistant L<sub>3</sub> were *pgp-9.3*, *abcf-1*, *mrp-5*, *abcf-2*, *pgp-3*, and *pgp-10*. The resistant isolate showed significantly higher transcription of *pgp-1*, *pgp-9.1* and *pgp-9.2* compared to the susceptible isolate. Five P-gp genes and the *haf-6* gene showed significantly higher transcription (up to 12.6-fold) after 3 h exposure to IVM in the resistant isolate. Similarly, five P-gp genes, *haf-6* and *abcf-1* were transcribed at significantly higher levels (up to 10.3-fold) following 3 h exposure to LEV in this isolate. On the other hand, there were no significant changes in transcriptional patterns of all transporter genes in the susceptible isolate following 3 and 6 h exposure to IVM or LEV. In contrast to these isolate-specific transcription changes, both isolates showed an increase in R-123 efflux following exposure to the drugs, suggesting that the drugs stimulated activity of existing transporter proteins in both isolates. Exposure of resistant larvae to IVM or LEV resulted, in some instances, in an increase in the proportion of the population able to migrate at the highest IVM concentrations in subsequent migration assays. The significant increase in transcription of some ABC transporter genes following 3 h exposure to both IVM and LEV in the resistant isolate only, suggests that an ability to rapidly upregulate protective pathways in response to drugs may be a component of the resistance displayed by this isolate.

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### 1. Introduction

*Haemonchus contortus* is one of the most pathogenic gastrointestinal nematodes (GINs) of small ruminants, causing substantial economic losses to livestock industries worldwide. Owing to the unavailability of effective vaccines and/or other alternate control methods for GINs, anthelmintics are the cornerstone of control programs (Kaplan and Vidyashankar, 2012). However, resistance has developed to all the major classes of anthelmintics and this situation threatens the sustainability of many livestock enterprises

(Kaplan, 2004; Sutherland and Leathwick, 2011).

A number of studies have shown that nematode ATP binding cassette (ABC) transport proteins (including P-glycoproteins (P-gps), multi-drug resistance proteins (MRPs) and Half (HAF) transporters) have a protective function through their role in the efflux of anthelmintics (Kerboeuf and Guegnard, 2011; Lespine et al., 2012; Janssen et al., 2013, 2015; Kaschny et al., 2015). Increased expression of P-gps in nematodes after *in vitro* as well as *in vivo* exposure to anthelmintics suggests a role for P-gps in the efflux of anthelmintics (Dicker et al., 2011; Williamson et al., 2011; De Graef et al., 2013). The reported increase in sensitivity to some anthelmintics, particularly IVM, in different P-gp knock-out strains of *Caenorhabditis elegans*, also provides evidence for their role in protection from anthelmintics (Ardelli and Prichard, 2013; Bygarski

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et al., 2014). Furthermore, P-gps have been implicated in resistance to anthelmintics, with a number of studies describing an increased transcription of specific transporter genes in drug-resistant nematodes (Dicker et al., 2011; Williamson et al., 2011; Sarai et al., 2014). Increased expression levels of several P-gps and MRP genes have been reported in *C. elegans* post-exposure to IVM (James and Davey, 2009; Ardelli and Prichard, 2013). Similarly, in parasitic nematodes, overexpression of *pgp-11*, *pgp-16* and *mrp-1* was observed in *Cooperia oncophora* recovered from animals treated with IVM (De Graef et al., 2013; Tydén et al., 2014), while Lloberas et al. (2013) reported that treatment of infected lambs with IVM increased the transcription levels of *pgp-2* in resistant worms of *H. contortus* compared to worms collected from untreated control animals. The use of multi-drug resistance inhibitors (MDRIs) to increase the toxicity of anthelmintics to nematodes further indicates a role for drug transporters in resistance (Bartley et al., 2009; Heckler et al., 2014; Raza et al., 2015).

Nematodes possess a greater diversity of MDR transporters compared to mammals. *C. elegans* is known to possess 15 P-gp genes, eight MRP and nine HAF genes (Sheps et al., 2004). In parasitic nematodes, 11 P-gp genes, one HAF gene and two MRP genes have been reported in *H. contortus* (Williamson and Wolstenholme, 2012; Laing et al., 2013). Furthermore, eight P-gp genes, five MRP genes and eight HAF genes were identified in *Brugia malayi* (Ardelli et al., 2010), two P-gp genes in cyathostomins (Drogemüller et al., 2004) and 11 partial sequences of P-gp genes have been reported in *Teladorsagia circumcincta* (Dicker et al., 2011). The diversity of nematode P-gps suggests that they may play a protective role in the efflux of a wide range of environmental toxins and internal metabolites (Prichard and Roulet, 2007).

The aim of the present study was to examine transcription patterns of ABC transporters in the third-stage larvae (L<sub>3</sub>) of *H. contortus*. Firstly, we examined the relative transcription levels of the various transporter genes in L<sub>3</sub> of susceptible and -resistant isolates of the parasite, and identified differences between transcription levels of the various genes within each isolate, and between the two isolates. We then measured gene transcription patterns in both susceptible and -resistant L<sub>3</sub> following exposure to IVM and LEV *in vitro*. Finally, we examined the functional consequences of this drug exposure by measuring efflux of the dye rhodamine-123 (R-123) from drug-exposed and control worms, and also by using migration assays to measure the sensitivity of larvae to IVM and LEV following pre-treatment with lower concentrations of these drugs.

## 2. Materials and methods

### 2.1. Parasites

Two isolates of *H. contortus* were used for the present study:

- (i) Kirby: isolated from the field at Kirby Research Farm, University of New England in 1986; susceptible to all commercially available anthelmintics (Albers and Burgess, 1988).
- (ii) Wallangra (WAL): isolated in 2003 from the New England region of Northern New South Wales (NSW). At the time of isolation from the field, WAL was resistant to benzimidazoles, closantel, LEV and IVM (Love et al., 2003). The isolate has further been selected against moxidectin (Cydectin®) for at least five generations.

Infected animals were housed at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Agriculture FD McMaster laboratory at Armidale, NSW. All animal procedures were approved by the FD McMaster Animal Ethics Committee, CSIRO

Agriculture Flagship (Animal Ethics Approval Number AEC 13/23).

Faeces was collected from infected sheep, placed into large ziplock bags, and sent by courier to the CSIRO Agriculture laboratories at the Queensland Bioscience Precinct, Brisbane, Queensland. For recovery of L<sub>3</sub>, the faecal samples were placed into 2L glass jars and most of the faecal pellets were broken up by hand. The faecal mixture was slightly moistened with tap water, and the jars were then placed in an incubator at 27 °C. After approximately one week, the L<sub>3</sub> moving up the sides of the jar were flushed out with tap water, placed onto a cloth (20 µm) suspended in water, and allowed to migrate into a collection jar overnight. Collected L<sub>3</sub> were stored at 15 °C for the later use in migration and molecular assays within three to four weeks.

### 2.2. RNA extraction and generation of complementary (c)DNA

Approximately 30,000 L<sub>3</sub> were used for each RNA preparation. Total RNA was extracted using RNeasy mini kit (Qiagen®, Germany) following the manufacturer's protocol. Briefly, the samples were homogenised in buffer RLT using a bead-based homogenizer (Power-lyzer® 24, Mo-Bio Laboratories, USA) followed by washing, binding and elution of total RNA. The extracted RNA was treated with Turbo-DNase (Ambion®, USA) to remove genomic DNA. RNA samples were quantified using a spectrophotometer (Nanodrop 8000, Thermo Scientific®, USA) and stored at –80 °C for further use. cDNA was synthesised using DNase-treated RNA with Superscript III™ reverse transcriptase (Invitrogen®, USA) according to the manufacturer's instructions. The final volume of cDNA was diluted to a concentration of 4 ng/µL for downstream applications. For each of the isolate and/or treatment, cDNA was generated in three distinct replicates using separate worm samples for subsequent gene expression analysis.

### 2.3. Quantitative PCR (qPCR)

Primer sequences for some of the ABC-transporters used herein were previously reported by Sarai et al. (2013) (GAPDH, actin, *pgp-1*, *pgp-2*, *pgp-3*, *pgp-10*, *pgp-12* and *pgp-14*) and Issouf et al. (2014) (*pgp-9.2* and *pgp-16*). Primers for β-tubulin, *pgp-9.1*, *pgp-9.3*, *pgp-11*, *mrp-1*, *mrp-5*, *haf-6*, and ABCF transporters were designed using Primer-3 from the sequence information available on Nembase-4 (see Supplementary Table 1). Three housekeeping genes, GAPDH, actin and β-tubulin, were used as reference genes for the qPCR analyses. Approximately 18 ng of cDNA was added to other standard PCR reagents in a total reaction volume of 25 µL and then split into four replicates, each of 5 µL. A Vii A7 thermocycler® (Applied Biosystems, USA) was used with SYBR® Green dye system (Applied Biosystems, USA) under the following PCR cycling conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, followed by a melt curve stage (95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s). Each sample was represented by three biological replicates and run in four technical replicates in the real-time PCR. Amplification efficiency for each primer set was determined by performing PCR using either 2-fold or 1.5-fold cDNA dilutions. Standard curves for all primers showed an efficiency range between 80 and 99%. Melt-curves were analysed for each primer sequence to ensure the specificity of the primers. Furthermore, the PCR products were run on an electrophoresis gel and viewed under UV illumination after SYBR safe staining to ensure that a single band without any non-specific product was visible. In order to confirm the identity of the PCR products generated using primers designed for the present study (*pgp-9.1*, *pgp-9.3*, *mrp-1*, *mrp-5*, *abcf-1* and *abcf-2*), the products were cloned into Top-10 cells (Invitrogen®, USA) and sequenced (Big-Dye terminator, V3.1; Applied Biosystems, USA) using M13 forward/reverse primers. In each case,

the PCR product corresponded to the specific targeted gene.

Expression values for all genes in each sample were normalised to the three housekeeping genes using REST 2009 (version v2.0.13) to: (1) determine the transcription levels of each gene within an isolate using the transcription level of *pgp-1* as control; (2) compare the transcription profiles of ABC transporter genes between resistant and susceptible isolates, and (3) measure the transcriptional patterns of ABC transporters post-exposure to IVM and LEV (drug exposure regimen described below in section 2.4) using DMSO-treated samples as controls for each isolate. In order to compare the transcription profiles of control and test samples, the triplicate expression values were  $\log_{10}$ -transformed and analysed using repeated measures ANOVA with Fischer's LSD as multiple comparison test in GraphPad Prism® software (GraphPad Prism, USA version 6.01).

#### 2.4. Drug exposure assays

Technical grade IVM and LEV were purchased from Sigma–Aldrich. For each anthelmintic, a stock solution was prepared at 10 mg/mL in dimethyl sulfoxide (DMSO) followed by two-fold serial dilutions in DMSO to produce multiple separate anthelmintic solutions.

To measure the effects of drug exposure on the expression profiles of ABC transporters, groups of approximately 30,000 L<sub>3</sub> were exposed to IVM (0.2 and 0.8 µg/mL), LEV (0.4 µg/mL) or DMSO (vehicle control) for two different time periods (3 and 6 h). These selected concentrations were based on the dose–response curves described previously (Raza et al., 2015). The lower (0.2 µg/mL) concentration of IVM was non-toxic to resistant isolate, but inhibited about 12% of the larval migration for the susceptible isolate, whereas, the higher concentration of IVM (0.8 µg/mL) showed about 12% and 25% inhibition of larval migration in the resistant and susceptible isolates, respectively. The LEV concentration (0.4 µg/mL) showed about 10% inhibition of larval migration with both the isolates (Raza et al., 2015). The concentration of DMSO across all the treatments was 1% (v/v).

The worms were kept on a roller-mixer (BTR-5, Ratek®, Australia) for the entire duration of the drug-exposure period. Both the susceptible and resistant isolates were examined in three separate experiments. After exposure, the worms were snap frozen in liquid nitrogen and kept at –80 °C for further use. The transcriptional profiles of 11 P-gps, two MRPs, one HAF and two ABCF genes were measured by qPCR as described above (Section 2.3).

#### 2.5. Larval migration assay (LMA)

An LMA was used to determine the sensitivity of Kirby and WAL L<sub>3</sub> larvae to a range of concentrations of IVM and LEV following 3 h drug-exposure for Kirby and 3 and 6 h drug-exposure for WAL as described in Section 2.4. The LMA was modified from Kotze et al. (2006) as follows: (1) the assays used short incubation/migration periods (30 min each) compared to 48 and 24 h periods in the earlier paper, (2) filter-mesh plates were used without any agar, and (3) receiver plate wells received only 300 µL water and no drug. Final concentration ranges used for IVM were 100–0.195 µg/mL and 12.5–0.024 µg/mL for LEV. Three separate experiments (each with assays in triplicate) were run for each combination of pre-exposure treatment (IVM at 0.2 µg/mL and 0.8 µg/mL or LEV at 0.4 µg/mL) and subsequent dose response (range of IVM or LEV concentrations) with the resistant Wallangra isolate. Assays with the Kirby isolate were only performed using L<sub>3</sub> pre-exposed to the lower IVM concentration (0.2 µg/mL), as well as LEV (0.4 µg/mL), as a previous study had shown that the migration of Kirby larvae was reduced by 25% at the higher IVM concentration compared to

controls (Raza et al., 2015), hence making it difficult to accurately measure the specific effects of subsequent drug exposure.

The data were analysed using non-linear regression in GraphPad Prism® software (GraphPad Prism, USA version 6.01). IC<sub>50</sub> values and 95% confidence intervals were calculated based on the pooled data from each set of nine assays, and significant differences were determined by the overlap of 95% confidence intervals. For assays with LEV, the larvae showed % migration ranging from approximately 100% (equivalent to controls) down to 0% migration, and hence we used a normalised dose–response model in GraphPad (dose–response from 100% to 0%), and a variable slope. On the other hand, the dose response to IVM in the migration assay showed the presence of a plateau in response at the highest drug concentrations. The % migration remained at a constant level (above 0%) over the highest 3 to 4 drug concentrations. Hence, for the analysis of the IVM dose–response data we used a non-normalised model in GraphPad ('top to bottom'), with a variable slope. The output of this analysis provided us with two parameters with which to compare populations: firstly, the % migration at the dose–response plateau (that is, the % of the population unaffected by the highest concentrations of IVM in the assay), and secondly, the IC<sub>50</sub> in the remaining proportion of the population that had shown a dose–response to the drug (for instance, if the plateau existed at a level of 40% migration, then the IC<sub>50</sub> value defined the response to the drug in the remaining 60% of the worm population alone).

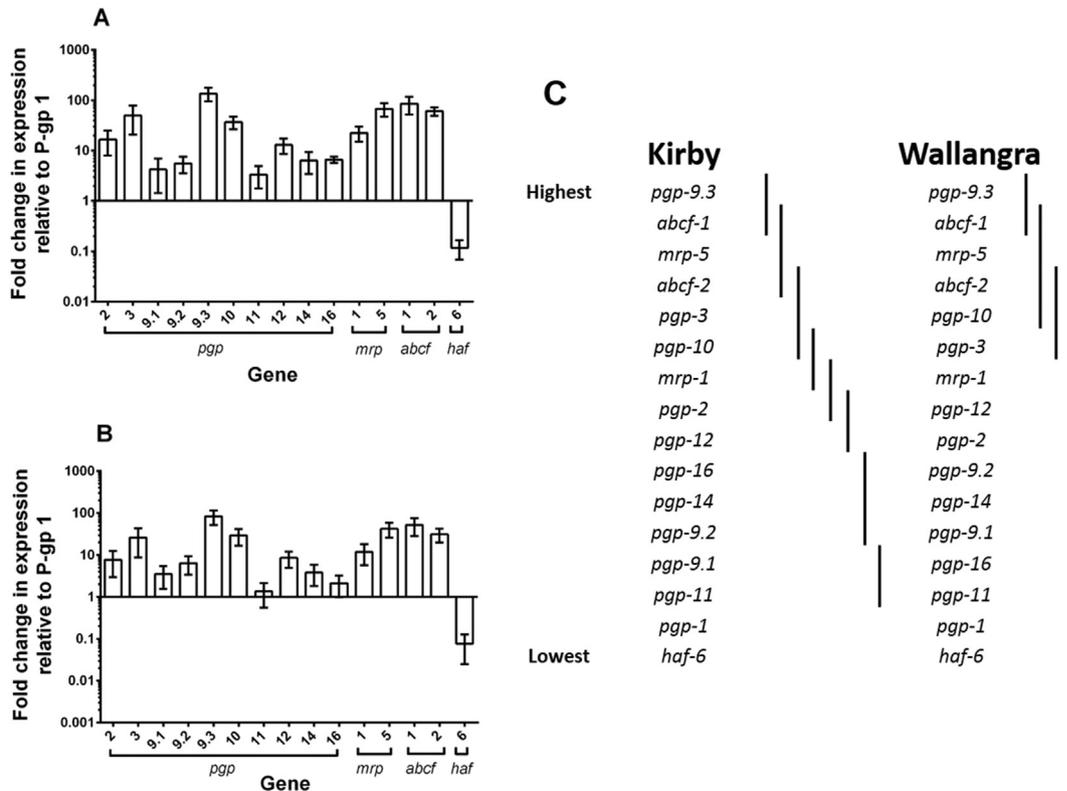
#### 2.6. Rhodamine-123 efflux assay

Efflux of rhodamine-123 from larvae following exposure to IVM and LEV was measured using an assay modified from Kerboeuf and Guegnard (2011). Briefly, for each isolate, approximately 20,000 L<sub>3</sub> were exposed to IVM (at 0.2 µg/mL and 0.8 µg/mL), LEV at 0.4 µg/mL or 1% DMSO for 3 h, as described above in Section 2.4. Following exposure to drugs, the worms were centrifuged (3000g, 1 min), the supernatant was discarded, and the worm pellet washed using 1 mL distilled water. R-123 solution (2 mL of 1.5 µM) was added to the worm pellet which was then placed on a roller for 15 min in the dark at room temperature (= R-123 accumulation period). At the end of the accumulation period, the worms were centrifuged (3000g, 1 min), followed by washing in 1 mL of water. The worm pellet was resuspended in water (1 mL) and placed on a roller-mixer (BTR-5, Ratek®, Australia) in the dark for 60 min (= R-123 efflux period). The worms were sedimented by centrifugation (3000g, 1 min) and the supernatant was collected and stored at room temperature for 60 min in the dark before analysis. R-123 was detected using a fluorescence spectrophotometer (Spectra Max M3, Molecular Devices®, USA) ( $\lambda$  for excitation = 495 nm and  $\lambda$  for emission = 525 nm). Three separate experiments were performed, each with duplicate assays. The concentration of R-123 in each experimental sample was calculated from a standard curve determined using a range of concentrations of R-123. The pooled data ( $n = 6$  for each treatment) were  $\log_{10}$ -transformed and analysed using repeated measures ANOVA followed by Fischer's LSD (GraphPad Prism, USA version 6.01).

### 3. Results

#### 3.1. Basal transcription levels of ABC transporters in resistant and susceptible isolates of *H. contortus*

In the susceptible isolate, transcription of all the P-gp, MRP and ABCF genes was significantly higher ( $P < 0.05$ ) than for the *pgp-1* gene (Fig. 1A and C). The pattern observed in the resistant isolate was similar, except that *pgp-11* and *pgp-16* were at equivalent levels



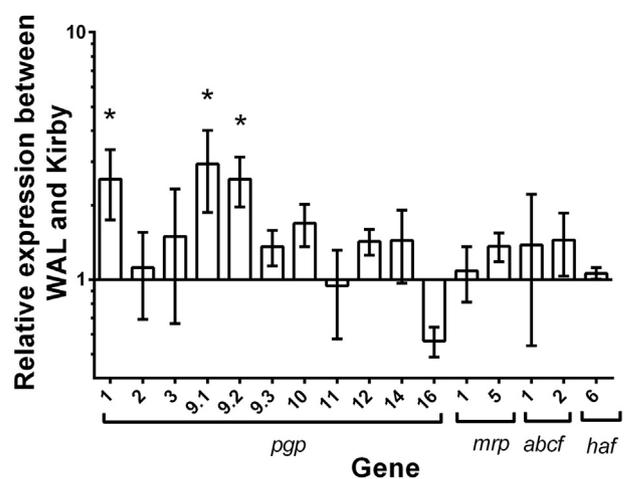
**Fig. 1.** Basal transcription levels of ABC transporter genes in susceptible (A) and resistant (B) isolates of *Haemonchus contortus* compared to the transcription levels of *pgp-1*. Data shown as mean ± SEM, n = 3 separate experiments each with four technical replicates. Significant differences in the relative transcription of the genes within each isolate are indicated in part (C). Gene transcription levels relative to *pgp-1* are ranked from the highest to the lowest; genes grouped together with vertical lines did not show significant differences in transcription levels (at P < 0.05).

to *pgp-1* (Fig. 1B and C). Transcription of *haf-6* was significantly lower (P > 0.05) than *pgp-1* in both isolates. Compared to *pgp-1*, transcription of *pgp-9.3* was increased by 136-fold and 83-fold in the susceptible and resistant isolates, respectively. The other markedly higher transcription levels, relative to *pgp-1*, were for *abcf-1* (85-fold higher in susceptible, and 52-fold higher in resistant), *mrp-5* (68- and 42-fold), *abcf-2* (61- and 31-fold), *pgp-3* (50-fold and 26-fold), and *pgp-10* (37- and 29- fold).

A comparison of the transcription levels of the ABC transporter genes in resistant (WAL) and susceptible (Kirby) isolates showed that *pgp-1*, *pgp-9.1* and *pgp-9.2* were transcribed at significantly higher levels in the former isolate (P = 0.04, 0.03, and 0.02, respectively) (Fig. 2). Transcription levels were increased in WAL by 2.5-fold for *pgp-1* and *pgp-9.2*, and 3-fold for *pgp-9.1*. There were no significant differences in transcription levels for all the other transporters, including MRPs, ABCFs and the *haf-6* gene, between the two isolates.

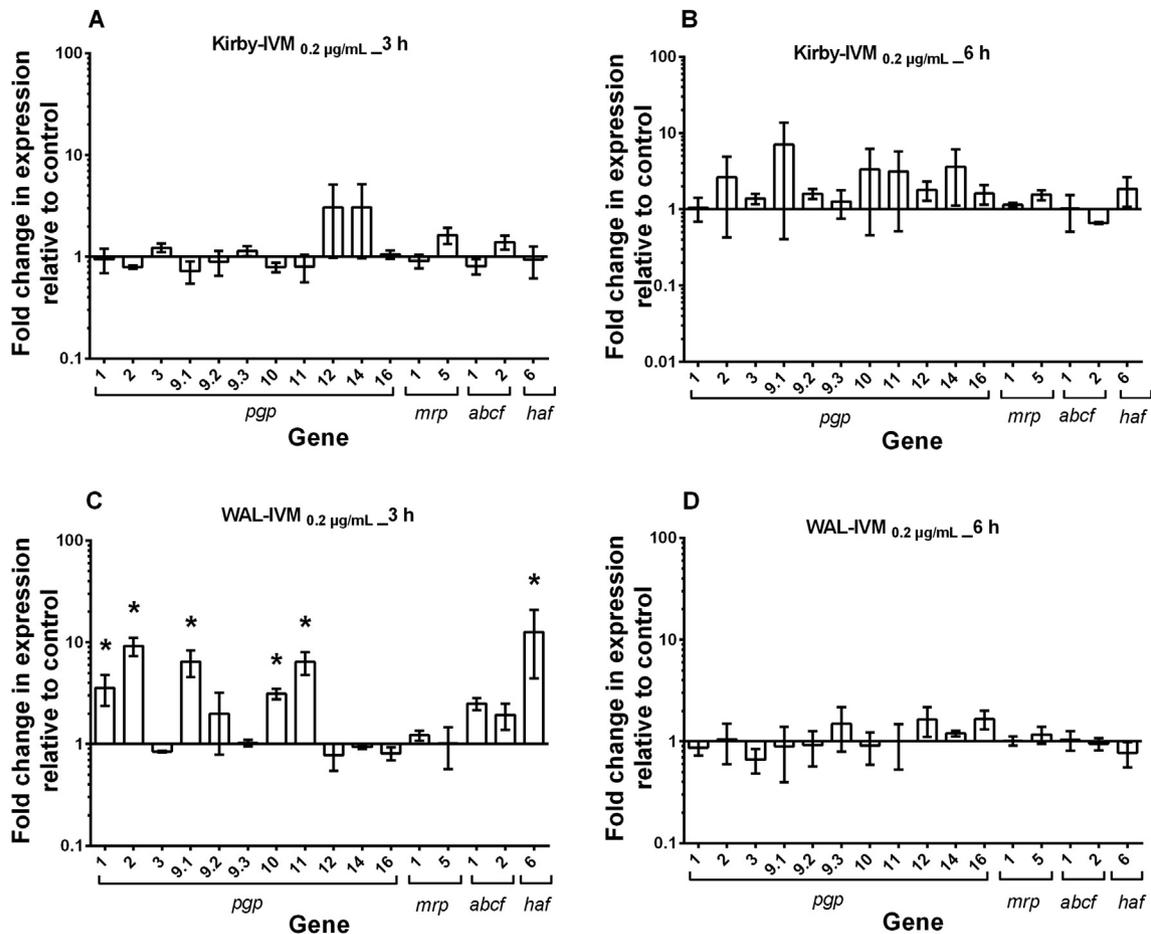
**3.2. Transcription levels of ABC transporters in resistant and susceptible isolates of H. contortus following exposure to IVM**

There were no significant changes in transcription profiles of all 16 ABC transporter genes in the susceptible isolate following 3 or 6 h exposure to IVM at 0.2 µg/mL compared to control worms (exposed to DMSO alone) (Fig. 3A and B). The data at the 6 h time point revealed a degree of variability among different replicates as reflected in the large standard error (SE) bars in some cases in Fig. 3B, however, none of the genes showed a statistically significant change relative to controls. In contrast, five P-gp genes and the *haf-6* gene were significantly up-regulated in the resistant isolate



**Fig. 2.** Relative transcription levels of ABC transporter genes in resistant (WAL) compared to susceptible (Kirby) isolates of *Haemonchus contortus*. Significant differences in the transcription between the two isolates are indicated by \* (P < 0.05). Data shown as mean ± SEM, n = 3 separate experiments, each with four technical replicates.

after exposure to IVM at 0.2 µg/mL for 3 h (Fig. 3C). The magnitude of these increases were as follows: 12.6-fold for *haf-6* (P < 0.0001), 9.1-fold for *pgp-2* (P < 0.0001), 6.4-fold for *pgp-9.1* (P = 0.0006), 6.4-fold for *pgp-11* (P = 0.0004), 3.6-fold for *pgp-1* (P = 0.02), and 3.1-fold for *pgp-10* (P = 0.02). These increased transcription levels were however quite temporary, as by 6 h of exposure to this concentration of IVM, the resistant isolate showed no changes in transcription of any of the transporter genes relative to DMSO-



**Fig. 3.** Effects of IVM (0.2 µg/mL) on transcription patterns of ABC transporter genes in the third-stage larvae of susceptible (A at 3 h and B at 6 h) and resistant (C at 3 h and D at 6 h) isolates of *Haemonchus contortus* compared to DMSO controls. Y-axis shows fold-change in gene expression levels in drug-treated vs DMSO-treated larvae. Data shown as mean ± SEM,  $n = 3$  separate experiments, each with four technical replicates. Significant differences in gene transcription levels are indicated by \* ( $P < 0.05$ ).

treated controls (Fig. 3D).

The results of IVM-exposure experiments at a concentration of 0.8 µg/mL are shown in Fig. 4. Again, as seen at 0.2 µg/mL, the only significant instances of altered gene transcription were increases in IVM-exposed resistant isolate larvae at the 3 h time point (Fig. 4C). There were no significant changes in any of the genes for the susceptible isolate at 3 or 6 h, however, as noted above for the 0.2 µg/mL data, there was a degree of variability between replicate experiments which resulted in large SEs in some cases, particularly for *pgp-9.1* at the 6 h time point (Fig. 4B). The magnitudes of the increases observed for the resistant isolate at 3 h were as follows: *pgp-2* 4.5-fold ( $P = 0.0001$ ), *pgp-9.1* 2.6-fold ( $P = 0.005$ ), *pgp-11* 2.3-fold ( $P = 0.01$ ) and *haf-6* 2.4-fold ( $P = 0.03$ ). By the 6 h time point, transcription levels in the resistant isolate had returned to control levels (Fig. 4D), as was observed for the 0.2 µg/mL treatment. Comparison of the 0.2 and 0.8 µg/mL data sets showed that the increases in gene transcription were 2–3 fold lower for the P-gp genes (*pgp-2*, *9.1* and *11*) and 6-fold lower for the *haf-6* gene at the higher IVM concentration.

### 3.3. Transcription levels of ABC transporters in resistant and susceptible isolates of *H. contortus* following exposure to LEV

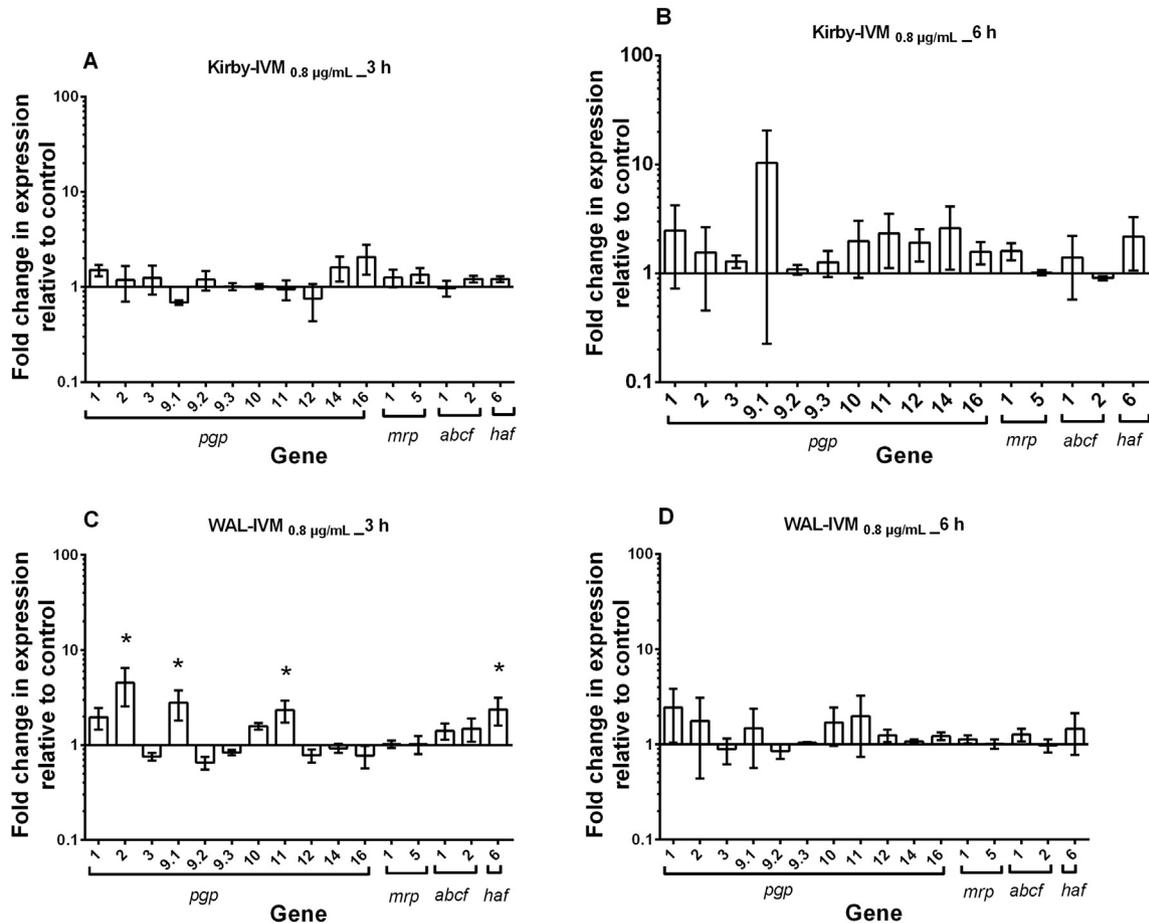
Exposure to LEV at 0.4 µg/mL did not affect transcription levels of any of the transporter genes in the susceptible isolate for both the 3 and 6 h exposure treatments (Fig. 5A and B). In contrast, 3 h

exposure of the resistant isolate resulted in significant up-regulation of a number of genes: *pgp-1* 3.5-fold ( $P = 0.0003$ ), *pgp-2* 9.6-fold ( $P < 0.0001$ ), *pgp-9.1* 6.5-fold ( $P < 0.0001$ ), *pgp-10* 3-fold ( $P = 0.0007$ ), *pgp-11* 6-fold ( $P < 0.0001$ ), *abcf-1* 2-fold ( $P = 0.03$ ), and *haf-6* 10.3-fold ( $P < 0.0001$ ) (Fig. 5C). There was a single instance of down-regulation, with *pgp-16* showing a significant decrease (2-fold) ( $P = 0.04$ ) at 3 h exposure relative to the DMSO control. Interestingly, *pgp-11* remained up-regulated (2.6-fold) after 6 h pre-exposure to LEV at 0.4 µg/mL (Fig. 5D), representing the only instance of significantly altered transcription ( $P = 0.04$ ) at this time point across the entire set of IVM and LEV exposure experiments.

### 3.4. Functional consequences of increased transcription of transporter genes

#### 3.4.1. Larval migration assay

The functional consequences of increased transcription of transporter genes following exposure to IVM and LEV were examined using larval migration assays to measure the ability of IVM and LEV to inhibit migration in larvae of the susceptible and resistant isolates that had previously been exposed for 3 or 6 h to low levels of the two drugs (the same concentrations as used for the gene expression experiments) compared to DMSO-treated controls (Figs. 6 and 7; Tables 1 and 2). A feature of the IVM dose-responses in these experiments was the presence of a plateau in the response



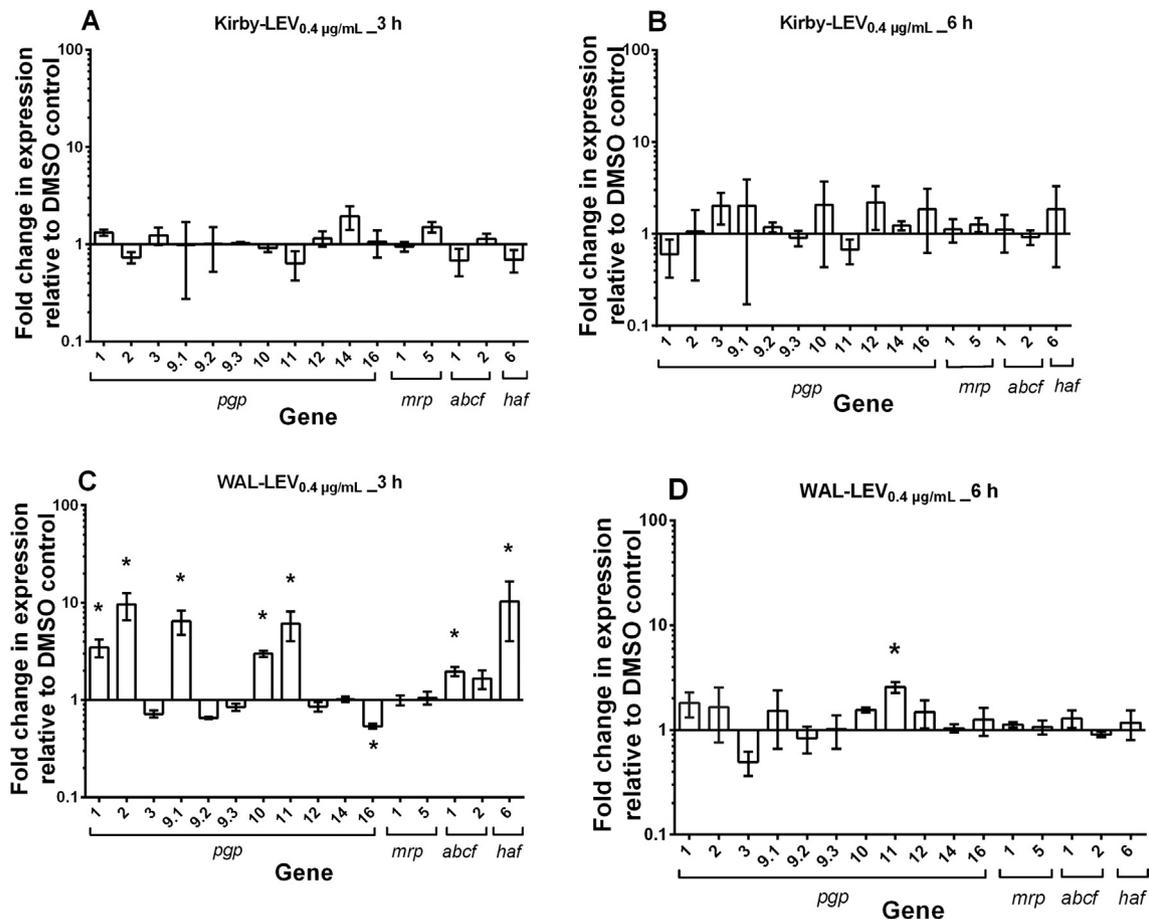
**Fig. 4.** Effects of IVM (0.8 µg/mL) on transcription patterns of ABC transporter genes in the third-stage larvae of susceptible (A at 3 h and B at 6 h) and resistant (C at 3 h and D at 6 h) isolates of *Haemonchus contortus* compared to DMSO controls. Y-axis shows fold-changes in gene expression levels in drug-treated vs DMSO-treated larvae. Data shown as mean  $\pm$  SEM,  $n = 3$  separate experiments, each with four technical replicates. Significant differences in gene transcription levels are indicated by \* ( $P < 0.05$ ).

at the highest IVM drug concentrations (Figs. 6A, B and 7A). A proportion of the worm population remained able to migrate through the filter-mesh system at the highest drug concentrations. Our analysis of these dose response curves provided two parameters for comparing the effects of the drug pre-treatments, namely, the % of the population that was unaffected by the high IVM concentrations (the dose–response plateau), and the  $IC_{50}$  value derived from the dose–response shown by the remainder of the population. Pre-exposure for 3 h to IVM at 0.8 µg/mL resulted in a significant increase in the % of larvae able to migrate at the highest drug concentrations (plateau increased from 17.6% to 48.1%), alongside no change in the  $IC_{50}$  of the remaining population (Fig. 6A, Table 1). Pre-exposure to IVM at the lower concentration (0.2 µg/mL) resulted in no change to the migration plateau, alongside a decrease in the  $IC_{50}$  (to 0.63 of the control). Pre-exposure to LEV (0.4 µg/mL) resulted in a significant increase in the proportion of the population unaffected by the highest drug concentrations (from 17.6 to 30.7%), alongside a decrease in the  $IC_{50}$  in the remainder of the population (to 0.57 of the control). The effects of 6 h pre-exposure to IVM at 0.8 µg/mL and LEV at 0.4 µg/mL, were similar to those seen after 3 h, with significant increases in the IVM dose response plateau (from 15.5% in controls to 25.4 and 31.3% in  $L_3$  pre-treated with IVM and LEV, respectively), although in this case there was no decrease in the LEV  $IC_{50}$  as had been observed after 3 h pre-exposure (Fig. 6B, Table 1). The response to IVM after 6 h pre-exposure to the same drug at 0.2 µg/mL was similar to that observed after 3 h, with no change in the

dose response plateau and a decrease in the  $IC_{50}$  for the remaining population (to 0.60 of the control). Overall, two effects were apparent: an increase in the proportion of the population able to migrate at the highest IVM concentrations following exposure to IVM at 0.8 µg/mL and LEV at 0.4 µg/mL, alongside a number of instances of decreased  $IC_{50}$  in the remainder of the population, indicating the presence of instances of increased drug tolerance (higher plateau) as well as increased drug sensitivity (lower  $IC_{50}$ ) in different components of the worm populations. After 3 h pre-exposure to LEV, these aspects of increased and decreased sensitivity to IVM in the two separate components of the population occurred simultaneously.

LEV  $IC_{50}$  values were significantly decreased following 3 h pre-exposure to IVM (0.2 or 0.8 µg/mL) (to 0.49 or 0.33 of control) and LEV (0.4 µg/mL) (to 0.28 of control) (Fig. 6C; Table 2). Similarly, following 6 h pre-exposure to IVM or LEV, the  $L_3$  showed increased sensitivity to LEV ( $IC_{50}$  decreased to 0.43 of control for IVM pre-exposure, and 0.33 of control for LEV pre-exposure) (Fig. 6D; Table 2).

In the susceptible isolate, pre-exposure to IVM (0.2 µg/mL) and LEV (0.4 µg/mL) did not result in any significant changes to the IVM dose response plateau, although the  $IC_{50}$  was reduced following pre-treatment with IVM at 0.2 µg/mL (to 0.72 of control) (Fig. 7A;  $IC_{50}$  and plateau data not shown). Pre-exposure to IVM (0.2 µg/mL) resulted in increased sensitivity to LEV ( $IC_{50}$  reduced to 0.29 of control), alongside no change in LEV response following pre-exposure to this same drug (Fig. 7B).



**Fig. 5.** Effects of LEV (0.4 µg/mL) on transcription patterns of ABC transporter genes in the third-stage larvae of susceptible (**A** at 3 h and **B** at 6 h) and resistant (**C** at 3 h and **D** at 6 h) isolates of *Haemonchus contortus* compared to DMSO controls. Y-axis shows fold-change in gene expression levels in drug-treated vs DMSO-treated larvae. Data shown as mean  $\pm$  SEM,  $n = 3$  separate experiments each with four technical replicates. Significant differences in gene transcription levels are indicated by \* ( $P < 0.05$ ).

#### 3.4.2. Rhodamine-123 efflux assay

The functional consequences of drug pre-exposure were further evaluated by observing the effects of the drug treatments on the ability of L<sub>3</sub> to efflux the fluorescent dye R-123 (Fig. 8). In both resistant and susceptible isolates, exposure to IVM (0.8 µg/mL) and LEV (0.4 µg/mL) for 3 h significantly increased ( $P < 0.05$ ) the efflux of R-123 compared to the DMSO-treated control worms. In contrast, pre-exposure to the lower concentration of IVM (0.2 µg/mL) did not result in any change in R-123 efflux in both the isolates (Fig. 8).

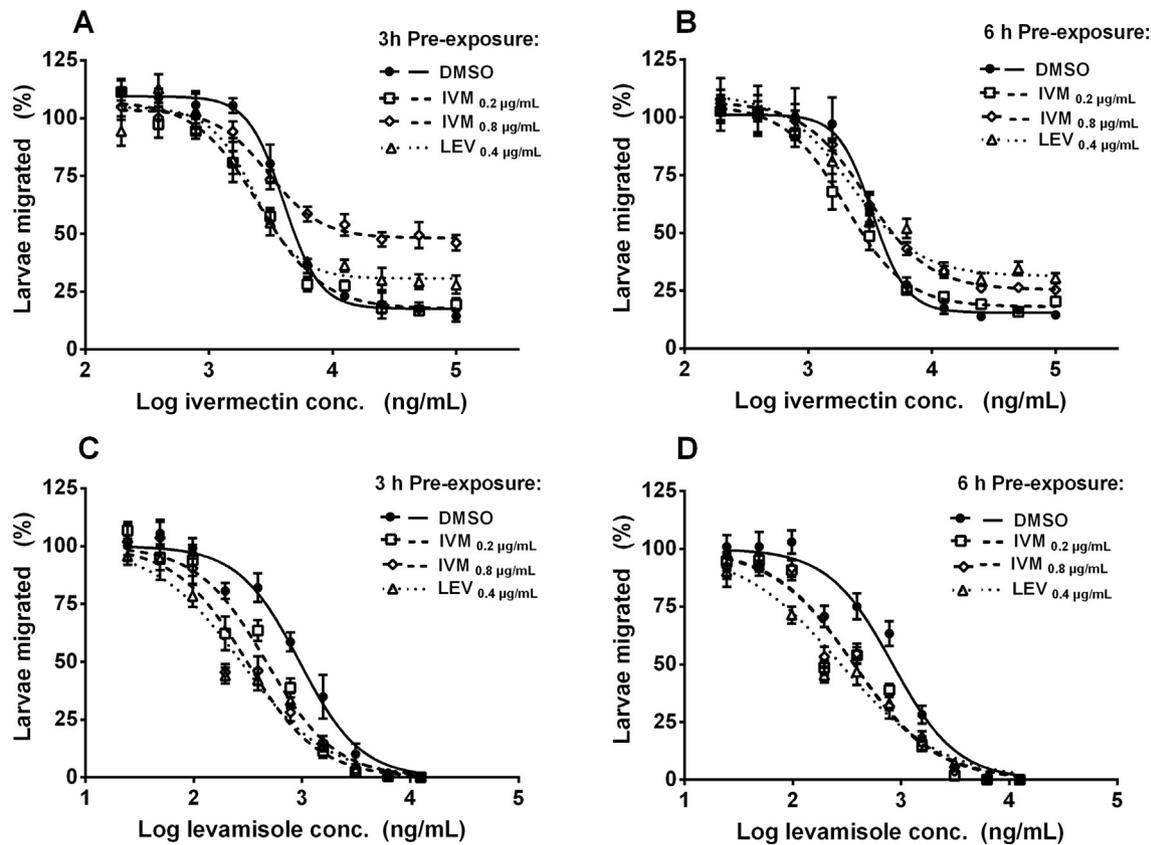
The levels of R-123 efflux were equivalent in resistant and susceptible L<sub>3</sub> in the absence of any drug exposure (DMSO-treated) ( $P = 0.07$ ).

## 4. Discussion

The present study represents the first description of the relative transcription levels of the suite of drug transporter genes in a parasitic nematode. We measured the transcription levels for each ABC transporter gene within a susceptible and resistant isolate and then described these levels relative to *pgp-1* within each isolate. The ranking of transcription levels from the highest to the lowest was similar, but not identical, in the two isolates. The same set of genes (*pgp-9.3*, *abcf-1*, *mrp-5*, *abcf-2*, *pgp-10* and *pgp-3*) were transcribed at the highest levels in both isolates. The *haf-6* gene was transcribed at the lowest levels in both isolates. The protective functions of several of these ABC transporters have been reported previously.

*Pgp-3* acts in protection against natural toxins in *C. elegans* (Broeks et al., 1995). Issouf et al. (2014) described the specific induction of *pgp-3* in *H. contortus* exposed to sheep eosinophil granules as suggesting a role in detoxification of host immune cell products. In addition, the expression of some P-gp genes (including *pgp-3* and *pgp-9*) in intestinal excretory cells of the closely related model organism *C. elegans*, further suggests a role for them in the protection of worms against toxic substances (Ardelli and Prichard, 2013). We also observed relatively high expression levels of ABCF transporters (*abcf-1* and *abcf-2*) compared to many of the other genes. ABCF transporters lack the transmembrane domains (TMDs) present in other transporter proteins, and their function as transporters is currently unclear. It has been suggested that ABCF transporters are involved in cell physiology (ribosome assembly, translational control and mRNA transport) in arthropods (Dermauw and Van Leeuwen, 2014). Furthermore, it has been reported that absence of ABCF transporters in arthropods results in physical abnormalities as shown by Broehan et al. (2013) who observed the death of L<sub>3</sub> and arrested growth, as well as the death as pharate adults, in RNA interference studies with L<sub>3</sub> and pupae of *Tribolium castaneum*, respectively. However, the role of these transporters in gastrointestinal nematodes remains to be determined.

The transcription levels for *pgp-1*, *pgp-9.1* and *pgp-9.2* were significantly greater in the drug-resistant WAL isolate compared to the drug-susceptible Kirby isolate. This finding is in agreement with the earlier report by Sarai et al. (2013) who described an up-regulation of *pgp-1* and *pgp-9* in L<sub>3</sub> of the WAL isolate compared



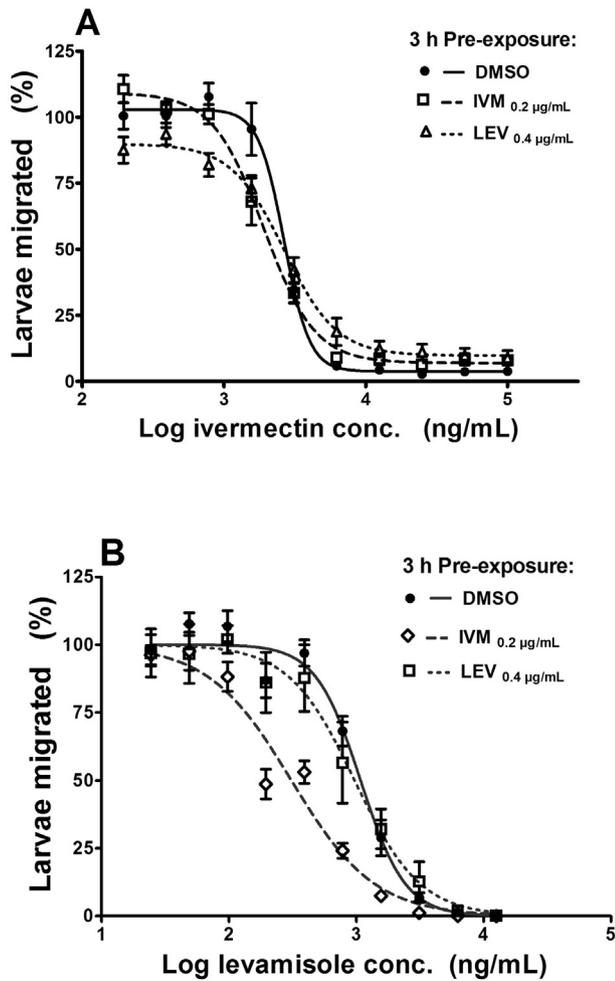
**Fig. 6.** Effects of IVM (0.2 and 0.8 µg/mL) and LEV (0.4 µg/mL) pre-exposure on tolerance to IVM (A after 3 h pre-exposure and B after 6 h pre-exposure) and LEV (C at 3 h pre-exposure and D at 6 h pre-exposure) in the third-stage larvae of the resistant isolate of *Haemonchus contortus* in larval migration assays. Each data point represents mean  $\pm$  SE,  $n = 9$  (pooled data from three experiments, each with assays in triplicate).

to Kirby. This earlier study did not consider the three different homologues of *pgp-9* (as their existence was unknown at that time) and hence the present study has extended the earlier findings by showing that the increase in transcription of *pgp-9* reported earlier was most-likely associated with increases for *pgp-9.1* and *9.2*, alongside no change in *pgp-9.3*. The expression patterns of P-gps in nematodes seem to be quite variable, with some reports linking them to anthelmintic resistance and other studies finding no association. Williamson et al. (2011) reported a significantly increased expression of *pgp-2* and *pgp-9* in a multi-drug resistant isolate compared to a susceptible isolate, alongside a significant decrease in *pgp-1* transcription. In contrast, Williamson and Wolstenholme (2012) found no changes in P-gp transcription in a laboratory selected IVM-resistant isolate compared to its drug-sensitive parent. In addition, there have been reports describing increased transcription of *pgp-9* in ML-resistant *T. circumcincta* adult worms, and of *haf-9* and *mrp-1* in eggs of IVM resistant *C. oncophora* (Dicker et al., 2011; De Graef et al., 2013). The increased transcription of several P-gps in resistant L<sub>3</sub> in the present study suggests that they may play a role in the anthelmintic resistance shown by the multi-drug resistant WAL isolate. However, confirmation of any such association requires studies measuring actual drug efflux from larval and adult life-stages of susceptible and resistant isolates. Our R-123 efflux measurements showed that the rate of efflux was equivalent in the susceptible and resistant L<sub>3</sub>. This may be expected, despite the up-regulation of several transporter genes in the resistant isolate, as these specific transporters (*pgp-1*, *pgp-9.1* and *pgp-9.2*) were expressed at low levels compared to most of the other transporter genes (from Fig. 1C). Hence, increased transcription of

just these three genes would likely have little or no impact on the total efflux activity measured using a general substrate such as R-123.

Substrates of ABC transporters are known to regulate the expression levels of P-gps and other ABC transporters through transcriptional or post-transcriptional mechanisms (Schrenk et al., 2001). There is a great deal of literature available on the inducing effects of various agents on P-gp expression levels in mammals (Lespine et al., 2012; Menez et al., 2012; Yu et al., 2013). The experimental evidence of up-regulation in nematodes in response to exposure to anthelmintics is limited and inconsistent. Up-regulation of several P-gp genes has been observed in ML resistant *C. elegans* (James and Davey, 2009; Yan et al., 2012; Ardelli and Prichard, 2013). Induction of P-gps was observed in *H. contortus* (*pgp-2*) and *C. oncophora* (*pgp-16* and *mrp-1*) adult worms after *in vivo* exposure to IVM (Lloberas et al., 2013; Tydén et al., 2014). Furthermore, De Graef et al. (2013) also reported a significant increase (3–5 fold) in transcription levels of *pgp-11* in *C. oncophora* adult worms 14 days after treatment with IVM or moxidectin compared to non-exposed adults. However, there were no significant differences observed between the expression patterns of P-gp genes before and after IVM treatment in *C. oncophora* and *H. contortus* (Williamson and Wolstenholme, 2012; Areskog et al., 2013).

We examined the effects of exposure to low levels of IVM and LEV on the transcription profiles of ABC transporters in *H. contortus* L<sub>3</sub>. Exposure to IVM and LEV for 3 h resulted in increased transcription of multiple ABC transporter genes in the resistant isolate only. The patterns of up-regulation were very similar for IVM and



**Fig. 7.** Effects of IVM (0.2 µg/mL) and LEV (0.4 µg/mL) pre-exposure for 3 h on tolerance to IVM (A) and LEV (B) in the third-stage larvae of the susceptible isolate of *Haemonchus contortus* in larval migration assays. Each data point represents mean ± SE, n = 9 (pooled data from three experiments, each with assays in triplicate).

LEV, with both drugs resulting in increased transcription of *pgp-1*, *-2*, *-9.1*, *-10*, *-11*, and *haf-6*, while LEV exposure also resulted in up-regulation of *abcf-1*. Given the resistance shown by this isolate towards both IVM and LEV, it is noteworthy that two of the genes that responded to drug exposure (*pgp-1*, and *pgp-9.1*) were also shown to be constitutively over-expressed in this isolate compared to the susceptible Kirby isolate (Fig. 2). Surprisingly, the increases in transcript levels following IVM treatment were greater at the lower IVM concentration (0.2 µg/mL) than at the higher concentration (0.8 µg/mL) (compare Figs. 3C and 4C). There are two possible explanations for this. Firstly, as noted in Section 2.4, the lower IVM concentration did not affect migration of the resistant larvae, as reported by Raza et al. (2015), while the higher IVM concentration resulted in 12% inhibition of migration. Thus, the larvae exposed to this higher concentration may have been compromised to some degree in their fitness, resulting in a lower gene induction response compared to the larvae exposed to the lower concentration. Secondly, it may be possible that greater increases in transcription occurred with 0.8 µg/mL IVM at an earlier time than the 3 h time point examined in our experiments. Ardelli et al. (2009) showed that *glc-1* transcript increases in *C. elegans* were much more pronounced after exposure to 10 nM IVM compared to 2.5 nM IVM at the 0.5 h time point, while the pattern was reversed after 2.5 h of drug exposure, with the fold increases in transcription being much greater at the lower IVM concentration by that time.

The up-regulation response of the resistant larvae to drug exposure was quite short-lived. By the 6 h time point, gene transcription had returned to the same levels as shown by DMSO-treated controls. The only exception to this was the continued up-regulation of *pgp-11* after 6 h exposure to LEV in resistant L3. Previously, it was observed that more P-gp genes were upregulated after a short period of IVM treatment (0.5 h) compared to the number up-regulated after longer periods of treatment (1.5 and 2.5 h) in *C. elegans* (Ardelli and Prichard, 2013). These transient responses suggest that the worm acts to quickly increase transcription of transporter genes in response to drug exposure, and then returns transcription to basal levels, having initiated the process of synthesising transporter proteins.

The effects of exposure to IVM and LEV were very different for

**Table 1**

Response of third-stage larvae of the resistant isolate of *Haemonchus contortus* (Wallangarra) to ivermectin following pre-exposure for 3 or 6 h to IVM (0.2 µg/mL and 0.8 µg/mL) or LEV (0.4 µg/mL) in larval migration assays.

Pre-exposure	Ivermectin dose–response											
	3 h pre-exposure						6 h pre-exposure					
	IC <sub>50</sub>		Dose–response plateau				IC <sub>50</sub>		Dose–response plateau			
	Drug conc. <sup>a</sup> µg/mL	95% CI	Drug/DMSO <sup>a,b</sup>	Migration <sup>c</sup> (%)	95% CI	Drug/DMSO <sup>c,d</sup>	Drug conc. <sup>a</sup> µg/mL	95% CI	Drug/DMSO <sup>a,b</sup>	Migration <sup>c</sup> (%)	95% CI	Drug/DMSO <sup>c,d</sup>
DMSO	4.03	3.51–4.63	–	17.6	12.7–22.5	–	3.31	2.78–3.93	–	15.5	9.6–21.4	–
IVM 0.2 µg/mL	2.55*	2.11–3.07	0.63*	17.6	13.0–22.0	1	2.00*	1.56–2.55	0.60*	18.0	12.6–23.5	1.2
IVM 0.8 µg/mL	3.04	2.21–4.19	0.75	48.1*	42.6–53.7	2.7*	2.94	2.49–3.47	0.89	25.4*	21.5–29.2	1.6*
LEV 0.4 µg/mL	2.31*	1.79–2.99	0.57*	30.7*	24.7–36.5	1.7*	2.21	1.44–3.40	0.67	31.3*	22.9–39.7	2.0*

<sup>a</sup> Within either the 3 or 6 h pre-exposure data sets: \* denotes that the IC<sub>50</sub> following pre-exposure to anthelmintic was significantly lower than the IC<sub>50</sub> following pre-exposure to DMSO, as determined by non-overlap of 95% Confidence Intervals.

<sup>b</sup> Drug/DMSO = IC<sub>50</sub> for IVM following pre-exposure to anthelmintics/IC<sub>50</sub> for IVM following pre-exposure to DMSO.

<sup>c</sup> Within either the 3 or 6 h pre-exposure data sets: \* denotes that L3 migration (%) at the dose–response plateau following pre-exposure to anthelmintic was significantly higher than L3 migration (%) at the plateau following pre-exposure to DMSO, as determined by non-overlap of 95% Confidence Intervals.

<sup>d</sup> Drug/DMSO = L3 migration (%) at the dose–response plateau for IVM following pre-exposure to anthelmintics/L3 migration (%) at the plateau for IVM following pre-exposure to DMSO.

**Table 2**  
Response of third-stage larvae of the resistant isolate of *Haemonchus contortus* (Wallangarra) to levamisole following pre-exposure for 3 or 6 h to IVM (0.2 µg/mL and 0.8 µg/mL) or LEV (0.4 µg/mL) in larval migration assays.

Pre-exposure	Levamisole dose–response					
	3 h pre-exposure			6 h pre-exposure		
	IC <sub>50</sub> <sup>a</sup>	95% C.I.	Drug/DMSO <sup>a,b</sup>	IC <sub>50</sub> <sup>a</sup>	95% C.I.	Drug/DMSO <sup>a,b</sup>
DMSO	0.95	0.81–1.11	–	0.83	0.71–0.98	–
IVM 0.2 µg/mL	0.47*	0.39–0.55	0.49*	0.36*	0.31–0.42	0.43*
IVM 0.8 µg/mL	0.31*	0.25–0.37	0.33*	0.36*	0.31–0.42	0.43*
LEV 0.4 µg/mL	0.27*	0.22–0.32	0.28*	0.27*	0.23–0.32	0.33*

<sup>a</sup> Within either the 3 or 6 h pre-exposure data sets: \* denotes that the IC<sub>50</sub> following pre-exposure to anthelmintic was significantly lower than the IC<sub>50</sub> following pre-exposure to DMSO, as determined by non-overlap of 95% Confidence Intervals.

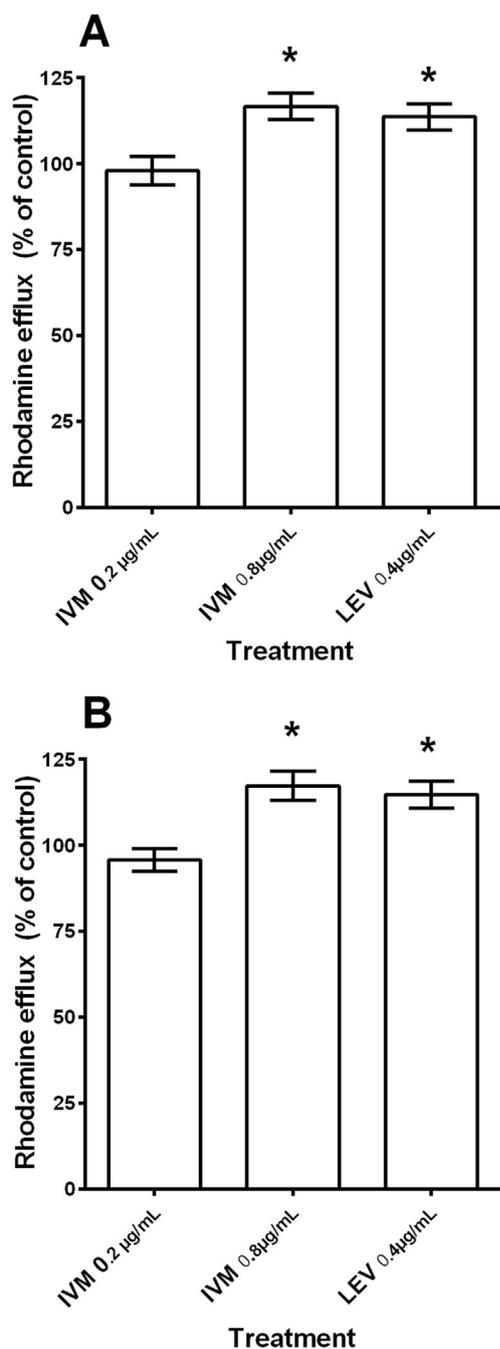
<sup>b</sup> Drug/DMSO = IC<sub>50</sub> for LEV following pre-exposure to anthelmintics/IC<sub>50</sub> for LEV following pre-exposure to DMSO.

susceptible compared to resistant L<sub>3</sub>. The former showed no changes in transcription for any of the transporter genes at either the 3 or 6 h time points. Of note however was the marked variability between replicate experiments for the susceptible L<sub>3</sub> that resulted in large SE bars, particularly at the 6 h time point. Hence, although there were no significant changes in transcription patterns across the three replicate experiments, the non-significant increases in mean gene transcription values for many of the transporters at the 6 h time point in the susceptible isolate, alongside the greater variability at 6 h compared to the resistant isolate, suggests that some up-regulation may have been occurring in one or more of our separate experiments. It remains possible that the transcriptional response observed at 3 h with resistant L<sub>3</sub> is delayed in susceptible L<sub>3</sub> such that the 6 h time point represents the early stages of transcriptional response in this isolate. However, this is speculative, and would require an examination of transcription patterns at later time points to confirm. Another possible explanation for the observed variation between replicate experiments for the susceptible isolate might be the slightly toxic effects of the drugs under the experimental conditions. While the lower concentration of IVM and LEV showed about 12% inhibition of larval migration (from Raza et al., 2015), the higher concentration of IVM (0.8 µg/mL) inhibited larval migration by approximately 25%. Hence, the larvae exposed to IVM at these concentrations may have shown a degree of variability in their fitness at the time of sampling for transcription measurements.

We examined the effects of exposure to IVM and LEV on the rate of R-123 efflux by the L<sub>3</sub>. Efflux was increased following exposure to the higher concentration of IVM (0.8 µg/mL) and LEV in both resistant and susceptible L<sub>3</sub> despite the fact that the gene up-regulation effects had only been observed with resistant L<sub>3</sub>. There were no effects on R-123 efflux in both the isolates following exposure to the lower concentration of IVM (0.2 µg/mL) (Fig. 8), in contrast to the effect of this drug treatment in increasing the transcription levels of a number of transporter genes in resistant L<sub>3</sub> (Fig. 3C). These differences in the two measurements may be explained by consideration of two aspects of the effects of exposure to xenobiotics on ABC transporters, at the gene and protein levels: firstly, at the gene level, the effects of drug exposure in increasing transcription of transporter genes resulting in the generation of an increased number of efflux proteins (Seelig, 1998), and, secondly, the direct effect of xenobiotics in stimulating the activity of pre-existing transporter proteins (Kerboeuf and Guegnard, 2011). This latter study reported an increase in R-123 transport within a few minutes after addition of anthelmintics in worm eggs. The authors suggested that this was not compatible with up-regulation of P-gp genes in such a short period, but rather was most likely due to increased activity of transporter proteins present in the organism at the start of the drug exposure period. As described above, our gene transcription and R-123 experiments show a number of differences

in the responses of the two worm isolates to the two drugs at the different time points. This indicates that the effects of drug exposure on gene transcription and on R-123 efflux are not directly correlated, thus suggesting that the increases in R-123 efflux measured under our experimental conditions were at least partly due to increases in the activity of existing transporter proteins, as observed by Kerboeuf and Guegnard (2011), rather than as a consequence of increased transcription.

We also studied the consequences of exposure to low levels of IVM and LEV in terms of whether such exposure equipped the L<sub>3</sub> with an ability to survive subsequent exposure to higher levels of these same anthelmintics in migration assays. The assays were performed using short incubation/migration periods (30 min for both) compared to the 48 and 24 h periods usually used for this assay (Kotze et al., 2006) in order to avoid any effects of the drugs in possibly inducing transcription of the transporter genes during such long incubation periods. The dose response curves showed the presence of a plateau at the highest IVM concentrations. This was most likely due to the short nature of both the drug incubation and migration phases of the assay, and the subsequent inability to inhibit migration completely in all larvae in this short time frame. Such plateaus are not observed using standard longer time periods (24–48 h) (Kotze et al., 2006; Raza et al., 2015). The presence of this plateau allowed us to describe the response of the larvae to IVM in terms of both the % migration at the plateau (the % of the population able to migrate at the high IVM concentrations), as well as the IC<sub>50</sub> for the remainder of the population. We found that resistant L<sub>3</sub> pre-exposed to IVM (at 0.8 µg/mL) or LEV showed an increased tolerance to subsequent treatment with IVM in a proportion of the population, as indicated by an increase in the percentage of the population able to migrate at the highest drug concentrations. This apparent tolerance to the drug only occurred in the proportion of the population represented by the dose response plateau, as the IC<sub>50</sub> in the remainder of the population was either unchanged or decreased compared to L<sub>3</sub> pre-treated with DMSO alone (Table 1). On the other hand there was no increase in this dose response plateau at the lower IVM concentration (0.2 µg/mL) even though this pre-treatment had resulted in greater fold increases in transporter gene transcription. This contradiction may be at least partly explained by examining the drug exposure data alongside both the gene transcription and R-123 efflux data. Exposure to the higher IVM concentration (0.8 µg/mL) and LEV resulted in increases in both gene transcription and R-123 efflux, alongside an increased ability to tolerate subsequent IVM exposure in a proportion of the worm population (higher dose response plateau). On the other hand, exposure to the lower IVM concentration resulted in increased transcription levels, but no change in R-123 efflux, alongside no increased drug tolerance. This suggests that, under our experimental conditions, both the transcription and protein activity responses are required in order to equip a proportion of the



**Fig. 8.** The effects of IVM (0.2 and 0.8 µg/mL) and LEV (0.4 µg/mL) pre-exposure for 3 h on R-123 efflux in third-stage larvae of susceptible (A) and resistant (B) isolates of *Haemonchus contortus*. R-123 efflux is expressed relative to controls (DMSO-treated). Significant differences in R-123 efflux compared to controls, within each isolate, are indicated by \* ( $P < 0.05$ ). Data shown as mean  $\pm$  SE,  $n = 3$  separate experiments, each with assays in duplicate.

L<sub>3</sub> population with an ability to tolerate subsequent drug exposure. Increases in transcription alone, in the absence of increased activity of existing transporter proteins, as was observed with IVM at the lower concentration, may not provide protection against drugs to a degree measurable in our migration assays. We did not assess the effects of exposure to IVM at 0.8 µg/mL on the subsequent ability of susceptible L<sub>3</sub> to the drug as exposure to this drug concentration was previously shown to reduce migration by 25% (Raza et al., 2015), hence making it difficult to accurately measure the specific

effects of subsequent drug exposure. The lack of drug tolerance observed in susceptible L<sub>3</sub> pre-exposed to IVM at 0.2 µg/mL (Fig. 7A) would be expected given the lack of any increases in both gene transcription and R-123 efflux (Figs. 3, 4, 5 and 8).

In contrast to the protective effects towards IVM of drug pre-exposure in a proportion of the larval population for the resistant isolate, as described above in terms of increases in the dose response plateau, there were a number of instances of either no change or a decrease in the IVM IC<sub>50</sub> of the remainder of the population. An increase in sensitivity to LEV following pre-treatment with IVM or LEV was also observed in most cases. In the absence of any ABC transporter-mediated protection for at least a portion of the population, as suggested above for IVM, a decreased tolerance to a second drug exposure may be expected due to the cumulative effects of sequential drug treatments. This may be particularly so for sequential treatments with IVM and LEV given that macrocyclic lactones have been reported to interact with nAChRs in vertebrates, arthropods and nematodes (Krause et al., 1998; Raymond et al., 2000; Carmichael et al., 2013; Abongwa et al., 2016). Hence, the increased sensitivity of larvae to IVM or LEV following an initial period of drug exposure to one of these drugs, compared to controls exposed to the second drug alone, may be expected. It is the reversal of this expected outcome, as observed in the cases of increased tolerance to IVM as the second drug (Fig. 6A and B, Table 1), that suggests the influence of a protective pathway allowing the worms to show a degree of tolerance towards the second drug. As mentioned above, tolerance to IVM in a component of the larval population of the resistant isolate was accompanied by either no change or an increase in sensitivity in the remainder of the population. This suggests a degree of heterogeneity in the response of the larvae to the drug pre-treatment. If, as we suggested above, increased transporter activity is at least partly responsible for the increased tolerance seen at the dose response plateau, then the unchanged or decreased IVM IC<sub>50</sub> values observed in the other component of the worm population suggests that the gene expression and R-123 efflux increases may also be quite heterogeneous across the worm population.

The difference in the responses of the resistant isolate to IVM and LEV in the migration assay phase of these experiments (some increased tolerance for IVM, none for LEV) suggests that, despite the fact that pre-exposure to both drugs clearly leads to approximately equivalent up-regulation of a number of transporter genes, and equivalent increases in R-123 efflux, the induced transporters are able to subsequently provide protection only against IVM and not LEV. This may suggest that IVM is a better substrate for the *H. contortus* P-gs than LEV as reported earlier for mammalian P-gps (Efferth and Volm, 1993) and, hence, the induced P-gps are more effective in preventing the IVM interacting with its target site compared to LEV.

In conclusion, the present study provides further evidence that P-gps play a role in the interaction of anthelmintics with *H. contortus*. We have shown that there is a great deal of variation in the relative transcription levels of the different transporter genes in this worm species, thereby suggesting an existence of variation in their specific roles in protecting the worms from a range of xenobiotics. The constitutive over-expression of *pgp-1*, *pgp-9.1* and *pgp-9.2* in Wallangra L<sub>3</sub> suggests that these specific P-gps may play a role in the anthelmintic resistance shown by this isolate. Furthermore, the increased transcription of some ABC transporters following exposure to anthelmintics (IVM and LEV) indicates that these anthelmintics interact with a number of specific *H. contortus* ABC transporters and hence are likely to be the substrates for these transporters. The presence of significant levels of increased transcription following 3 h exposure to both IVM and LEV in the resistant isolate only, as well as stimulation of the existing

transport proteins, alongside the subsequent ability of a greater proportion of the worm population to tolerate high IVM concentrations in larval migration assays, suggest that the ability to rapidly up-regulate protective pathways in response to drugs may be a component of the drug resistance displayed by this isolate, and possibly by other drug-resistant nematodes. The variability seen in the effects of pre-treatment with the two drugs on the sensitivity of larvae in subsequent migration assays, with instances of increased tolerance as well as increased sensitivity within the various treatment groups, and even within single experimental populations, suggests the presence of some heterogeneity in the observed responses to drug exposure in the resistant isolate. This indicates that simple measurements of ABC transporter gene transcription levels and efflux activities in preparations from whole larvae are not necessarily indicative of the ability of transporter pathways to protect worms from anthelmintics.

### Conflicts of interest

The authors have no conflicts of interest concerning the work reported in this paper.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ijpddr.2016.03.001>.

### References

- Abongwa, M., Buxton, S.K., Robertson, A.P., Martin, R.J., 2016. Curiouser and curiouser: the macrocyclic lactone, abamectin, is also a potent inhibitor of pyrantel/tribendimidine nicotinic acetylcholine receptors of gastro-intestinal worms. *PLoS One* 11, e0146854.
- Albers, G.A., Burgess, S.K., 1988. Serial passage of *Haemonchus contortus* in resistant and susceptible sheep. *Vet. Parasitol.* 28, 303–306.
- Ardelli, B.F., Prichard, R.K., 2013. Inhibition of P-glycoprotein enhances sensitivity of *Caenorhabditis elegans* to ivermectin. *Vet. Parasitol.* 191, 264–275.
- Ardelli, B.F., Stitt, L.E., Tompkins, J.B., 2010. Inventory and analysis of ATP-binding cassette (ABC) systems in *Brugia malayi*. *Parasitol* 137, 1195–1212.
- Ardelli, B.F., Stitt, L.E., Tompkins, J.B., Prichard, R.K., 2009. A comparison of the effects of ivermectin and moxidectin on the nematode *Caenorhabditis elegans*. *Veterinary Parasitol.* 165, 96–108.
- Areskog, M., Engström, A., Tallkvist, J., Samson-Himmelstjerna, G., Höglund, J., 2013. PGP expression in *Cooperia oncophora* before and after ivermectin selection. *Parasitol. Res.* 112, 3005–3012.
- Bartley, D.J., McAllister, H., Bartley, Y., Dupuy, J., Menez, C., Alvinerie, M., Jackson, F., Lespine, A., 2009. P-glycoprotein interfering agents potentiate ivermectin susceptibility in ivermectin sensitive and resistant isolates of *Teladorsagia circumcincta* and *Haemonchus contortus*. *Parasitol* 136, 1081–1088.
- Broehan, G., Kroeger, T., Lorenzen, M., Merzendorfer, H., 2013. Functional analysis of the ATP-binding cassette (ABC) transporter gene family of *Tribolium castaneum*. *BMC Genomics* 14, 6.
- Broeks, A., Janssen, H.W., Calafat, J., Plasterk, R.H., 1995. A P-glycoprotein protects *Caenorhabditis elegans* against natural toxins. *EMBO J.* 14, 1858–1866.
- Bygarski, E.E., Prichard, R.K., Ardelli, B.F., 2014. Resistance to the macrocyclic lactone moxidectin is mediated in part by membrane transporter P-glycoproteins: implications for control of drug resistant parasitic nematodes. *Int. J. Parasitol. Drugs Drug Resist* 4, 143–151.
- Carmichael, S.N., Bron, J.E., Taggart, J.B., Ireland, J.H., Bekaert, M., Burgess, S.T., Skuce, P.J., Nisbet, A.J., Gharbi, K., Sturm, A., 2013. Salmon lice (*Lepeophtheirus salmonis*) showing varying emamectin benzoate susceptibility differ in neuronal acetylcholine receptor and GABA-gated chloride channel mRNA expression. *BMC Genomics* 14, 408.
- De Graef, J., Demeler, J., Skuce, P., Mitreva, M., Von Samson-Himmelstjerna, G., Vercruyse, J., Claerebout, E., Geldhof, P., 2013. Gene expression analysis of ABC transporters in a resistant *Cooperia oncophora* isolate following *in vivo* and *in vitro* exposure to macrocyclic lactones. *Parasitol* 140, 499–508.
- Dermauw, W., Van Leeuwen, T., 2014. The ABC gene family in arthropods: comparative genomics and role in insecticide transport and resistance. *Insect biochem. Mol. Biol.* 45, 89–110.
- Dicker, A.J., Nisbet, A.J., Skuce, P.J., 2011. Gene expression changes in a P-glycoprotein (Tci-ppg-9) putatively associated with ivermectin resistance in *Teladorsagia circumcincta*. *Int. J. Parasitol.* 41, 935–942.
- Drogemüller, M., Schnieder, T., von Samson-Himmelstjerna, G., 2004. Evidence of p-glycoprotein sequence diversity in cyathostomins. *J. Parasitol.* 90, 998–1003.
- Efferth, T., Volm, M., 1993. Reversal of doxorubicin-resistance in sarcoma 180 tumor cells by inhibition of different resistance mechanisms. *Cancer Lett.* 70, 197–202.
- Heckler, R.P., Almeida, G.D., Santos, L.B., Borges, D.G., Neves, J.P., Onizuka, M.K., Borges, F.A., 2014. P-gp modulating drugs greatly potentiate the *in vitro* effect of ivermectin against resistant larvae of *Haemonchus placei*. *Vet. Parasitol.* 205, 638–645.
- Issouf, M., Guegnard, F., Koch, C., Le Vern, Y., Blanchard-Letort, A., Che, H., Beech, R.N., Kerboeuf, D., Neveu, C., 2014. *Haemonchus contortus* P-glycoproteins interact with host eosinophil granules: a novel insight into the role of ABC transporters in host-parasite interaction. *PLoS One* 9, e87802.
- James, C.E., Davey, M.W., 2009. Increased expression of ABC transport proteins is associated with ivermectin resistance in the model nematode *Caenorhabditis elegans*. *Int. J. Parasitol.* 39, 213–220.
- Janssen, I.J., Krucken, J., Demeler, J., Basiaga, M., Kornas, S., von Samson-Himmelstjerna, G., 2013. Genetic variants and increased expression of *Parascaris equorum* P-glycoprotein-11 in populations with decreased ivermectin susceptibility. *PLoS One* 8, e61635.
- Janssen, I.J., Krucken, J., Demeler, J., von Samson-Himmelstjerna, G., 2015. Transgenically expressed *Parascaris* P-glycoprotein-11 can modulate ivermectin susceptibility in *Caenorhabditis elegans*. *Int. J. Parasitol. Drugs Drug Resist* 5, 44–47.
- Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. *Trends Parasitol.* 20, 477–481.
- Kaplan, R.M., Vidyashankar, A.N., 2012. An inconvenient truth: global worming and anthelmintic resistance. *Vet. Parasitol.* 186, 70–78.
- Kaschny, M., Demeler, J., Janssen, I.J., Kuzmina, T.A., Besognet, B., Kanellos, T., Kerboeuf, D., von Samson-Himmelstjerna, G., Krucken, J., 2015. Macrocyclic lactones differ in interaction with recombinant P-Glycoprotein 9 of the parasitic nematode *Cylicocyclus elongatus* and ketoconazole in a yeast growth assay. *PLoS Pathog.* 11, e1004781.
- Kerboeuf, D., Guegnard, F., 2011. Anthelmintics are substrates and activators of nematode P-glycoprotein. *Antimicrob. Agents Chemother.* 55, 2224–2232.
- Kotze, A.C., Le Jambre, L.F., O'Grady, J., 2006. A modified larval migration assay for detection of resistance to macrocyclic lactones in *Haemonchus contortus*, and drug screening with *Trichostrongylidae* parasites. *Vet. Parasitol.* 137, 294–305.
- Krause, R.M., Buisson, B., Bertrand, S., Corringier, P.J., Galzi, J.L., Changeux, J.P., Bertrand, D., 1998. Ivermectin: a positive allosteric effector of the alpha7 neuronal nicotinic acetylcholine receptor. *Mol. Pharmacol.* 53, 283–294.
- Laing, R., Kikuchi, T., Martinelli, A., Tsai, I.J., Beech, R.N., Redman, E., Holroyd, N., Bartley, D.J., Beasley, H., Britton, C., Curran, D., Devaney, E., Gilbert, A., Hunt, M., Jackson, F., Johnston, S.L., Kryukov, I., Li, K., Morrison, A.A., Reid, A.J., Sargison, N., Saunders, G.I., Wasmuth, J.D., Wolstenholme, A., Berriman, M., Gilleard, J.S., Cotton, J.A., 2013. The genome and transcriptome of *Haemonchus contortus*, a key model parasite for drug and vaccine discovery. *Genome Biol.* 14, R88.
- Lespine, A., Ménez, C., Bourguinat, C., Prichard, R.K., 2012. P-glycoproteins and other multidrug resistance transporters in the pharmacology of anthelmintics: prospects for reversing transport-dependent anthelmintic resistance. *Int. J. Parasitol. Drugs Drug Resist* 2, 58–75.
- Lloberas, M., Alvarez, L., Entrocasso, C., Virkel, G., Ballent, M., Mate, L., Lanusse, C., Lifschitz, A., 2013. Comparative tissue pharmacokinetics and efficacy of moxidectin, abamectin and ivermectin in lambs infected with resistant nematodes: impact of drug treatments on parasite P-glycoprotein expression. *Int. J. Parasitol. Drugs Drug Resist* 3, 20–27.
- Love, S.C., Neilson, F.J., Biddle, A.J., McKinnon, R., 2003. Moxidectin-resistant *Haemonchus contortus* in sheep in northern New South Wales. *Aust. Vet. J.* 81, 359–360.
- Menez, C., Sutra, J.F., Prichard, R., Lespine, A., 2012. Relative neurotoxicity of ivermectin and moxidectin in Mdr1ab (-/-) mice and effects on mammalian GABA(A) channel activity. *PLoS Negl. Trop. Dis.* 6, e1883.
- Prichard, R.K., Roulet, A., 2007. ABC transporters and beta-tubulin in macrocyclic lactone resistance: prospects for marker development. *Parasitol* 134, 1123–1132.
- Raymond, V., Mongan, N.P., Sattelle, D.B., 2000. Anthelmintic actions on homomer-forming nicotinic acetylcholine receptor subunits: chicken alpha7 and ACR-16 from the nematode *Caenorhabditis elegans*. *Neuroscience* 101, 785–791.
- Raza, A., Kopp, S.R., Jabbar, A., Kotze, A.C., 2015. Effects of third generation P-glycoprotein inhibitors on the sensitivity of drug-resistant and -susceptible isolates of *Haemonchus contortus* to anthelmintics *in vitro*. *Vet. Parasitol.* 211, 80–88.
- Sarai, R.S., Kopp, S.R., Coleman, G.T., Kotze, A.C., 2013. Acetylcholine receptor subunit and P-glycoprotein transcription patterns in levamisole-susceptible and -resistant *Haemonchus contortus*. *Int. J. Parasitol. Drugs Drug Resist* 3, 51–58.
- Sarai, R.S., Kopp, S.R., Coleman, G.T., Kotze, A.C., 2014. Drug-efflux and target-site gene expression patterns in *Haemonchus contortus* larvae able to survive increasing concentrations of levamisole *in vitro*. *Int. J. Parasitol. Drugs Drug Resist* 4, 77–84.
- Schrenk, D., Baus, P.R., Ermel, N., Klein, C., Vorderstemann, B., Kauffmann, H.M., 2001. Up-regulation of transporters of the MRP family by drugs and toxins.

- Toxicol. Lett. 120, 51–57.
- Seelig, A., 1998. A general pattern for substrate recognition by P-glycoprotein. *Eur. J. Biochem.* 251, 252–261.
- Sheps, J.A., Ralph, S., Zhao, Z., Baillie, D.L., Ling, V., 2004. The ABC transporter gene family of *Caenorhabditis elegans* has implications for the evolutionary dynamics of multidrug resistance in eukaryotes. *Genome Biol.* 5, R15.
- Sutherland, I.A., Leathwick, D.M., 2011. Anthelmintic resistance in nematode parasites of cattle: a global issue? *Trends Parasitol.* 27, 176–181.
- Tydén, E., Skarin, M., Höglund, J., 2014. Gene expression of ABC transporters in *Cooperia oncophora* after field and laboratory selection with macrocyclic lactones. *Mol. Biochem. Parasitol.* 198, 66–70.
- Williamson, S.M., Storey, B., Howell, S., Harper, K.M., Kaplan, R.M., Wolstenholme, A.J., 2011. Candidate anthelmintic resistance-associated gene expression and sequence polymorphisms in a triple-resistant field isolate of *Haemonchus contortus*. *Mol. Biochem. Parasitol.* 180, 99–105.
- Williamson, S.M., Wolstenholme, A.J., 2012. P-glycoproteins of *Haemonchus contortus*: development of real-time PCR assays for gene expression studies. *J. Helminthol.* 86, 202–208.
- Yan, R., Urdaneta-Marquez, L., Keller, K., James, C.E., Davey, M.W., Prichard, R.K., 2012. The role of several ABC transporter genes in ivermectin resistance in *Caenorhabditis elegans*. *Vet. Parasitol.* 190, 519–529.
- Yu, M., Ocana, A., Tannock, I., 2013. Reversal of ATP-binding cassette drug transporter activity to modulate chemoresistance: why has it failed to provide clinical benefit? *Cancer Metastasis Rev.* 32, 211–227.