

# Gene expression analysis of ABC transporters in a resistant *Cooperia oncophora* isolate following *in vivo* and *in vitro* exposure to macrocyclic lactones

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(Received 20 July 2012; revised 26 September 2012; accepted 27 September 2012; first published online 2 January 2013)

## SUMMARY

Members of the ATP-binding cassette (ABC) transporter family (P-glycoproteins, Half-transporters and Multidrug Resistant Proteins) potentially play a role in the development of anthelmintic resistance. The aim of this study was to investigate the possible involvement of ABC transporters in anthelmintic resistance in the bovine parasite, *Cooperia oncophora*. Partial sequences of 15 members of the ABC transporter protein family were identified, by mining a transcriptome dataset combined with a degenerate PCR approach. Reverse transcriptase PCR showed that most of the ABC transporters identified were constitutively transcribed throughout the life cycle of *C. oncophora*. Constitutive differences in gene transcript levels between a susceptible and resistant isolate were only observed for *Con-haf-9* and *Con-mrp-1* in eggs of the resistant isolate, while no differences were observed in L3 or the adult life stage. Analysis of resistant adult worms, collected from calves 14 days after treatment with either ivermectin or moxidectin, showed a significant 3- to 5-fold increase in the transcript levels of *Con-pgp-11* compared to non-exposed worms. Interestingly, a 4-fold transcriptional up-regulation of *Con-pgp-11* was also observed in L3 of the resistant isolate, after *in vitro* exposure to different concentrations of ivermectin, whereas this effect was not observed in exposed L3 of the susceptible isolate. The results suggest that the worms of this particular resistant isolate have acquired the ability to up-regulate *Con-pgp-11* upon exposure to macrocyclic lactones. Further work is needed to understand the genetic basis underpinning this process and the functional role of PGP-11.

Key words: *Cooperia oncophora*, ivermectin resistance, ABC transporters, PGP, HAF, MRP.

## INTRODUCTION

*Cooperia oncophora* is a nematode species parasitizing the small intestines of cattle. In temperate climatic regions, co-infections of *C. oncophora* species with other gastrointestinal parasites such as *Ostertagia ostertagi* cause important production losses. The control of these helminth infections relies heavily on the use of broad-spectrum anthelmintics such as the macrocyclic lactones (MLs), benzimidazoles (BZs) and levamisole (LEV). However, the intensive and continuous administration of such products have led to the development of resistance in livestock parasites against all anthelmintic classes (Kaplan, 2004).

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The majority of the reports on ML-resistance in cattle nematodes involve *C. oncophora* (Coles *et al.* 2001; Demeler *et al.* 2009; Edmonds *et al.* 2010; El-Abdellati *et al.* 2010a, b). Although the molecular mechanisms underlying the development of ML-resistance in helminth parasites remain elusive, the members of the ATP-binding cassette (ABC) transporter family, such as P-glycoproteins (PGPs), Half-transporters (HAFs) and Multidrug Resistant Proteins (MRPs), are thought to play an important role, since they are suspected to affect the absorption, distribution and elimination of xenobiotics inside these worms. A large number of ABC transporter genes has been identified in nematodes. In the free-living model nematode, *Caenorhabditis elegans*, 15 *pgp* genes, 9 *haf* genes and 8 *mrp* genes have been identified (Zhao *et al.* 2007; Lespine *et al.* 2008). *Pgp* expression has been observed in all developmental life stages of *C. elegans* (Lincke *et al.* 1993), in

particular in the intestinal cells, but also in the pharynx, the excretory cells and the chemosensory AWA neurons in the head (Lincke *et al.* 1992, 1993; Broeks *et al.* 1995; Nunes *et al.* 2005). Functional analyses of *mrp-1* and *pgp-1* in *C. elegans* describe a protective role against the heavy metal ions, cadmium and arsenite (Broeks *et al.* 1996). *Cel-pgp-2* has a function in the biogenesis of a lysosome-related fat storage organelle (Schroeder *et al.* 2007) and *Cel-pgp-3* is important in defence against the natural toxins, colchicine and chloroquine (Broeks *et al.* 1995). In parasitic nematode species, the number of identified ABC transporter genes is still expanding. So far, 9 *pgp* genes, 1 *haf* gene and 2 *mrp* genes have been described in *Haemonchus contortus* (Lespine *et al.* 2008; Williamson *et al.* 2011) and 11 partial *pgp* sequences were recently identified in *Teladorsagia circumcincta* (Dicker *et al.* 2011b). In the human filarial worms, 8 *pgp* genes, 8 *haf* genes and 5 *mrp* genes are reported for *Brugia malayi* (Ardelli *et al.* 2010) and, for *Onchocerca volvulus*, 4 *pgp* genes and 3 *haf* genes have been described (Huang and Prichard, 1999; Ardelli *et al.* 2005, 2006; Bourguinat *et al.* 2008; Lespine *et al.* 2012). The extent to which the biological role of ABC transporters is conserved between nematode species is still unclear.

In the first reports that associated ML-resistance in nematodes with ABC transporters, higher *pgp* expression levels or changes in allelic diversity were documented in resistant *H. contortus* worms (Blackhall *et al.* 1998; Xu *et al.* 1998). More recently, a constitutive up-regulation of *Hcon-pgp-2* and *Hcon-pgp-9* was observed in a triple-resistant *H. contortus* isolate compared to a susceptible isolate (Williamson *et al.* 2011). In *T. circumcincta*, constitutive differences in gene expression between a susceptible and a triple-resistant isolate were most notable in *Tci-pgp-9*, which was up-regulated in all life-cycle stages of the resistant isolate. Also, high levels of polymorphisms in the partial *Tci-pgp-9* nucleotide sequence were identified between the isolates (Dicker *et al.* 2011b). The involvement of ABC transporters in the mechanism of ML resistance was recently also shown in *C. elegans*. In these studies, resistant isolates, either generated by IVM-receptor knock-down (*glc-1/avr-14/avr-15* triple mutant) or through step-wise exposure to non-lethal doses of ivermectin (IVM), were *in vitro* cultured with IVM or moxidectin (MOX). Ardelli and Prichard (2008) observed that IVM and MOX induced similar expression profiles with a marked overexpression of *mrp-3*, *mrp-5*, *mrp-7* and *mrp-8*. More recently Yan *et al.* (2012) described an IVM-induced up-regulation of *pgp-1*, *pgp-2*, *pgp-4*, *pgp-12*, *pgp-14*, *mrp-1*, *mrp-2*, *mrp-4*, *mrp-5*, *mrp-6*, *mrp-7*, *haf-1*, *haf-2* and *haf-3*. Additionally, the role of PGPs, HAFs and MRPs in protecting *C. elegans* from anthelmintic toxicity was investigated in mutant strains (through deletion mutations or RNAi) exposed to IVM. *Cel-mrp-3*,

*Cel-mrp-4* and *Cel-mrp-8* may play a role in protecting the worm from paralysis induced by IVM (Ardelli and Prichard, 2008), while knock-down of *Cel-mrp-1* and *Cel-pgp-2* appeared to have the greatest effects in terms of reduced pharyngeal pumping and/or egg production and motility in response to IVM (Yan *et al.* 2012).

Despite the fact that resistance is widespread in *C. oncophora*, no reports have yet been published on the potential role of ABC transporters in the development of anthelmintic resistance in this species. For this reason, the aim of the present study was to identify members of the ABC transporter family in *C. oncophora* and subsequently to investigate their possible involvement in the resistance mechanism by analysing constitutive and inducible changes in gene transcription levels between a susceptible and an IVM-resistant field isolate.

## MATERIALS AND METHODS

### Parasite material

The anthelmintic-susceptible *C. oncophora* isolate (CoIVS<sub>Sus</sub>) is a lab-maintained isolate, which has never been exposed to drug treatment (El-Abdellati *et al.* 2010b, 2011). The IVM-resistant *C. oncophora* isolate (CoIVR08) was collected from a Belgian farm in 2008 (El-Abdellati *et al.* 2010b). Both isolates are maintained in the laboratory by regular passages, without treatment, through helminth-free calves. For the collection of L1, *C. oncophora* eggs were purified from faecal samples (Coles *et al.* 1992) and incubated at 28 °C in deionized water. Sixteen hours later, the hatched L1 were placed on a Baermann apparatus with tap water and collected the next morning. For L2 and L3, coprocultures were incubated for 72 h or 14 days, respectively, at 25 °C and then placed on a Baermann apparatus. L4 and adult worms were recovered live at necropsy, 8 and 21 days post-infection, respectively. The washings of the small intestines were poured over a 116 µm sieve, worms were retained and then placed on a Baermann apparatus.

*In vivo*-exposed resistant adult worms were recovered live at necropsy, 14 days after subcutaneous treatment with IVM or MOX (0.2 mg/kg body-weight). The controlled efficacy test revealed a 38% and 31% reduction in worm burden, respectively for IVM and MOX (De Graef *et al.* 2012).

To obtain *in vitro*-exposed *C. oncophora* L3, fresh larvae were harvested from coprocultures and exsheathed in 0.5% sodium hypochloride (Demeler *et al.* 2010). Third stage larvae were then incubated at 28 °C in deionized water or in 10<sup>-8</sup>M IVM (8.7 ng/ml IVM) or 10<sup>-7</sup>M IVM (87 ng/ml IVM). A stock solution of 10<sup>-2</sup>M IVM was first prepared in 100% dimethyl sulphoxide (DMSO), while the final dilutions were made in water. After 24 h, the larvae were transferred onto 28 µm sieves suspended in rows

of a 24-well plate. Two hours later, the sieves were carefully lifted out of the rows, the migrated larvae were collected from the wells and washed 3 times with deionized water. Pools of larvae were stored at  $-80^{\circ}\text{C}$  until required.

#### RNA extraction and cDNA synthesis

Total RNA samples were extracted from all *C. oncophora* life stages, by grinding the parasites with 0.2 ml glass homogenizers (Wheaton), followed by a Trizol<sup>®</sup> extraction (Invitrogen). Residual genomic DNA was removed by DNase I treatment (Roche). The RNA quality was verified with the Experion<sup>™</sup> RNA StdSens Starter kit (Bio-Rad) and the RNA concentration was determined using a Nanodrop<sup>®</sup> ND-1000 spectrophotometer (NanoDrop Technologies). cDNA was synthesized from 1  $\mu\text{g}$  total RNA by random priming using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's recommendations.

#### Identification of *C. oncophora* ABC transporter genes

An expressed sequence tag (EST) dataset, generated by 454 sequencing, of *C. oncophora* worms and available on nematode.net v3.0 (PMID: 22139919) (Martin *et al.* 2012) was analysed for members of the ABC transporter protein family by sequential TBLASTN searches using the protein sequences of known *C. elegans* ABC transporter genes. Isotig numbers and sequence information of the identified *C. oncophora* ABC transporter homologues were saved and gene-specific primers (listed in the Supplementary Table 1, online version only) were designed using the online Primer3 software (<http://frodo.wi.mit.edu/primer3/>). PCR amplifications were carried out by combining 1  $\mu\text{l}$  of cDNA template (CoIVSsus adults) and 2.5  $\mu\text{l}$  each of the forward and reverse primer (10  $\mu\text{M}$ ) with the Promega PCR reagents. All reactions were run as follows: 2 min at  $95^{\circ}\text{C}$ , followed by 35 cycles of  $95^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 30 s, followed by a final extension at  $72^{\circ}\text{C}$  for 10 min and then held at  $10^{\circ}\text{C}$ . PCR products were visualized on a 1.5% agarose gel and stained with 0.5  $\mu\text{g}/\text{ml}$  ethidium bromide. Bands were excised and purified with the GeneClean kit<sup>®</sup> (MPBio). Purified PCR products were cloned using the pGEM<sup>®</sup>-T easy vector (Promega) and *Escherichia coli* DH5a competent cells (Stratagene) according to the manufacturer's protocols. Plasmid products were sequenced bidirectionally with SP6 and T7 vector primers. The sequences were analysed with DNASTAR software (Lasergene version 8). To assign the *C. oncophora* ABC transporter genes with the correct nomenclature, the partial protein sequences were blasted against the *C. elegans* protein database on

NEMBASE4 (PMID: 21550347) (Elsworth *et al.* 2011) and consensus sequences were blasted against the *Caenorhabditis* taxid, using BLASTx on NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

#### Degenerate PCR approach

*Con-pgp-2*, *Con-pgp-3*, *Con-pgp-12* and *Con-pgp-16* were obtained, using degenerated primers followed by RACE-PCR (Demeler *et al.* unpublished data). In addition, *Con-pgp-9* was isolated with degenerated primers (fw: 5'-TGCHTTGGACGGTCTGTK-GAA-3'; rv: 5'-AGWAGTAGGATYTTTGGAT-TYC-3'), based on sequence homology between the *C. elegans* *pgp-9* (GenBank ID: NM\_075086) and *T. circumcincta* *pgp-9* (provided by Dr. P. Skuce) sequences. The identified sequences were analysed as described above.

#### Reverse transcription PCR

To investigate the transcription pattern of the individual ABC transporter genes throughout the life cycle of *C. oncophora*, reverse transcription PCRs (SuperScript<sup>™</sup> One-Step RT-PCR with Platinum<sup>®</sup> Taq, Invitrogen) was carried out. PCR mixtures had final concentrations of 200 ng RNA template, 0.8  $\mu\text{M}$  of both forward and reverse primer, 1 unit of RT/Platinum<sup>®</sup> Taq Mix, in a reaction buffer containing 0.2 mM of each dNTP and 1.2 mM  $\text{MgCl}_2$ . PCR conditions were set as follows: 30 min at  $50^{\circ}\text{C}$ , 2 min at  $94^{\circ}\text{C}$ , followed by 40 cycles of denaturing (15 s at  $94^{\circ}\text{C}$ ), annealing (30 s at  $60^{\circ}\text{C}$ ) and elongation (12 s at  $72^{\circ}\text{C}$ ), followed by a final elongation step at  $72^{\circ}\text{C}$  for 10 min, after which the PCR mixtures were kept at  $10^{\circ}\text{C}$ . Glyceraldehyde-3-phosphate dehydrogenase (*Con-gapdh*) was included as an internal standard.





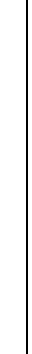


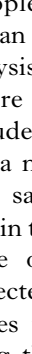
#### Quantitative Real-Time PCR

Quantitative real-time PCRs (qRT-PCRs) were performed to compare the constitutive (without drug exposure) and inducible (after exposure to IVM or MOX) transcriptional changes of ABC transporter genes between CoIVSsus and CoIVR08 parasite stages. For each biological sample, at least 2 independent RNA extractions were performed. Total RNA (1  $\mu\text{g}$ ) was converted to cDNA and diluted 1/5 or used undiluted for *Con-pgp-11*, *Con-pgp-12*, *Con-pgp-16* and *Con-mrp-7*. Real-time PCR reactions were prepared with the SYBR Green Master Mix (Applied Biosystems) using 6.4  $\mu\text{l}$  of  $\text{H}_2\text{O}$ , 0.8  $\mu\text{l}$  of each amplification primer (10  $\mu\text{M}$ ) and 2  $\mu\text{l}$  of cDNA to give a 20  $\mu\text{l}$  reaction volume. All amplification runs were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems), under the following conditions:  $95^{\circ}\text{C}$  for 20 s, followed by 40–50 cycles of  $95^{\circ}\text{C}$  for 5 s, optimal annealing temperature

Table 1. Sequence sizes, Accession numbers, blast analysis and reverse-transcription PCR results of the 15 partially identified *Cooperia oncophora* ABC-transporter genes to determine their correct annotation and to show the transcription pattern throughout the lice cycle of *C. oncophora*

Gene name	Transcriptome database <sup>a</sup>		Degenerate PCR		Accession number	2 best Nembase4 <sup>b</sup> BLAST <sup>p</sup> hits	e-value	Maximum identity	Transcription values throughout <i>C. oncophora</i> life cycle						
	Isogroup	Sequence size (bp)	Sequence size (bp)						E	L1	L2	L3	L4	♂	♀
<i>Con-pgp-1</i>	10197	129			HE855848	CE11932 WBGene00003995 locus:pgp-1 protein_id: CAB01232.1 CE31624 WBGene00004002 locus:pgp-8 protein_id: CAA94221.2	9,E-06 1,E-05	91% (22/24) 60% (26/43)							
<i>Con-pgp-2</i>	02710	612	3822 <sup>c</sup>		JX262229	CE29212 WBGene00003996 locus:pgp-2 protein_id: AAB52482.2 CE15714 WBGene00004003 locus:pgp-9 protein_id: CAB07855.1	0-0 0-0	69% (870/1258) 42% (543/1285)							
<i>Con-pgp-3</i>	11823	447	735		JX262228	CE03818 WBGene00003997 locus:pgp-3 protein_id: CAA91495.1 CE03308 WBGene00003998 locus:pgp-4 protein_id: CAA91463.1	1,E-103 1,E-101	73% (178/243) 50% (122/243)							
<i>Con-pgp-9</i>	-	-	278		HE855849	CE15714 WBGene00004003 locus:pgp-9 protein_id: CAB07855.1 CE11932 WBGene00003995 locus:pgp-1 protein_id: CAB01232.1	2,E-34 4,E-33	75% (69/91) 72% (66/91)							
<i>Con-pgp-11</i>	07488	735	-		HE855850	CE34788 WBGene00004005 locus:pgp-11 protein_id: CAA88940.3 CE03260 WBGene00004006 locus:pgp-12 protein_id: CAA91799.1	2,E-76 2,E-73	60% (145/240) 58% (140/240)							
<i>Con-pgp-12</i>	-	-	350		JX262226	CE03260 WBGene00004006 locus:pgp-12 protein_id: CAA91799.1 CE03262 WBGene00004008 locus:pgp-14 protein_id: CAA91801.1	5,E-44 3,E-43	68% (80/116) 64% (75/116)							
<i>Con-pgp-16<sup>d</sup></i>	-	-	298		JX262227	CE03263 WBGene00004009 locus:pgp-15 protein_id: CAA91802.1 CE03261 WBGene00004007 locus:pgp-13 protein_id: CAA91800.1	3,E-29 1,E-28	55% (54/97) 56% (55/97)							
<i>Con-haf-2</i>	03369	1248	-		HE855851	CE07240 WBGene00001812 locus:haf-2 protein_id: AAC71121.1 CE28355 WBGene00001814 locus:haf-4 protein_id: AAC68724.2	1,E-174 1,E-129	73% (297/402) 57% (230/402)							
<i>Con-haf-3</i>	06613	1044	-		HE855852	CE16149 WBGene00001813 locus:haf-3 protein_id: CAB09418.1 CE15650 WBGene00001811 locus:haf-1 protein_id: CAB02812.1	1,E-131 1,E-104	71% (235/330) 55% (184/331)							
<i>Con-haf-4</i>	09074	480	-		HE855853	CE28355 WBGene00001814 locus:haf-4 protein_id: AAC68724.2 CE27353 WBGene00001819 locus:haf-9 protein_id: AAK39394.1	7,E-54 3,E-45	78% (110/141) 71% (92/129)							
<i>Con-haf-7</i>	13744	234	-		HE855854	CE24404 WBGene00001817 locus:haf-7 protein_id: CAB60586.1 CE07240 WBGene00001812 locus:haf-2 protein_id: AAC71121.1	3,E-23 3,E-22	68% (50/73) 61% (47/76)							



<i>Con-haf-9</i>	11142	309	-	HE855855	CE27353 WBGene00001819 locus:haf-9 protein_id:AAK39394.1	7,E-38	88% (78/88)	
<i>Con-mrp-1</i>	04290	216	-	HE855856	CE28355 WBGene00001814 locus:haf-4 protein_id:AA C68724.2	7,E-35	80% (71/88)	
<i>Con-mrp-4</i>	09929	414	-	HE855857	CE39102 WBGene00003407 locus:mrp-1 protein_id:ABA03118.1	1,E-27	78% (55/70)	
<i>Con-mrp-7</i>	04382	480	-	HE855858	CE34565 WBGene00003407 locus:mrp-1 protein_id:AAP82650.1	1,E-27	78% (55/70)	
					CE09548 WBGene00003410 locus:mrp-4 protein_id:CAA88549.1	3,E-52	72% (99/137)	
					CE26370 WBGene00003408 locus:mrp-2 protein_id:AAA83299.2	2,E-46	64% (88/137)	
					CE32017 WBGene00003413 locus:mrp-7 protein_id:CAA21622.3	8,E-41	61% (83/135)	
					CE26370 WBGene00003408 locus:mrp-2 protein_id:AAA83299.2	1,E-39	56% (76/135)	

<sup>a</sup> PMID: 22139919 (Martin *et al.* 2012).

<sup>b</sup> PMID: 21550347 (Elsworth *et al.* 2011).

<sup>c</sup> Denotes a full-length cDNA sequence.

<sup>d</sup> Annotation based on *Caenorhabditis briggsae* according to Demeler *et al.* (2012).

(Supplementary Table 1, online version only) for 20 s and an extension of 72 °C for 12 s. A melting curve analysis was performed at the end of the reaction to ensure specificity of the primers. Each run also included a 5-point dilution series of pooled cDNA and a non-template control. Technical replicates of each sample were performed at least in duplicate within the same run. For each transcript, the mean Ct value of the replicates was calculated and then corrected for the run efficiency. Subsequently, Ct values were transformed in relative quantities (Q) using the delta Ct method:  $Q = E^{(\min Ct - sample Ct)}$ . Where E is the amplification efficiency and min Ct is the lowest Ct value. The relative quantities were then normalized with the normalization factor, obtained by the geNorm software for reference genes *Con-gapdh* and *C. oncophora*  $\beta$ -tubulin (*Con-tubb*) (Vandesompele *et al.* 2002; Van Zeveren *et al.* 2007). Transcript levels were statistically analysed using an independent-samples *t*-test (SPSS Statistics 19). Changes of minimum 2-fold with  $P < 0.05$  were regarded as being significant.

## RESULTS

### *ABC transporter genes in C. oncophora and their developmental transcription pattern*

Analysis of the *C. oncophora* transcriptome dataset resulted in the identification of 12 partial sequences encoding ABC transporters ranging in size from 129 bp to 1245 bp (Table 1). Based on the best BLASTp results on the NEMBASE4 server against the *C. elegans* protein database, the *C. oncophora* sequences were subsequently assigned the putative correct gene name. Four partial P-glycoprotein sequences were identified with highest homology to *pgp-1*, *pgp-2*, *pgp-3* and *pgp-11*, respectively. Furthermore, 5 partial Haf transporters were identified (i.e. *haf-2*, *haf-3*, *haf-4*, *haf-7* and *haf-9*) and 3 partial sequences encoding Mrps (i.e. *mrp-1*, *mrp-4* and *mrp-7*). An additional 3 *C. oncophora* *pgp* sequences were identified by a degenerate PCR approach with homology to *C. elegans* *pgp-9*, *pgp-12* and *pgp-16*. Reverse transcriptase PCR showed that all ABC transporter genes identified were constitutively transcribed throughout the life cycle of *C. oncophora*, except for only a very low transcript level of *Con-pgp-1* and no expression of *Con-pgp-9* in eggs (Table 1).

### *Analysis of constitutive transcriptional differences between susceptible and resistant parasites*

Constitutive differences in transcript levels of the ABC transporter genes between the susceptible and resistant isolates were examined in eggs, L3 and adults worm. The results of the qRT-PCRs are shown in Fig. 1 as the average fold change in mRNA

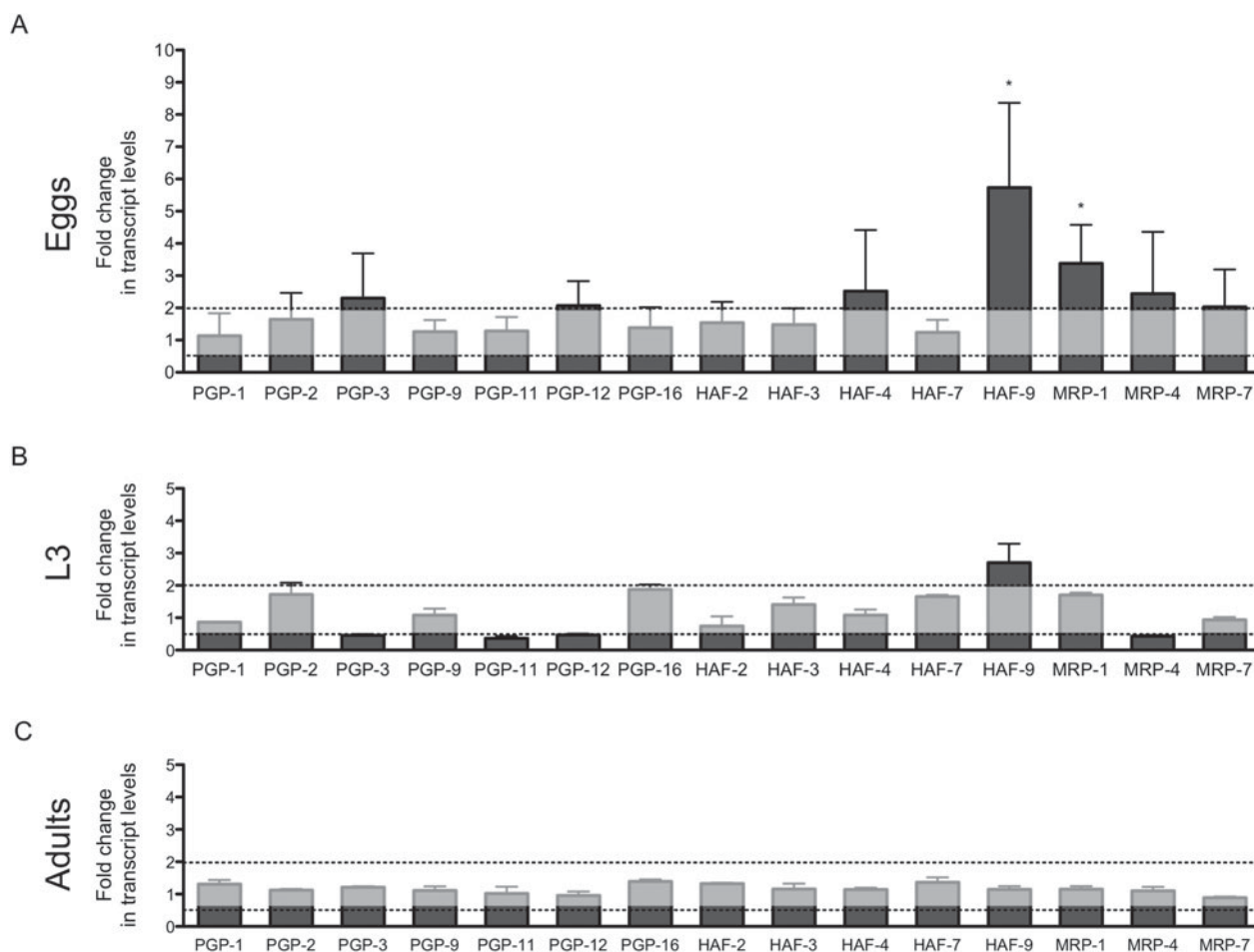


Fig. 1. Fold changes in constitutive mRNA transcript levels of *ABC transporter* genes in *Cooperia oncophora* eggs (A) L3 (B) and adult worms (C). The transcript levels in susceptible stages have been set at 1 and the transcript levels  $\pm$  s.d. in CoIVR08 eggs, L3 and adult worms expressed relative to this. Changes of a minimum 2-fold with  $P < 0.05$  were regarded as being statistically significant (\*  $P < 0.05$ , \*\*  $P < 0.01$ ).

levels compared to non-exposed susceptible eggs, L3 or adult worms. In the *C. oncophora* egg stage, there was considerable variation in the constitutive transcript levels of ABC transporter genes between biological replicates, but the overall trend showed a higher expression in the eggs of the resistant isolate compared to the susceptible eggs. The up-regulation was only significant in *Con-haf-9* (6-fold) and *Con-mrp-1* (3-fold). None of the genes analysed showed significant, constitutive differences in transcript levels between susceptible and resistant L3 and adult worms.

*Analysis of inducible transcriptional changes in adult worms after in vivo exposure to macrocyclic lactones*

Inducible changes in gene expression levels were investigated in adult worms of the resistant isolate after *in vivo* exposure to IVM and MOX. The results of the qRT-PCRs are shown in Fig. 2 and presented as the average fold change in mRNA levels compared to non-exposed susceptible worms. The analysis showed that both the IVM and MOX treatment induced a significant up-regulation of *Con-pgp-11*

transcript levels in the surviving worms, ranging between 3.1 and 4.7-fold increase compared to unexposed susceptible worms. Apart from *Con-pgp-12*, which was 2-fold up-regulated in the worms exposed to MOX, none of the other ABC transporter genes investigated showed transcriptional changes exceeding the 2-fold cut-off compared to unexposed susceptible worms.

*Analysis of inducible transcriptional changes after in vitro exposure of L3 to Ivermectin*

Inducible changes in the transcript levels of the ABC transporter genes were also analysed by exposing L3 stages of the susceptible and resistant isolates to 2 concentrations of IVM *in vitro*, i.e.  $10^{-8}$  M and  $10^{-7}$  M. The results of the qRT-PCR analyses on the worms after exposure are shown in Fig. 3. Significant up-regulations of at least 2-fold were observed for *Con-pgp-12* and *Con-pgp-16* in both the L3 of the susceptible and resistant isolate after exposure. Furthermore, significantly increased transcript levels of *Con-pgp-11* and *Con-mrp-1* were induced in resistant L3 and not in susceptible ones (Fig. 3).

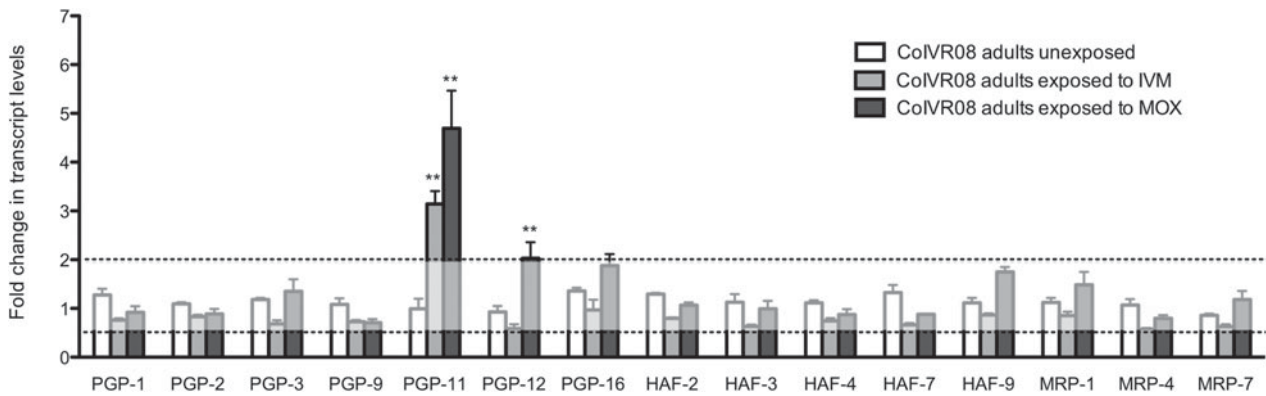


Fig. 2. Fold changes in inducible mRNA transcript levels of *ABC transporter* genes in *Cooperia oncophora* adult worms. The transcript levels in unexposed susceptible worms have been set at 1 and the transcript levels  $\pm$  s.d. in unexposed CoIVR08, CoIVR08 exposed *in vivo* to IVM and CoIVR08 exposed *in vivo* to MOX expressed relative to this. Changes of a minimum 2-fold with  $P < 0.05$  were regarded as being significant (\*  $P < 0.05$ , \*\*  $P < 0.01$ ).

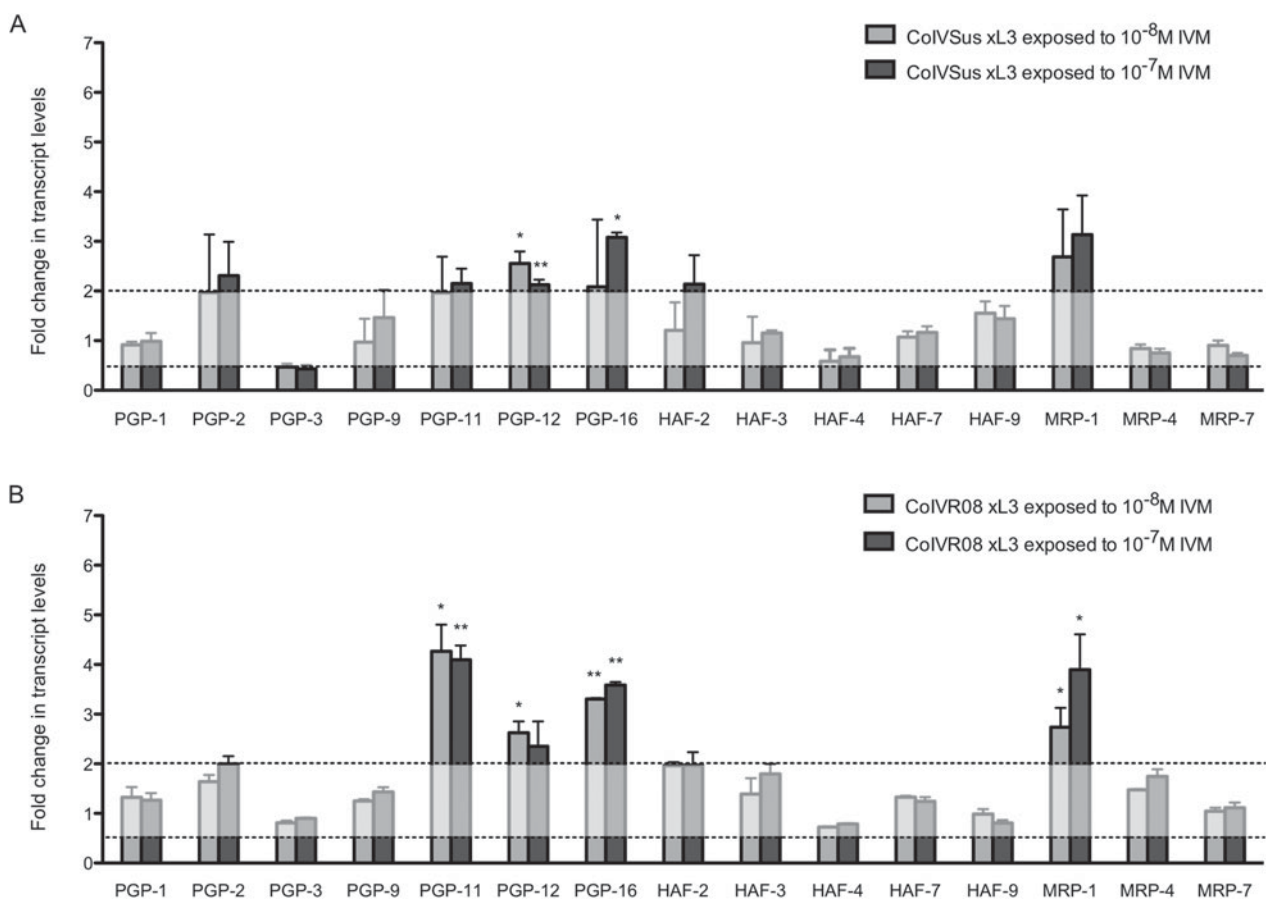


Fig. 3. Fold changes in inducible mRNA transcript levels of *ABC transporter* genes in *Cooperia oncophora* L3. The transcript levels in unexposed susceptible L3s have been set at 1 and the transcript levels  $\pm$  s.d. in CoIVSUs L3s exposed *in vitro* to  $10^{-8}$  M IVM and  $10^{-7}$  M IVM expressed relative to this (A). The transcript levels in unexposed resistant L3 have been set at 1 and the transcript levels  $\pm$  s.d. in CoIVR08 L3 exposed *in vitro* to  $10^{-8}$  M IVM and  $10^{-7}$  M IVM expressed relative to this (B). Changes of a minimum 2-fold with  $P < 0.05$  were regarded as being significant (\*  $P < 0.05$ , \*\*  $P < 0.01$ ).

DISCUSSION

IVM-resistance has been associated with changes in ABC transporter genes in several parasitic nematodes (Xu *et al.* 1998; Ardelli *et al.* 2005, 2006; Ardelli and Prichard, 2007; Prichard and Roulet, 2007; Blackhall

*et al.* 1998, 2008; Bourguinat *et al.* 2008; Dicker *et al.* 2011a; Williamson *et al.* 2011) but, until now, no reports are available on the potential role of ABC transporters in the development of ML-resistance in *C. oncophora*. In this study, 7 *pgp* genes, 5 *haf* genes and 3 *mrp* genes were identified, either by mining a

transcriptome dataset or by a degenerate PCR approach. It is important to note that since most of the identified ABC transporter genes were partial, the currently assigned gene names may still change once more sequence information becomes available. Twelve out of the 15 ABC transporter genes were identified in the transcriptome database (Martin *et al.* 2012), indicating that these are likely to be the most highly transcribed ABC transporter genes in *C. oncophora* under normal conditions. Transcripts of *Con-pgp-9*, *Con-pgp-12* and *Con-pgp-16* on the other hand were only identified by PCR, suggesting a lower constitutive transcript level. Most of the ABC transporter genes investigated were constitutively transcribed throughout the complete life cycle of *C. oncophora*, suggesting that they have a basic metabolic function in these worms.

In the present study, differences in constitutive transcript levels were compared between eggs, L3 and adult worms from a susceptible and resistant isolate. No significant differences in the transcription of ABC transporter genes were observed between the L3 and adult parasites of the susceptible and resistant isolates. In the eggs, however, *Con-haf-9* and *Con-mrp-1* transcript levels were significantly higher in the resistant isolate compared to the susceptible one. However, further information on the constitutive expression of these genes in other nematode species is limited. In laboratory-selected resistant *C. elegans* worms *mrp-1* transcription was highly up-regulated (20 to 36-fold), this was evident even after 3 months without drug exposure (James and Davey, 2009). In *H. contortus* and *O. volvulus*, a constitutive up-regulation of *pgp-2* transcription has been associated with IVM-resistance, whereas in *T. circumcincta* *pgp-9* was implicated (Blackhall *et al.* 1998; Prichard and Roulet, 2007; Bourguinat *et al.* 2008; Dicker *et al.* 2011b; Williamson *et al.* 2011). Interestingly, based on the results of this study, neither of these PGP are likely to be involved in the resistance mechanism of *C. oncophora*, further suggesting that the resistance mechanism might differ between species.

Although it is often hypothesized that anthelmintic exposure can induce the expression of ABC transporter genes in nematodes, the experimental evidence is still scarce. In *C. elegans*, resistance was associated with the inducible up-regulation of *pgp-1*, *pgp-2*, *pgp-4*, *pgp-12*, *pgp-14*, *mrp-1*, *mrp-2*, *mrp-3*, *mrp-4*, *mrp-5*, *mrp-6*, *mrp-7*, *mrp-8*, *haf-1*, *haf-2* and *haf-3* after culturing the worms on agar plates with IVM or MOX (Ardelli and Prichard, 2008; James and Davey, 2009; Yan, 2012). In *H. contortus*, on the other hand, no consistent pattern could be discerned in L3 after exposure to IVM or MOX (Williamson *et al.* 2011). In this study, the effect of anthelmintic exposure was investigated *in vivo* 14 days post-treatment, at which time the worms would still be exposed to active drug (Alvinerie *et al.* 1999; Lifschitz *et al.* 1999). The most

notable change in the surviving worms was observed for *pgp-11*, in which there was a significant up-regulation after both IVM and MOX treatment. A smaller but significant effect was also observed for *pgp-12* induced by MOX treatment, but not by IVM. As far as we are aware, only one other study (Prichard and Roulet, 2007) investigated the expression levels of ABC transporters in worms following *in vivo* exposure. The authors reported the over-expression of five *pgp* genes (termed *Hc-PgpA*, *-B*, *-C*, *-D* and *-E*) in adult *H. contortus* worms collected 24 h after the treatment of sheep with IVM, whereas MOX treatment only resulted in the up-regulation of 2 *pgp* genes (termed *Hc-PgpC* and *-E*). Interestingly, according to a phylogenetic analysis, *Hc-PgpB* showed most homology to the *C. elegans* *pgp-11*. Unfortunately, the study did not provide information regarding the levels of up-regulation of the genes analysed.

Although it is debatable to what extent the *in vitro* assays can mimic *in vivo* conditions, the inducible up-regulation of *pgp-11* was also observed in the L3 of the resistant isolate following *in vitro* exposure to 2 different concentrations of IVM. Importantly, this up-regulation was not observed in the larvae of the susceptible isolate, suggesting that the resistant worms have acquired the ability to up-regulate *pgp-11* upon exposure to MLs. On the other hand, *pgp-12*, *pgp-16* and, to a lesser extent, also *mrp-1* were transcriptionally up-regulated in the larvae of both the susceptible and resistant isolate after exposure, suggesting they are part of a more general xenobiotic response. The molecular mechanisms involved in the transcriptional regulation of ABC transporter genes in nematodes are still largely unknown. In mammals, the transcriptional regulation of the multidrug-resistance ABC transporter gene *MDR-1* seems to be controlled by the nuclear pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) (Wei *et al.* 2000; Synold *et al.* 2001; Maglich *et al.* 2003). Several members of the nuclear receptor (NR) superfamily have been described in *C. elegans*, of which the nuclear hormone receptor-8 (NHR-8) seems to be involved in resistance to colchicine and chloroquine (Lindblom and Dodd, 2006; Zhao *et al.* 2007; Lespine *et al.* 2012). Since both of these natural toxins are substrates for PGP-3 (Broeks *et al.* 1995), NHR-8 may play some role in the transcriptional regulation of *Cel-pgp-3*. However, because of the broad substrate specificity of ABC transporters, it is plausible that a large number of NRs is involved in the transcriptional activation of each ABC transporter gene. Besides transcriptional activation, the regulation of ABC transporters can also occur by post-transcriptional mechanisms such as mRNA stabilization. Recently, in mouse hepatocytes IVM was shown to prolong the half-life of *MDR-1a* and *MDR-1b* mRNA, leading to the overexpression of P-glycoprotein through post-transcriptional mRNA stabilization (Ménez *et al.* 2012).



In summary, the data presented here indicate that resistant *C. oncophora* worms surviving exposure to IVM and MOX are able to induce *pgp-11* transcription, whereas this is not observed in susceptible worms. Whether the up-regulation of this particular P-glycoprotein actually helps to protect the parasites against the toxicity of both MLs is still unclear. Further work is needed to reveal the genetic basis underpinning this inducible up-regulation and to unravel the functional role of PGP-11.

## FINANCIAL SUPPORT

J.D.G. is funded by the Institute for the Promotion of Innovation through Science and Technology in Flanders (grant number SBO/81041). This work was in part supported by the National Institutes of Health (M.M., grant number AI081803).

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