



## Research paper

Ivermectin-induced changes in the expression of cytochromes P450 and efflux transporters in *Haemonchus contortus* female and male adults

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## ABSTRACT

*Haemonchus contortus*, one of the most pathogenic of all small ruminant parasites, have developed resistance to all used anthelmintics. Detoxification enzymes, e.g. cytochromes P450 (CYPs) and efflux transporters P-glycoproteins (P-gps), which represent the main defense system against harmful xenobiotics, have been suggested to contribute to drug resistance development. The present study was designed to compare the constitutive expression of individual CYPs and P-gps in females and males of *H. contortus* adults and to follow up on the changes in expression of these genes in nematodes exposed to sub-lethal concentrations of ivermectin (IVM), which might occur during inaccurate treatment. The adults of inbred susceptible-Edinburgh strain (ISE, MHco3) of *H. contortus* were used for this purpose. The nematodes were incubated *ex vivo* with or without IVM (1, 10 and 100 nM) in culture medium for 4, 12 and 24 h. After incubation, total RNA was isolated and expression levels of individual CYPs and P-gps were analyzed using qPCR. Our results showed a great variability in the constitutive expression of individual CYPs and P-gps in *H. contortus* adults. The constitutive expression as well as the inducibility of CYPs and P-gps significantly differed in males and females. Contact of adult nematodes with sub-lethal IVM concentrations led to only minor changes in expression of CYPs, while expression of several P-gps, particularly *pgp-9.2* in males and *pgp-10*, *pgp-11* in females was increased significantly in IVM-exposed nematodes. In conclusion, inaccurate treatment of sheep with IVM might contribute to drug resistance development via increased expression of efflux transporters in *H. contortus* adults.

## 1. Introduction

Helminthosis is a worldwide problem in human and animal health, with treatment mainly based on anthelmintic drugs such as benzimidazoles, macrocyclic lactones and salicylanilides (Getachew et al., 2007). However, the overuse of these drugs and improper management practices (Leathwick and Besier, 2014) have led to a rapid increase in anthelmintic resistance across all livestock hosts (Kaplan and Vidyashankar, 2012). Multi-resistance to all used anthelmintics have been detected in many isolates of *Haemonchus contortus*, one of the most pathogenic parasites of small ruminants (Besier et al., 2016; Kotze and Prichard, 2016). For this reason, the sources, causes and mechanisms of resistance development have been extensively studied in this nematode (Lanusse et al., 2016).

An example of risk management practices is usage of lower doses than recommended. The exposure of the parasites to sub-lethal doses of

anthelmintic can lead to adaption of their defense system via expression changes of specific detoxification proteins, e.g. biotransformation enzymes and/or drug transporters. Drug biotransformation enzymes and efflux transporters, which catalyze drug deactivation and elimination, represent an important defense system of all organisms, including helminths (Matoušková et al., 2018, 2016; Wolstenholme et al., 2004). Biotransformation enzymes such as cytochrome P450 (CYPs), a large family presented in almost all living organisms, are known to be associated with drug resistance in mammals (Rochat, 2005), insects (David et al., 2013) and have been implicated in helminths (Laing et al., 2015; Yilmaz et al., 2017). In the eggs and larvae of the gastro-intestinal nematodes *Cooperia oncophora* and *Ostertagia ostertagia*, increased toxicity to thiabendazole (TBZ) and ivermectin (IVM) after exposure to the CYP inhibitor piperonyl butoxide (PBO) were shown (AlGusbi et al., 2014). Similarly in *H. contortus* (adults and larvae) and *Trichostrongylus colubriformis* (larvae), PBO was shown to increase sensitivity to the

Abbreviations: CYP, cytochrome P450; ISE, inbred susceptible-Edinburgh strain (MHco3); IVM, ivermectin; P-gp, P-glycoprotein

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pesticide rotenone (AlGusbi et al., 2014; Kotze et al., 2006). In *Caenorhabditis elegans*, the genome encodes 80 CYPs, including four large xenobiotic-induced enzyme families: CYP35, CYP34, CYP33, CYP31 (Laing et al., 2010; Menzel et al., 2001, 2005). The CYPs of *H. contortus* cluster with members of all of these families; however, the parasite is apparently lacking the extensive duplications of genes as appear in *C. elegans* (Laing et al., 2015). Yilmaz et al. identified one gene – CYP34/35 (HCON\_00022640) – as showing high expression in a multi-resistant isolate of *H. contortus* fourth-stage larvae (L4) after TBZ exposure (Yilmaz et al., 2017). However, the present knowledge about the constitutive and anthelmintic-induced expression of CYPs in different *H. contortus* isolates is still insufficient and further examinations are needed.

In addition to biotransformation enzymes, the increased expression of efflux transporters might also contribute to drug resistance in nematodes (Matoušková et al., 2016). The most studied efflux transporters in nematodes are P-glycoproteins (P-gps) homologs belonging to the large family of ATP Binding Cassette (ABC) transporters. ABC transporters catalyze the efflux of a wide spectrum of endogenous and exogenous substrates in all living organisms. Human P-gp (ABCB1) is well-known transporter responsible for the chemotherapy resistance of many cancer cells (Eckford and Sharom, 2009; Leonard et al., 2003). In nematodes, several P-gp homologs have been identified, e.g. 14 P-gp homologs in *C. elegans* (Zhao et al., 2004), and 10 P-gp homologs in *H. contortus* (Laing et al., 2011, 2013). Their role in anthelmintic resistance has been studied extensively (Ardelli, 2013; Godoy et al., 2016; Prichard and Roulet, 2007). Nematode P-gps demonstrate a great ability to efflux macrocyclic lactones (James and Davey, 2009; James et al., 2009; Lespine et al., 2007) and are inducible by exposure to drugs (Janssen et al., 2013b; Lespine et al., 2012; Raza et al., 2016b). In particular, the up-regulation of Con-gpg-11 and Con-gpg-16 (De Graef et al., 2013; Tyden et al., 2014), of Hco-gpg-2 (Godoy et al., 2015b; Lloberas et al., 2013), Hco-gpg-13 (David et al., 2018) and Hco-gpg-16 (Godoy et al., 2015a) by macrocyclic lactones have all been described in *C. oncophora* and *H. contortus*. In *Parascaris equorum*, the induction of P-gpg11 has been reported (Janssen et al., 2015). However, recombinant proteins or larvae stages of the nematodes have been used in almost all studies, and direct identification of the P-gp responsible for anthelmintic resistance in adults is still lacking.

The present study was designed to compare the constitutive expression of individual CYPs and P-gps in female and male adult *H. contortus* and to follow up on the changes in expression of these genes in nematodes exposed to sub-lethal concentrations of ivermectin (IVM). In addition to CYPs and P-gps, the level of transcription factor SKN was analyzed as SKN has been found to mediate inducible detoxification and antioxidation defenses in nematodes (Glover-Cutter et al., 2013; Tang and Choe, 2015). The anthelmintic drug IVM has been widely and extensively used against nematodes and arthropods since 1981 (Omura, 2008). We seek to determine if the contact of adult nematodes with sub-lethal concentrations of IVM could strengthen the defense system of nematodes and thus contribute to drug resistance development.

## 2. Material and methods

### 2.1. Parasites

In this study we used a susceptible isolate of *H. contortus* ISE: inbred susceptible-Edinburgh strain (MHco3) (Roos et al., 2004). Five 3–4 months old lambs, free of parasites, were orally infected with 6000 third stage larvae (L3) of the *H. contortus* ISE strain. Approximately seven weeks after infection the animals were stunned and exsanguinated in agreement with Czech slaughtering rules for farm animals and according to the protocols which were evaluated and approved by the *Ethics Committee of the Ministry of Education, Youth and Sports* (Protocol MSMT-25908/2014-9). The agar method (Van Wyk et al., 1980) was used to isolate the adult nematodes from the sheep

hosts abomasum. The live parasites were washed with phosphate-buffered saline (pH 7.4), manually divided by gender based on morphology and were immediately placed into incubation.

### 2.2. Ivermectin exposure

Males and females were placed separately into RPMI 1640 media (Sigma–Aldrich, Prague, Czech Republic). Then, the adults were split into four groups (12 sample parallels, 10 nematodes per sample) and incubated with or without the drug at 37 °C. Three groups were exposed to different concentrations of IVM (1 nM, 10 nM, 100 nM), with one group placed into 0.1% DMSO as a control. A stock solution of 1 mM IVM (Sigma–Aldrich, Prague, Czech Republic) was prepared in dimethyl sulfoxide (DMSO) followed by serial dilutions in DMSO to produce 3 separate stock concentrations (1 µM, 10 µM and 100 µM).

The incubations lasted 4, 12 and 24 h. In all groups, the nematodes were alive (based on motility check) during all incubations regardless IVM presence or absence in medium. From each group, four nematodes (i.e. four biological replicates) were immediately placed into TriReagent® (Molecular Research Centre, OH, USA) after 4, 12 and 24 h IVM exposure, and stored at –80 °C for later use.

### 2.3. RNA extraction and cDNA synthesis

Total RNA was extracted using TriReagent® according to the manufacturer's protocol, following previous homogenization of the samples in the FastPrep-24 5 G Homogenizer (MP Biomedicals, France). RNA concentrations and purity were determined spectrophotometrically using the NanoDrop ND-1000 UV–vis Spectrophotometer (Thermo Fisher Scientific, MA, USA) at a wavelength of 260 and 280 nm. Samples were analyzed by the Agilent 2100 Bioanalyzer on RNA Nano chips (Agilent Technologies, CA, USA). 4 µg of RNA were treated with DNase I (NEB, UK) and diluted to a concentration of 0.1 µg/µl. One half microgram of the total RNA, random hexamers and Protoscript® II Reverse Transcriptase (NEB, UK) were used for reverse transcription (in 20 µl reaction mixture) following the manufacturer's protocol. Then, the obtained cDNA was diluted 10x and stored at –20 °C.

### 2.4. Quantitative PCR (qPCR)

The changes in expression of selected CYPs and ABC-transporters in *H. contortus* were analyzed by qPCR analyses performed in the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, CA, USA) with SYBR Green I detection. Approximately 12.5 ng of cDNA was added into a reaction mixture consisting of qPCR Xceed SG 1-step 2x Mix Lo-ROX (IAB, Czech Republic), both forward and reverse primers (final concentration 100 nM), in a final volume of 20 µl. The PCR cycling conditions were initiated by a denaturation step of 2 min at 94 °C, followed by 40 cycles of two step amplification as follows: denaturation for 10 s at 95 °C, annealing for 40 s at 60 °C. Fluorescence data were acquired during the last step. A dissociation protocol with a gradient (0.5 °C every 30 s) from 65 °C to 95 °C was used to investigate the specificity of the qPCR reaction and presence of primer dimers. Gene-specific amplification was confirmed by a single peak in the melting curve analysis. Samples were run in three biological and two technical replicates. Relative expression was calculated based on the “Delta-Delta Ct method” (Livak and Schmittgen, 2001). Two housekeeping genes, glyceraldehyde-3P-dehydrogenase (gpd) and nuclear-cap binding protein subunit 2-like (ncbp), were used as reference genes for the qPCR assay (Lecova et al., 2015). All *cyps* and *pyp-3*, *pyp-9.2*, *pyp-10*, *skn* primer sequences were designed in Primer3 software (Untergasser et al., 2012) with predicted melting temperature 60 ± 2 °C, lengths of 20–23 nucleotides (nt) and GC contents of > 45%. Each gene was checked by Mfold at 60 °C (Zuker, 2003) to avoid the region of hairpin structure. Some of the P-gps primers, that met our criteria for specificity and efficiency, were adopted from previous studies (*pyp-1*, *pyp-2* (Sara

et al., 2013) and *pgp-9.1* (Raza et al., 2016b). All primer sequences were synthesized by Generi Biotech, Czech Republic. The specificity and efficiency of the primers were checked for used qPCR conditions. The primer sequences, amplicon sizes, and efficiencies are listed in Table 1. The phylogenetic trees of P-gps and CYPs are in Supplementary figures (Figs. S1 and S2).

## 2.5. Statistical analysis

The data for statistical analysis were expressed as the mean  $\pm$  S.D. (3–4 biological replicates of each sample). Statistical comparison of the gene constitutive expression level of target genes expression was analyzed using one-way ANOVA with Dunnett's multiple comparisons test, the differences between sexes calculated with multiple t test. Differences between treatment groups and incubation times were carried out using the two-way ANOVA with Dunnett's multiple comparisons test. All statistical tests were performed in GraphPad Prism® software 8.0.1 (GraphPad Prism, USA), with differences considered significant at  $P < 0.05$ .

## 3. Results

### 3.1. Comparison of constitutive transcription levels of individual CYPs and P-gps in *H. contortus* adults

To understand the physiological expression pattern of CYPs and P-gps in females and males of the susceptible isolate ISE of *H. contortus* without drug effect a qPCR analysis was performed between genes (Figs. 1 and 2, respectively). For a comparison of individual CYPs and P-gps, the expression levels of *cyp-1* and *pgp-2* (normalized to reference genes *gpd* and *ncbp*) were set as 1.

In the females (Fig. 1A), *cyp-5* is the primarily expressed CYPs gene, with its expression exceeding more than 20-fold that of other CYPs. The *Cyp-3* and *cyp-6* genes were slightly more expressed than *cyp-1*, *cyp-7* and *cyp-8*. In the males (Fig. 1B), the *cyp-3* and *cyp-6* genes were abundant (3.4- and 6.8-fold), with the transcription of *cyp-1* slightly higher than that of *cyp-5*, *cyp-7* and *cyp-8*.

In the females, the transcription of all P-gps was significantly lower ( $P < 0.05$ ) than for the *pgp-2* gene (Fig. 2A). In the males (Fig. 2B), the transcription levels of *pgp-3* a *pgp-9.1* were significantly higher (3- and 3.7- fold) than the *pgp-2* levels. On the other hand, the expression of *pgp-10*, *pgp-11*, *pgp-13* and *pgp-16* was very low in the males.

### 3.2. Comparison of CYPs and P-gps expression in males and females of *H. contortus*

A comparison of the transcription levels of CYP and P-gp genes in the females and males (see Fig. 3) showed that the expression of most genes in the males is higher than in females (with statistical significance for *cyp-3*, *cyp-6*, *cyp-7*, *cyp-8*, *pgp-9.1*, *pgp-9.2*, *pgp-11* and *pgp-16* genes). Only three genes, *cyp-5*, *pgp-2* and *pgp-10*, are transcribed to a lesser extent in the males than in females.

### 3.3. Transcriptional response of CYPs to exposure to sub-lethal IVM concentrations in *H. contortus* adults

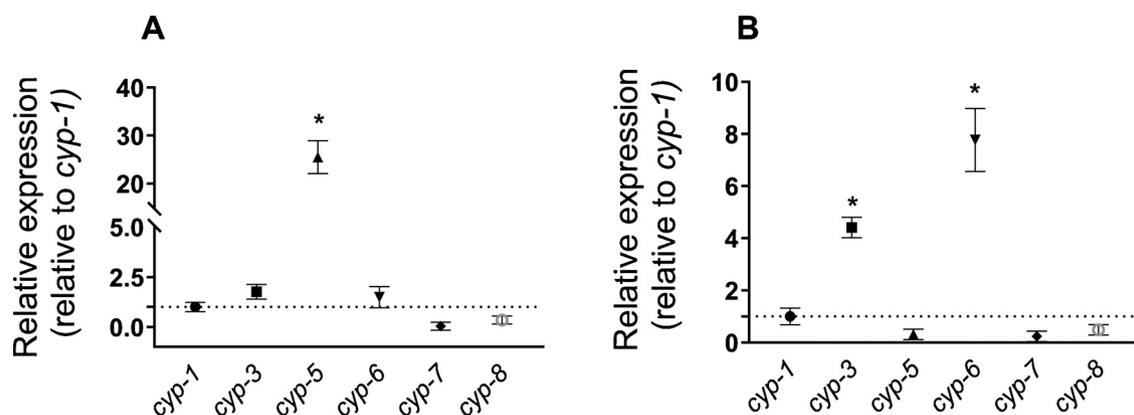
Fig. 4 shows the effect of IVM exposure on CYPs transcription in the adult *H. contortus*. There were no significant changes in the transcription profile of CYPs in the females except for *cyp-7*, which was significantly up-regulated, but only at the 10 nM IVM concentration after 12 h exposure time. In the males, three genes were significantly increased: *cyp-1* at 1 nM after 12 h, *cyp-3* at 100 nM for 4 h, and *cyp-5* (3.2-fold) at 100 nM IVM after 24 h exposure. No significant changes in the transcription of *cyp-6*, *cyp-7* or *cyp-8* were revealed in the males.

### 3.4. Transcription response of P-gps to exposure to sub-lethal IVM concentrations in *H. contortus* adults

The effects of low doses of IVM on P-gps transcription in *H. contortus* adults are shown in Fig. 5. The transcription of each P-gps reacts inconsistently to different IVM concentrations exposure and time points. Furthermore, there is also variation between females and males. In the females, IVM treatment for 4 h and 24 h caused an increased expression of *pgp-10*, *pgp-11* and *pgp-9.2*, respectively. On the other hand, the expression of *pgp-9.1* and *pgp-13* decreased significantly after IVM exposure for 12 h. In the males, after 24 h IVM treatment the expressions of *pgp-9.1* (at 1 nM), *pgp-9.2* (4.7-fold at 1 nM, 2.8-fold at 10 nM and 3.7 at 100 nM), *pgp-10* (at 100 nM) and *pgp-13* (at 100 nM) were significantly higher. The expression of *pgp-10* and *pgp-16* was also significantly increased after 12 h at different IVM concentrations. Contrary to the females, in the males IVM did not cause the down-regulation of any P-gps. There were no significant changes in the expression of *pgp-16* in the females, *pgp-11* in the males and *pgp-2* and *pgp-3* in both females and males.

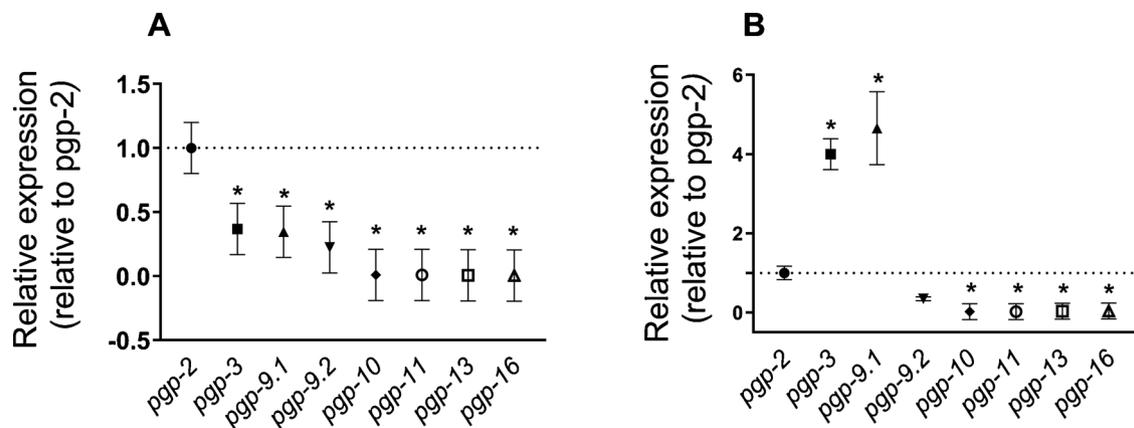
### 3.5. Transcription response of *Skn-1* to exposure to sub-lethal IVM concentrations in *H. contortus* adults

The expression level of transcription factor *Skn-1* (see Fig. 6) in both



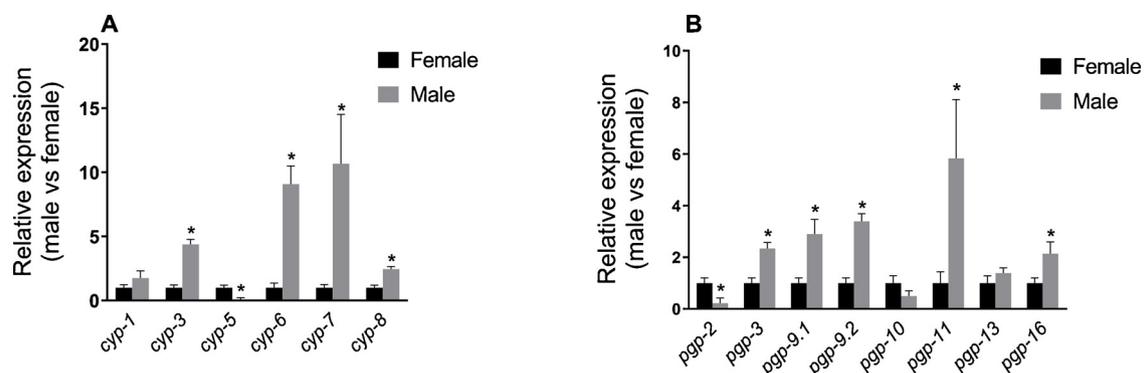
**Fig. 1.** The comparison of constitutive transcription levels of individual cytochrome P450 (CYP) mRNA in adult *Haemonchus contortus* females (A) and males (B). The housekeeping genes glyceraldehyde-3P-dehydrogenase (*gpd*) and, nuclear-cap binding protein subunit 2-like (*ncbp*) were used as reference genes. The gene expression of individual CYPs was related to the *cyp-1* gene.

\* =  $P < 0.05$ .



**Fig. 2.** The comparison of constitutive transcription levels of individual *P*-glycoprotein (*P*-gp) mRNA in adult *Haemonchus contortus* females (A) and males (B). The housekeeping genes glyceraldehyde-3P-dehydrogenase (*gpd*) and, nuclear-cap binding protein subunit 2-like (*ncbp*) were used as reference genes. The gene expression of individual *P*-gps was related to the *pgp-2* gene.

\* =  $P < 0.05$ .



**Fig. 3.** Relative transcription level of cytochrome P450 (CYP) (A) and *P*-glycoprotein (*P*-gp) (B) genes in males compared to females of *Haemonchus contortus*. The housekeeping genes glyceraldehyde-3P-dehydrogenase (*gpd*) and, nuclear-cap binding protein subunit 2-like (*ncbp*) were used as reference genes. The gene expression of individual CYPs and *P*-gps was related to females.

\* =  $P < 0.05$ .

the females and males displayed no significant changes following 4, 12 and 24 h exposure to sub-lethal IVM concentrations.

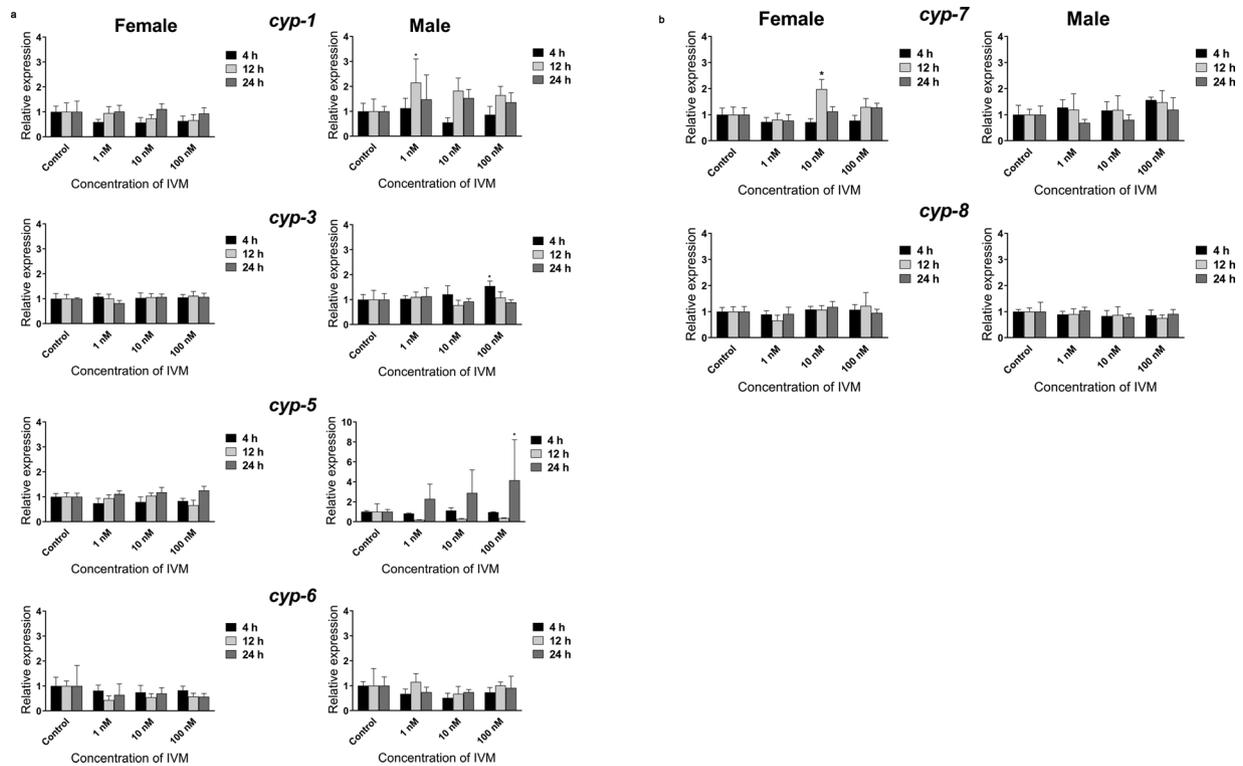
#### 4. Discussion

Parasites ability to develop drug resistance is driven by expanding agriculture as well as inappropriate therapy management and anthelmintics practices (Leathwick and Besier, 2014). Among them, the use of doses lower than recommended is considered a risk factor for the development of resistance. In *Caenorhabditis elegans*, it has been described that stepwise exposure to sub-lethal doses of IVM or moxidectin leads to acquired tolerance to the anthelmintic macrocyclic lactone family (Menez et al., 2016). The present study was designed to explore the changes induced by sub-lethal concentrations of anthelmintic IVM in expression of detoxification proteins in adults of *H. contortus* from ISE strain (MHco3), which is susceptible to all anti-nematode drugs. Nematode defense mechanisms against toxic anthelmintics depend on the expression and activities of enzymes responsible for detoxification metabolism such as biotransformation enzymes and efflux transporters. As the up-regulation of these enzymes might result in worm protection and subsequently in resistance development (Ardelli and Prichard, 2008), it is in our interest to deeply understand the regulation machinery involved in these processes.

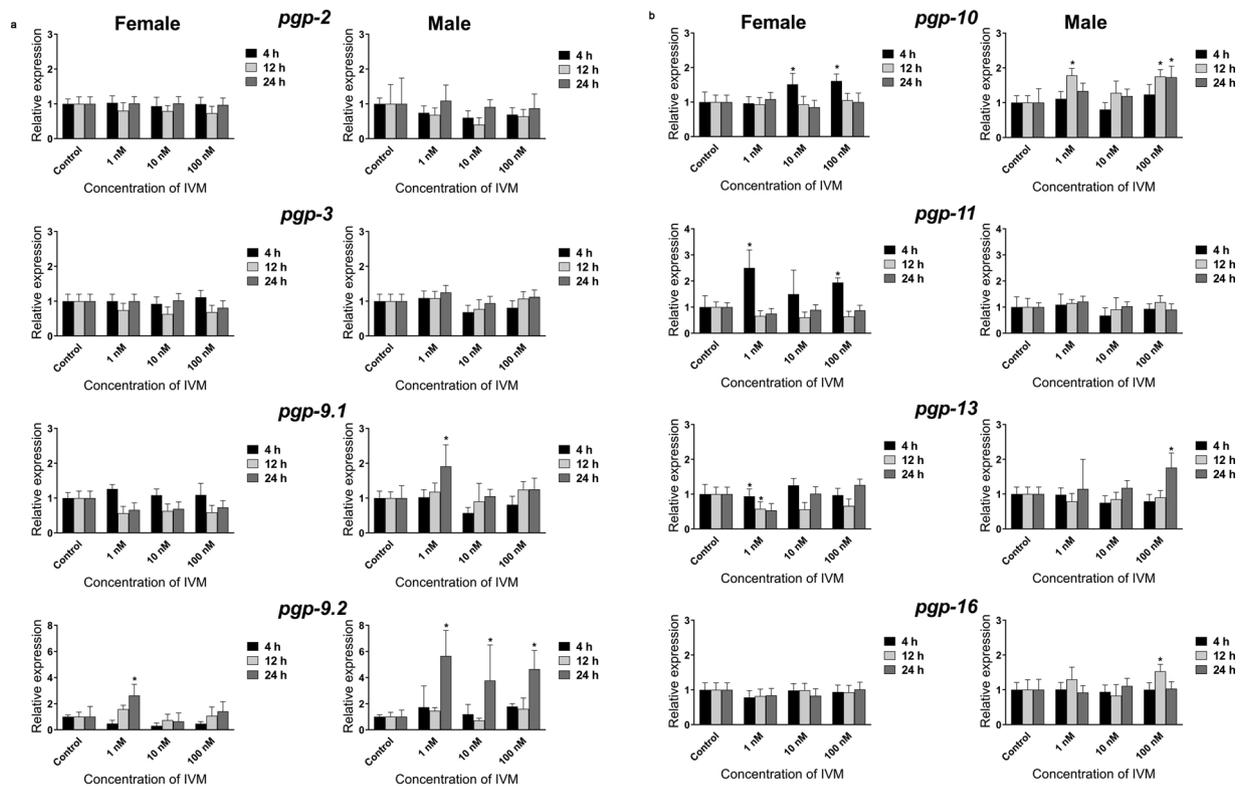
In our study we firstly focused on CYPs, the main family of biotransformation enzymes, which have been demonstrated to play a significant role in drug resistance development in several species. In nematodes, CYPs have been studied to the greatest extent in *C. elegans*.

Recently, however, the CYPs superfamily have also been characterized in *H. contortus* (Laing et al., 2015; Yilmaz et al., 2017). In the present study, we focused on those *H. contortus* CYPs which clustered with the xenobiotic inducible CYP families of *C. elegans*: CYP1 (HCON\_00143950) relates to the Ce-CYP33E subfamily; CYP3 (HCON\_00022670) and CYP8 (HCON\_00022640) cluster with the Ce-CYP34/35 family, CYP5 (HCON\_00038960) and CYP6 (HCON\_00024010) are homologs to Ce-CYP31A2/3 and Ce-CYP14A5, respectively, and CYP7 (HCON\_00045430) clusters with Ce-CYP37A1.

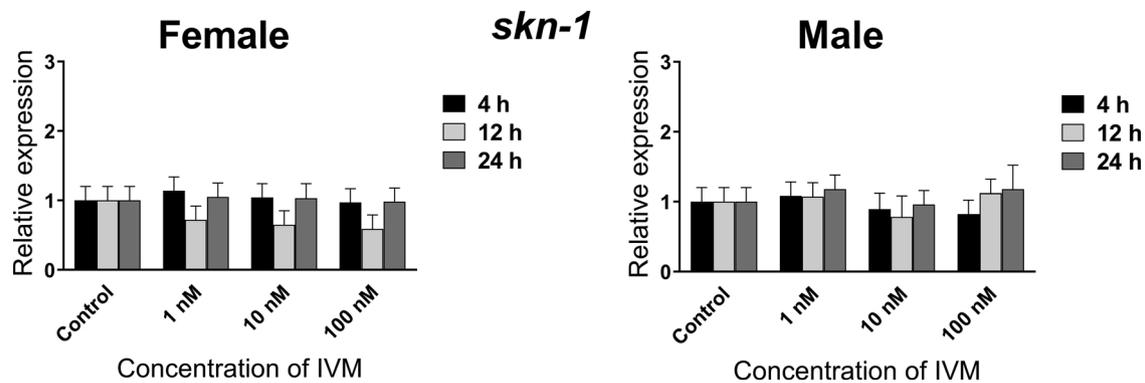
Our comparative analysis of these CYPs revealed significant sex-differences in the basal transcription levels. Interestingly, the expressions of the majority of CYPs were higher in the males than in the females. As both sexes were exposed to xenobiotics in the same extent, there is no reason for elevation of the level of xenobiotic-metabolizing enzymes in the males only. However, CYPs are also involved in the metabolism of many endogenous compounds and thus participation of CYPs in the male specific physiological processes might be supposed. Concerning the levels of individual CYPs in *H. contortus*, *cyp-5* was the only overexpressed CYP in females, whereas in the males, transcription of two genes, *cyp-3* and *cyp-6*, predominated. These results were also shown in a study by Laing et al. (Laing et al., 2015). Considering the fact that the putative orthologues of *cyp-5* in *C. elegans*, Ce-CYP31A2 and Ce-CYP31A3, are expressed in gonads, oocytes and embryos and are involved in eggshell formation (Benenati et al., 2009), CYP5 might be a crucial enzyme in the biological processes in *H. contortus* females. Main CYPs in *H. contortus* males, *cyp-3* and *cyp-6*, have been shown to be highly expressed in the intestine of *H. contortus* (Laing et al., 2015).



**Fig. 4.** The comparison of expression levels of cytochromes P450 (CYPs) mRNA in male and female adult *Haemonchus contortus* ISE isolates in response to low IVM concentrations. Adults were exposed to IVM (1 nM, 10 nM and 100 nM) for 4, 12 and 24 h. Expression of the gene was analyzed using the housekeeping genes glyceraldehyde-3P-dehydrogenase (gpd) and, nuclear-cap binding protein subunit 2-like (ncbp) as reference genes. Data represent the mean  $\pm$  S.D. (n = 3). \* = P < 0.05.



**Fig. 5.** Comparison of expression levels of P-glycoprotein (P-gp) mRNA in male and female *Haemonchus contortus* ISE isolates in response to low IVM concentrations. Adults were exposed to IVM (1 nM, 10 nM and 100 nM) for 4, 12 and 24 h. Expression of the gene was analyzed using the housekeeping genes glyceraldehyde-3P-dehydrogenase (gpd) and, nuclear-cap binding protein subunit 2-like (ncbp) as reference genes. Data represent the mean  $\pm$  S.D. (n = 3). \* = P < 0.05.



**Fig. 6.** The comparison of expression levels of *Skn-1* mRNA in male and female *Haemonchus contortus* ISE isolates in response to low IVM concentrations. Adults were exposed to IVM (1 nM, 10 nM and 100 nM) for 4, 12 and 24 h. Expression of the gene was analyzed using the housekeeping genes glyceraldehyde-3P-dehydrogenase (*gpd*) and, nuclear-cap binding protein subunit 2-like (*ncbp*) as reference genes. Data represent the mean  $\pm$  S.D. (n = 3).

\* =  $P < 0.05$ .

After 6-h TBZ exposure, *cyp-3* together showed a high inducibility in the resistant TBZ isolate (Yilmaz et al., 2017). The *cyp-6* putative of orthologue Ce-CYP14A5 was inducible by b-naphthoflavone in *C. elegans* (Menzel et al., 2001), and its transcription was increased in the IVM-selected IVR10 and MOX-selected strains of *C. elegans* (Menez et al., 2016).

In addition to CYPs, we also focused on P-gp transporters from the superfamily of ABC transporters (see Supplementary Table 1), which can efflux structurally unrelated drugs including IVM in mammals (Lespine et al., 2007; Roulet et al., 2003). In *H. contortus*, several genes of P-gps were identified and significant changes of expression levels of P-gps during all life stages of *H. contortus* have been reported (Issouf et al., 2014). In our study, the comparison of constitutive transcription mRNA levels of eight P-gps in adult *H. contortus* revealed significant differences between females and males. Similarly to CYPs, expression of most P-gps was also higher in the males than in the females. Nevertheless, the reason of this phenomenon remains unclear. In the females, all P-gps were significantly less transcribed than *pgp-2*, whereas in the males, *pgp-10*, *pgp-11*, *pgp-13*, and *pgp-16* showed significantly lower expression than *pgp-2*, but *pgp-3* and *pgp-9.1* were significantly more transcribed than *pgp-2*. In another study, *pgp-3* showed a higher expression level than *pgp-1* in the L3 stage of the Kirby isolate (Raza et al., 2016b) and L4 and adults of the Weybridge isolate (Issouf et al., 2014). Surprisingly, *pgp-9.1* had a higher expression in the males than in females of *H. contortus*, although its expression in *C. oncophora* was almost five times higher in females as compared to males (Areskog et al., 2013).

To elucidate the effect of low doses of IVM on CYPs and P-gps expression, we exposed *H. contortus* adults of ISE strain to three different doses of IVM (1, 10 and 100 nM). While the highest used concentration (100 nM) is comparable with the plasma concentrations in sheep after recommended dose of IVM (Canga et al., 2009), the lower doses represent the concentrations reached or assumed in inaccurate treatment e.g. when pour-on administrations of IVM were used (Gokbulut et al., 2016). As time-differences in inducibility of drug-metabolizing enzymes were observed (Lnenickova et al., 2018), three different incubation periods (4, 12 and 24 h) were used not to miss any transcription response to IVM-induced stress.

Concerning CYPs, the IVM-induced changes in their expression levels were none or inconsistent. Only in the case of *cyp-5*, clear dose-dependent increase of expression was observed in the males incubated with IVM for 24 h. The increased expression of CYP5 is in agreement with the observed inducibility of its orthologues Ce-CYP31A2 and Ce-CYP31A3 by xenobiotics in *C. elegans* (Menzel et al., 2001). Interestingly, *cyp-5* is highly expressed in the females under physiological conditions, however, drug exposure induced only *cyp-5* in the males. Therefore *cyp-5* might represent candidate xenobiotic metabolizing

enzyme which is induced after substrate exposure in the males, but it is also possible that in the females the *cyp-5* gene has dual function for reproduction and detoxification, respectively as similar to *C. elegans* (Benenati et al., 2009; Menzel et al., 2001). Being highly expressed in the females at physiological condition, the low concentrations of ivermectin did not induce *cyp-5*, but it would might get induced by stronger IVM doses. In *C. elegans*, also *cyp-6* orthologue Ce-CYP14A5 was inducible by b-naphthoflavone (Menzel et al., 2001), and its transcription was increased in the IVM-selected IVR10 and MOX-selected strains of *C. elegans* (Menez et al., 2016). However no IVM-induced increase of *cyp-6* was observed in our experiments. Anyway, increased CYPs expression probably cannot contribute to IVM-resistance in *H. contortus* as IVM is not metabolized in this species at all (Vokral et al., 2013a). However, CYPs might contribute to resistance to other anthelmintics e.g. albendazole, monepantel, which are oxidized in *H. contortus* (Stuchlikova et al., 2014; Vokral et al., 2013b). We assumed that nematodes which were in contact with sub-lethal dose of IVM might be less sensitive to consequent treatment with other anthelmintics. If verified, this mechanism might be involved in multi-drug resistance development. However, the changes in CYPs expression in IVM-exposed nematodes are so mild that the importance of CYPs in IVM-induced multi-drug resistance seems to be low.

On the other hand, the efflux transporters P-gps have been suggested to play an important role in IVM-resistance. Several P-gps have been described either as transporters of IVM (Godoy et al., 2015a, 2016; Godoy et al., 2015b), or as having raised mRNA transcription level in response to IVM exposure. In *H. contortus*, larvae L3 of Wallangra multi-resistant isolate, *pgp-1*, *pgp-2*, *pgp-9.1*, *pgp-10* and *pgp-11* were over-expressed after 3 h treatment with IVM (0.2  $\mu$ g/ml i.e. 230 nM) and after 6 h the mRNA levels were comparable with controls (Raza et al., 2016b). However, the same IVM concentration did not affect transcription of any P-gps in the L3 susceptible Kirby strain of *H. contortus*. In our experiments, even the lower concentrations of IVM increased the expression of some P-gps in adults of the susceptible ISE isolate. Similarly, a significant increase in expression of *pgp-10* in the males was observed after 4 h treatment and not the later time points, which supports the idea that gene expression strongly depends on the specific form of the mRNA lifetime distribution (Deneke et al., 2012). The mRNA can be quickly degraded after synthesis of sufficient amount of the protein, e.g. functional transporter. However, based on our results we cannot exclude the possibility that the mRNA upregulation is not followed on the translational level.

The upregulation of *pgp-11* in the females, *pgp-9.2* in the males could indicate a different response to IVM in different sexes of the nematodes. Although, sex-differences in the expression of drug-metabolizing enzymes is common in mammals (Howard et al., 2015; Waxman and Holloway, 2009) and reported in the expression of the drug efflux

transporter P-gp in parasitic sea lice (Igboeli et al., 2014), in *Haemonchus contortus* it is newly observed phenomenon. Recently, two studies observed great sex-differences in the metabolism of anthelmintics (Stuchlikova et al., 2018) and significant sex-differences in the constitutive expression levels of several UDP – glycosyltransferases (Matoušková et al., 2018). Our observation of female and male differences in IVM-induced gene expression support a hypothesis of gender specific detoxification mechanisms.

Anyway, several P-gps contributing to drug resistance in *H. contortus* were up-regulated by low doses of IVM in adults. Particularly, the expression of *pgp-9.2* were strongly increased in the male adults. Similarly, in the male worms *C. oncophora*, the expression profile of *pgp-9* gene tended to be increased by IVM treatment with 70% higher mean expression in treated than in untreated nematodes (Areskog et al., 2013). IVM-induced *pgp-9* expression was also found in the sheep parasite *Teladorsagia circumcincta* (Dicker et al., 2011). Moreover, in L3 of the *H. contortus* resistant isolate a significantly higher transcription of *pgp-9.1* and *pgp-9.2* was reported compared to the susceptible isolate (Raza et al., 2016b). In *H. contortus* females, the expression of *pgp-11* was significantly induced by IVM. Considering the facts that *pgp-11* was elevated by IVM and levamisole in L3 of the *H. contortus* multi-resistant isolate (Raza et al., 2016b), and by monepantel in L3 of the susceptible isolate (Raza et al., 2016a), as well as in other helminth species (Janssen et al., 2013a, 2015), *pgp-11* appears to be one of the efflux transporters with the highest inducibility.

As in other studies (Raza et al., 2016b), during our experiments with *H. contortus* adults, expression of the *pgp-1* gene was also analyzed using the *pgp-1* primers design by Sarai et al. (2013). However, after a sequenced analysis of the *pgp-1* mRNA transcripts available in NCBI, we observed mismatching sequencing which revealed the possible misamplification of *pgp-1* primers, the product of which might not represent the *pgp-1* gene. These potentially problematic findings require further examinations which are beyond the aim of the presented work. Due to these technical issues with *pgp-1* primers, our data regarding *pgp-1* expression were not presented.

To determine more information about the mechanisms of the IVM induction effect, the expression of transcription factor SKN has been analyzed in nematodes incubated with IVM. Transcription factor SKN has been found to mediate inducible detoxification and antioxidation defenses in nematodes (Glover-Cutter et al., 2013; Tang and Choe, 2015). Our results, however, did not show any IVM-induced changes in the expression of *skn-1* in the ISE susceptible isolate of *H. contortus*. Therefore, further studies are needed in order to clarify the mechanism of regulation of P-gps expression by IVM in nematodes.

## 5. Conclusions

Our data showed the great variability in the constitutive expression of individual CYPs and P-gps in *H. contortus* adults. In addition, significant sex-differences in CYPs and P-gps were revealed, with *cyp-5* and *pgp-2* showing the highest constitutive expression in females, whereas *cyp-3*, *cyp-6*, *pgp-3* and *pgp-9.1* predominated in the males. Also significant sex-differences occur in the inducibility of these genes by IVM. The contact of adults with sub-lethal IVM concentrations caused only minor and inconsistent changes in expression of CYPs, with exception of *cyp-5* in males. The IVM-induced changes in P-gps expression were more pronounced, particularly expression of *pgp-9.2* in males and *pgp-10*, *pgp-11* in females was increased significantly. These results indicate that inappropriate treatment might strengthen the defense system of nematodes and thus contribute to resistance development in *H. contortus* adults.

## Conflict of interest

The authors declared no conflict of interest. All experiments with animals were done according to the protocols which were evaluated

and approved by the *Ethics Committee of the Ministry of Education, Youth and Sports* of the Czech Republic (Protocol MSMT-25908/2014-9).

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetpar.2019.07.006>.

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