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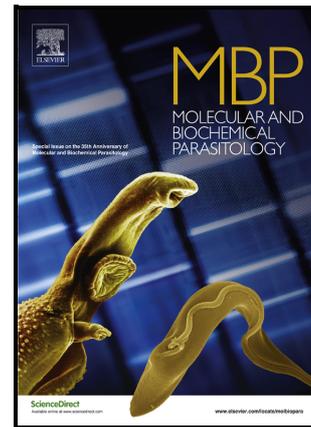
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Transporter gene expression and *Wolbachia* quantification in adults of *Dirofilaria immitis* treated *in vitro* with ivermectin or moxidectin alone or in combination with doxycycline for 12 hours.

Chiara Bazzocchi¹, Marco Genchi², Chiara Lucchetti², Alessandra Cafiso¹, Lavinia Ciuca³, John McCall⁴, Laura Helen Kamer², Alice Vismarra*²

1. Dipartimento di Medicina Veterinaria e Scienze Animali, Università degli Studi di Milano, via dell'Università 6, 26900 Lodi, Italy;

2. Dipartimento di Scienze Medico-Veterinarie, Università di Parma, via del Taglio, 10, 43126, Parma. Italy;

3. Dipartimento di Medicina Veterinaria e Produzioni Animali, Università di Napoli Federico II, Via Federico Delpino 1, 80137, Napoli, Italy;

4. TRS Labs Inc, 215 Paradise Blvd, Athens, GA, 30607, USA.

Corresponding author:

*Alice Vismarra, MSc, PhD

Dipartimento di Scienze Medico-Veterinarie

via del Taglio, 10

43126, Parma, Italy

Università di Parma

alice.vismarra@unipr.it

Abstract

Due to their marked larvicidal activity, macrocyclic lactones (MLs) are used for the prevention of heartworm disease (*Dirofilaria immitis*) in dogs. They have also been shown to eliminate adult parasites after long-term administration, with a so-called “slow-kill” effect. In addition, recent studies have established that a combination of doxycycline, which eliminates the endosymbiont *Wolbachia*, and MLs has superior adulticide effects when compared to MLs alone. It has been hypothesized that the apparent synergism between doxycycline/MLs may be due to interaction with drug efflux transport proteins. The aim of the present study was to evaluate gene expression of several transport proteins in *D. immitis* adults treated *in vitro* either with doxycycline alone, ivermectin alone, moxidectin alone, or a combination of ivermectin or moxidectin with doxycycline for 12 h. Quantitative PCR analysis showed a sex-dependent response to treatments. In female worms, *Dim-pgp-10*, *Dim-haf-1* and *Dim-haf-5* were upregulated compared to controls with doxycycline alone and when combined with ivermectin. Moxidectin did not induce any changes in gene expression. In males, moxidectin administered alone induced a slight increase in *Dim-pgp-10*, *Dim-pgp-11* and *Di-avr-14*, while ivermectin in combination with doxycycline produced significant upregulation of the ML receptor *Di-avr-14*. These results suggest possible synergism between the two drug classes and different susceptibility of males vs. females to adulticide effects.

Keywords: *Dirofilaria immitis*, ABC transporters, gene expression, Macrocyclic lactones, *Wolbachia*

1. Introduction

Dirofilariosis is caused by a group of parasites of the genus *Dirofilaria* that are transmitted by mosquitoes of the family Culicidae. *Dirofilaria immitis* is the agent of canine and feline heartworm disease (HWD). Melarsomine dihydrochloride (Immiticide®, Merial) is the only approved adulticidal drug for treatment of HWD globally [1]. However, treatment can often be followed by severe pulmonary thrombosis [1] and the drug is not registered for use in many countries making availability an issue when deciding the therapeutic approach.

Several studies have shown that a monthly, macrocyclic lactone (ML) heartworm preventive along with doxycycline for a 4-week period, which targets the bacterial endosymbiont *Wolbachia*, has been shown to be adulticidal [2]. The bacterial endosymbiont *Wolbachia* was first described in *D. immitis* [3] and has subsequently been identified in a number of filarial worm species, including several that cause human diseases like elephantiasis (*Wuchereria bancrofti*) and river blindness (*Onchocerca volvulus*) [4]. *Wolbachia* is required for normal parasite development, fertility and long-term survival of filarial worms that harbor the bacteria [5]. Recent sequencing of the *D. immitis*-derived *Wolbachia* genome has shown that the bacteria encode enzymes for anabolic pathways that are missing in the worm such as biosynthesis of heme, purine, and pyrimidines [6]. Thus, current research is aimed at the removal of *Wolbachia* through antibiotic treatment of infected hosts as an adulticide strategy.

Removal of *Wolbachia* through treatment with doxycycline has adulticidal activity against the parasite in dogs experimentally infected with *D. immitis* [7,8]. While MLs are highly efficacious against L3 and young fourth stage larvae (L4) of *D. immitis*, preventive doses have also been reported as having a so-called “slow kill” effect against adult parasites [9]. Furthermore, Bazzocchi et al [7] showed that, in experimentally infected dogs, a combination of ivermectin and doxycycline has a stronger adulticide

activity against *D. immitis* than either of the drugs administered alone. Doxycycline in combination with MLs have subsequently been evaluated in naturally-infected dogs and have been shown to be highly effective in clearing infection in a relatively short time [2,10,11] with reduced side effects (i.e., thromboembolism).

The nature of the apparent synergism between doxycycline and MLs is still unknown. Doxycycline is a bacteriostatic tetracycline which reduces endosymbiont *Wolbachia* populations in *D. immitis*, leading to inhibition of embryogenesis, larval development, microfilarial production, as well as long-term survival of adults [12,13]. Macrocyclic lactones like ivermectin and moxidectin bind to the glutamate-gated chloride (GluCl) ion channel, leading to an increase in membrane permeability, which induces worm paralysis and eventual death [14,15]. Recent studies have explored the potential role of cellular detoxification, by evaluating ATP-binding cassette (ABC) transporter gene expression (PgPs) in *D. immitis* treated *in vitro* with ivermectin, doxycycline and a combination of the two [16,17,18]. The authors reported differences in several PgPs' gene expression between males and females following 24 and 48 h of treatment; doxycycline more actively stimulated female PgP pumps, while ivermectin caused higher expression in male worms. Moreover, the combined treatment ivermectin+doxycycline induced an up-regulation in gene expression in both males and females, with higher values for the latter. Results, however, did not elucidate the potential role of PgPs in the higher efficacy of combined treatment observed in naturally infected dogs. The authors suggested that *Wolbachia* may be involved, particularly in the higher PgPs gene expression induced by doxycycline observed in females vs. males. Indeed, fecund female worms contain a greater number of *Wolbachia* compared to males and the bacteria may induce upregulation of PgPs in order to reduce antibiotic, thus protecting itself [19,20]. *Wolbachia* is maternally transmitted and is believed to supplement a

large array of nutrients in the nematodes, ranging from heme to riboflavin/flavin adenine dinucleotide (FAD) [13,21,22]. In this view, the possible role in modulating the activation of drug detoxification mechanisms is intriguing.

The aims of the present study were: i) evaluate gene expression of five selected Pgp pumps of *D. immitis*, involved in different mechanisms and with different locations, after 12 h treatment with two MLs (ivermectin, moxidectin) alone or in combination with doxycycline; ii) determine the *Wolbachia* loads in treated and non-treated adult worms in order to establish if doxycycline treatment affects bacterial load.

2. Materials and Methods

2.1 Dirofilaria immitis adults

A total of 21 female and 21 male adults of a ML-susceptible strain (*Georgia-2*) of *D. immitis* harvested from experimentally-infected dogs at approximately 6 months post-infection, were supplied by TRS® Labs Inc. (Athens, GA, USA). Worms were washed in Hank's balanced salt solution (HBSS) (Euroclone spa, Milano, Italy) at room temperature and then placed individually into tubes containing 50 ml of RPMI 1640 medium (w/o L-Glutamine with Phenol Red) (Euroclone spa, Milano, Italy) with the addition of 2 mM L-Glutamine (Euroclone spa, Milano, Italy) and 1% of a solution of antibiotic and antimycotic (Euroclone spa, Milano, Italy) and kept at 37°C, 5% CO₂.

2.2 In vitro treatment of adults

Drug concentrations (ivermectin, moxidectin and doxycycline) were chosen according to their reported peak plasma concentrations after *in vivo* treatments of dogs [23,24,25]. The basal medium

used for treatment of adult worms was a NI medium (NCTC/IMDM media mixture), see [16] for more details. Treatment protocols were as followed (Table 1): NI media + 1% DMSO (control; C); ivermectin (3.54 nM) + 1% DMSO (IVM); moxidectin (31nM) + 1% DMSO (MOX); doxycycline (56.5 μ M) + 1% DMSO (DOX); doxycycline (56.5 μ M) + ivermectin (3.54 nM) + 1% DMSO (DOX/IVM); doxycycline (56.5 μ M) and moxidectin (31 nM) + 1% DMSO (DOX/MOX).

Each treatment was performed in triplicates for both sexes. Tubes were maintained for 12 h at 37°C, 5% CO₂. Worms were checked for vitality, based on the observation of movement in RPMI medium following extraction [26]. Briefly, motility was scored as 0 (complete paralysis) – 3 (full motility). Worms were then washed in HBSS (Euroclone spa, Milano, Italy) and promptly frozen at – 80 °C.

2.3 RNA and DNA extraction and cDNA synthesis

RNA and DNA extractions were performed as described before [16,17]. Briefly, Trizol[®] reagent (Invitrogen, USA) was used for the simultaneous RNA and DNA isolation from the same worm respecting the proportionality between male and female in relation to their different size. After RNA isolation from the aqueous phase, concentration and quality of RNA samples were measured through spectrophotometer analysis with μ Cuvetta (BioSpectrometer[®], Eppendorf, Germany). The organic phase obtained, was then processed for DNA extraction; DNA was resuspended in 300 μ L of DEPC water and concentration and quality were evaluated through a spectrophotometer (BioSpectrometer[®], Eppendorf, Germany). 300 nanograms of RNA were used to produce cDNA according to manufacturer's instructions (OneScript[®] cDNA Synthesis Kit; Abm) after an initial step of AccuRT genomic DNA removal (Abm, Richmond, Canada). RNA, DNA and cDNA samples were stored at – 80 °C until subsequent use.

2.4 Quantitative PCR (q-PCR) for gene expression analysis

Two ABC-B transporters genes (*Dim-pgp-10* and *Dim-pgp-11*), two ABC-B half transporter genes (*Dim-haf-1* and *Dim-haf-5*) and the gene coding for a GluCl channel (*Di-avr-14*) were amplified according to literature [27,28]. Analyzed transporter genes were chosen based on previous reports [16] and on preliminary data obtained in our laboratory (only those genes showing variation compared to controls were analyzed further).

The optimization of the quantitative PCR (“q-PCR”) protocols in order to test the efficiency and the dynamic range of the reactions was done as described previously [16,17,18]. The 18S ribosomal subunit (18S rRNA) was selected as endogenous control [7]. Primer sequences are listed in Table 2. Three biological replicates of both sexes were analyzed in triplicate for each treatment group. Then, three technical replicates (three replicates of the same cDNA sample analyzed in qPCR) were analyzed per each biological replicate. Cycle threshold (Ct) values were determined for each gene and normalized using 18S rRNA gene as reference. The results of relative gene expression for each treatment (calculated using the $\Delta\Delta C_t$ -method) were compared to the treatment control (NI alone) considered as calibrator. The BrightGreen 2× qPCR Mastermix (Abm, Richmond, Canada) was used according to the manufacturer’s instructions. The amplification protocols were those described before [16,18].

2.5 *Wolbachia* quantification

The *Wolbachia* loads in each worm were quantified in the extracted DNA samples as described in Bazzocchi et al. [7]. Briefly, a fragment of the *ftsZ* gene of *Wolbachia* and of the genomic 18S ribosomal subunit (18S rDNA) of *D. immitis* were amplified. Primers and PCR conditions were

described in Bazzocchi et al. [7]. Copy numbers of the two genes were subsequently normalized and bacterial loads were presented as *ftsZ*/18S rDNA (*Wolbachia*/nematode) ratios.

2.6 Statistical analyses

Results of relative quantification were presented as the relative change in gene expression ($2^{-\Delta\Delta Ct}$) \pm standard deviation (SD) of the three biological replicates. All Ct values were managed by CFX Manager software (Bio-Rad, Hercules, CA, USA) and $2^{-\Delta\Delta Ct}$ was calculated according to the Livak method [29]. Three biological samples were analyzed per sex. The standard deviation between each technical replicate was calculated. All standard deviations were lower than 0.3, which is the threshold value for a Ct standard deviation to be considered accurate, as described in the “Guide to performing relative quantitation of gene expression using real-time quantitative PCR” [30]. The standard deviation per each $2^{-\Delta\Delta Ct}$ was calculated according to the same manual.

Data normality and distribution were tested respectively with Pearson test and two-way ANOVA using GraphPad Prism v.9.0.1 (GraphPad Software, San Diego, CA, USA; <http://www.graphpad.com>) and *P* values < 0.05 were considered significant.

An unpaired T-test to evaluate possible statistically significant differences in *Wolbachia* quantity between male and female treated with the drugs regimen described was performed using GraphPad Prism v.9.0.1 (GraphPad Software, San Diego, CA, USA; <http://www.graphpad.com>) comparing mean average values + standard deviation.

3. Results

3.1 Constitutive gene expression in males and females

Figure 1 shows results of gene expression for the five transporter genes in untreated males and females. The constitutive expression of *Dim-pgp-10* and *Dim haf-1* was higher in females vs. males, while *Dim-pgp-11* and *Di-Avr-14* was higher in males vs. females.

3.2 Modulation of female worm gene expression following treatment

Figure 2 reports the $2^{-\Delta\Delta Ct}$ values recorded in female worms following 12 h incubation with the different treatments.

When compared to controls, there was higher expression of *Dim-pgp-10*, *Dim-haf-1* and *Dim-haf-5* in worms treated with DOX (2.11, 2.30 and 14.03 fold higher) and when treated with IVM/DOX (3.22 and 2.59 fold for *Dim-pgp-10* and *Dim-haf-5*, respectively). The increase in *Dim-haf-5* expression was significant in female worms treated with DOX if compared to all other PgP genes (Fig. 2). Moreover, there was also a marked increase in the gene expression of *Dim-haf-5* when treated with DOX/MOX with a significant increase compared to *Di-avr-14* (P value: 0.002) (Fig. 2).

3.3 Modulation of male worm gene expression following treatment.

Figure 3 reports the $2^{-\Delta\Delta Ct}$ values recorded in male worms following 12 h incubation with the different drugs and combinations. Treatment protocols with IVM or with MOX/DOX did not induce marked changes in gene expression, with the notable exception of *Di-avr-14*, whose increase was highly significant compared to the other treatment protocols ($P < 0.0001$) (Fig. 3). It is also notable how *Di-avr-14* is the only gene with a marked increase of expression when males were treated with DOX (4.24 fold higher). MOX induced an increase in the expression of *Dim-pgp-10*, *Dim-pgp-11*, *Dim-haf-*

1 and *Di-avr-14* (4.66, 4.3, 2.21 and 4.26 fold higher, respectively), even if none showed a statistical significance.

3.4 *Wolbachia* quantification

Results of “q-PCR” are reported in Figure 4 as the ratio between the number of gene copies of *Wolbachia* (*ftsZ* gene) and the relative adult worm (18S rDNA) x 1000. No statistically significant differences were observed for the total amount of *Wolbachia* among treatment groups or between male and female worms.

4. Discussion

The results of the present study highlight a gender-dependent pattern of constitutive expression and ML-inducibility of several *D. immitis* ABC transporter genes. Untreated females had higher expression of *Dim-pgp-10* and of *Dim-haf-1* compared to males. A previous study of PgP-9 expression in *Cooperia oncophora* [31] reported that female worms had an approximately five times higher constitutive gene expression compared to male worms. On the contrary, Kellerova et al [32] reported higher expression of several PgPs in *Haemonchus contortus* males compared to females. The reasons for gender-based differences in constitutive expression are not known.

The only significant change in gene expression in females following treatment was observed for the *Dim-haf-5* gene in worms treated with doxycycline. Sheps et al [33] reported that *haf-5* in the nematode *Caenorhabditis elegans* is involved in the transport of heme from mitochondria to the cytosol, suggesting that it could play a critical role in iron homeostasis in mitochondria. Furthermore,

it has been shown that the use of tetracycline results in up-regulation of components of the mitochondrial respiratory chain in *Litomosoides sigmodontis*, a filarial nematode [34].

The only significant change in gene expression in males following treatment was observed for the *Di-avr-14* gene in *D. immitis* treated with ivermectin in combination with doxycycline. *Di-avr-14* is a gene encoding for a GluCl which is highly conserved in nematodes [28]. GluCl is an ML target and marked rise in expression in male worms may explain the greater susceptibility of male worms to ivermectin treatment [7]. Furthermore, considering the higher expression of *Di-avr-14* after treatment with ivermectin + doxycycline, it is clear that the combined protocol favors the activation of these channels and potentially increasing the influx of ivermectin.

Results of the present study suggest differences in gene expression when comparing ivermectin with moxidectin treatments. We observed upregulation of *Dim-pgp-10*, *Dim-haf-1* and *Dim-haf-5* genes in females treated with ivermectin (alone or in combination with doxycycline), even though the increase was not statistically significant. However, when females were treated with moxidectin (alone or in combination with doxycycline), expression of all genes considered remained near baseline level apart from *Dim-haf-5*. In males, on the other hand, while treatment with ivermectin alone or in combination with doxycycline did not induce significant changes in gene expression apart from *Di-avr-14*, moxidectin (alone or in combination with doxycycline) induced upregulation of three out of five genes evaluated (*Dim-pgp-10*, *Dim-pgp-11* and *Di-avr-14*). These results would suggest a sex-dependent nature of Pgp's interaction with moxidectin in *D. immitis*. Interestingly, Stitt et al. [35] showed that in *Brugia malayi*, a human nematode causing lymphatic filariasis, the administration of moxidectin together with ABC transporter inhibitors tended to influence the sensitivity to the drug in females and

microfilariae, while no important effects were recorded in males. Even though the study did not evaluate gene expression, results suggest possible sex-dependent responses.

Several studies have evaluated the role of moxidectin in modulating PgP genes in mammalian tissues and nematodes like *D. immitis* and *C. elegans* [36, 37, 38, 39, 40], suggesting that moxidectin kinetics are not totally dependent on PgPs or other ABC transporters and differences among molecules of the same family in terms of their ability to interact with PgPs likely exist.

The present study, similar to that reported by Lucchetti et al [16], shows that combination with doxycycline affects MLs-induced expression of transporter protein genes. Doxycycline has been reported as both up-regulating [16] and down-regulating transporter gene expression [41]. This may explain the mechanism by which doxycycline increases the effect of ivermectin, but not that of moxidectin.

It is important to remember that down-regulation of PgPs like *Dim-pgp-10* and *-11* is associated with increased susceptibility to ivermectin [42]. In female worms, *Dim-pgp-10*, was up-regulated following treatment with doxycycline and doxycycline + ivermectin (suggesting a defensive response on the part of the parasite). Indeed, the few studies which evaluated worm populations at necropsy following MLs/doxycycline combination protocols have shown that female worms were often found to survive drug treatment, suggesting a certain tolerance towards the adulticide effects of this treatment protocol [7,11].

Even though it has been reported that up-regulation of PgP genes is related to drug-resistance phenotypes of *H. contortus*, *C. elegans* and equine cyathostomins [42,43,44], the adult worms used in

the present study were a “susceptible” strain, so we can exclude the presence of drug resistance phenomenon.

The significant up-regulation of the *Dim-haf-5* gene, involved in heme transport from mitochondria, in females after 12 hours of doxycycline treatment suggested possible involvement of *Wolbachia*. Synthesis of heme has been identified as one of the most important functions of *Wolbachia* endosymbiosis, together with nucleotide synthesis [13,21,22,34] and the authors hypothesized that higher *Wolbachia* loads in female adult worms may have been associated with a differential expression of *Dim-haf-5* gene when females were exposed to doxycycline. However, results showed that males had similar or superior loads of *Wolbachia* compared to females, in agreement with that reported by other authors [45,46]. Indeed, the number of bacteria in adult worms not only depends on gender, but also on the age of the parasite. McGarry et al [47] reported that 6-month old worms of both sexes had similar *Wolbachia*:nematode genome ratios. Only when females reach 12-15 months of age do *Wolbachia* numbers increase, due to embryonic and microfilarial development. As reported in the methods, female worms used in the present study were harvested at approximately 6 months post-infection. Finally, 12 h *in vitro* doxycycline did not have any effect on *Wolbachia* numbers in either males or females. It must be remembered however that doxycycline is bacteriostatic, and treatment time likely was insufficient to decrease bacterial load in any significant way.

5. Conclusions

The results from the present study confirm the “female-male” dichotomy in *D. immitis* transporter gene expression following exposure to MLs and doxycycline. The most intriguing result of the present study was the marked effect of ivermectin and doxycycline on *Di-avr-14*, the gene encoding for a

GluCl, in particular in male worms. Is it possible that increased expression of this ML receptor when treatment with ivermectin (or other MLs) is combined with doxycycline could explain the synergism in adulticide activity?.

The present study has several limitations: it would have been interesting to include other post-treatment time points (4, 6 hrs) in order to determine a time-dependent expression pattern.

Furthermore, drug concentrations within the worms treated with doxycycline together with ivermectin or moxidectin would have strengthened our hypothesis that the combinations lead to greater or lesser influx of drugs within the parasite.

The use of doxycycline/MLs combinations for the treatment of canine heartworm disease leads to a reduction in pro-inflammatory responses to either dead or dying worms and in this way there is a reduction in respiratory complications as well as mortality rates [48]. On the other hand, in some cases, the high doses of doxycycline used are not always well tolerated in dogs [11] and there is the risk of possible drug- resistance in *Wolbachia*. It has been suggested that the current use of doxycycline in dogs with HWD is likely favoring the emergence of resistant strains in other bacteria, such as *Staphylococcus* or *Enterococcus* [49] and we cannot exclude that the same might happen also in *Wolbachia*. A better understanding of the molecular mechanisms involved in combination treatment protocols may aid in the development of new pharmacological approaches.

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Ethical approval

Not applicable

Author contributions

Chiara Bazzocchi: Investigation Data Curation, Funding, Resources, Writing, Review & Editing.

Marco Genchi: Investigation, Methodology, Data Curation.

Chiara Lucchetti and Alessandra Cafiso: Investigation, Methodology.

Lavinia Ciuca: Data Curation, Review & Editing.

John McCall: Data Curation, Review & Editing.

Laura Helen Kamer: Conceptualization, Supervision, Funding, Resources, Data Curation, Writing, Review & Editing.

Alice Vismarra: Investigation, Methodology, Data Curation, Writing, Review & Editing.

Declaration of competing interests

The authors declare no conflicts of interest.

Figure legend

Fig.1 Values of $2^{-\Delta\Delta Ct}$ recorded in female and male worms following 12 h incubation with the NI Medium+1%DMSO compared to NI media alone as calibrator (basal gene expression in non-treated worms).

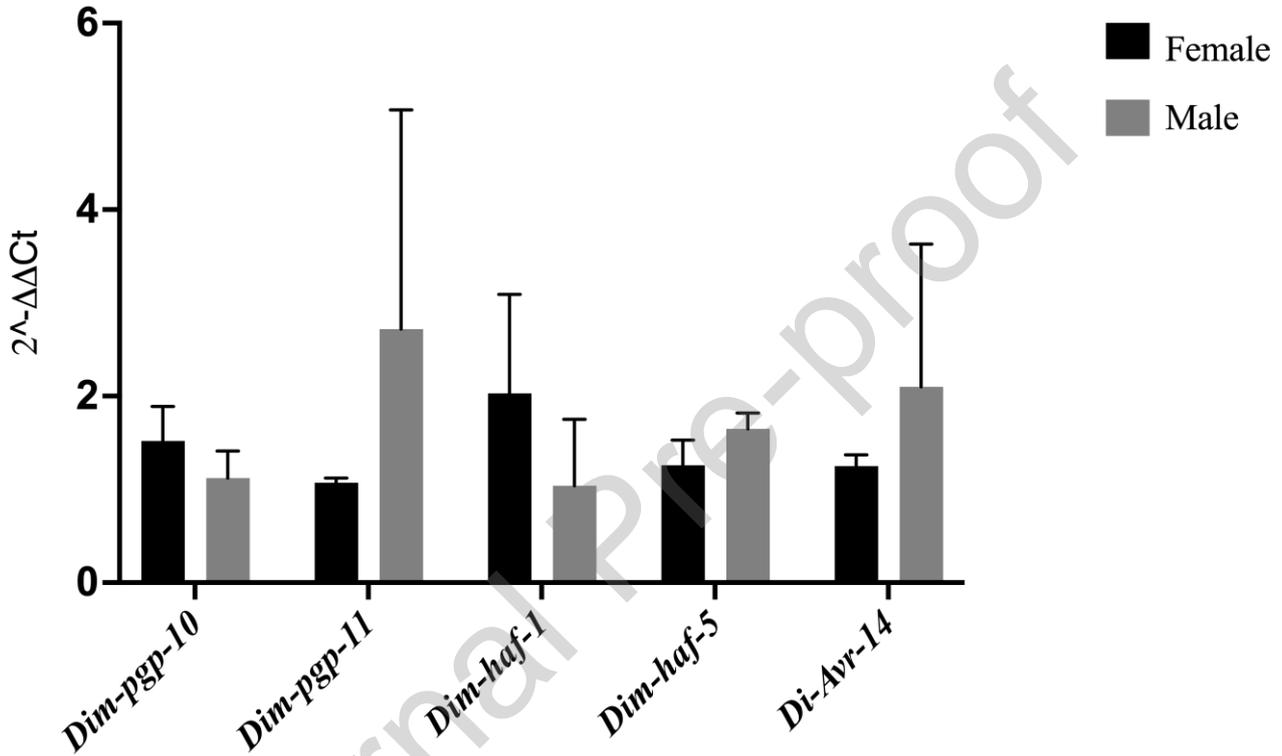


Fig. 2 Values of $2^{-\Delta\Delta Ct}$ recorded in female worms following 12 h incubation with the different drugs and combinations. (P values in DOX treatment: Dim-*pgp-10* vs. Dim-*haf-5* *** P=0.0010; Dim-*pgp-11* vs. Dim-*haf-5* **** P=0.0006; Dim-*haf-1* vs. Dim-*haf-5* ***P=0.0011; Di-*avr-14* vs. Dim-*haf-5* **** P=0.0006. P value in DOX/MOX treatment Di-*avr-14* vs. Dim-*haf-5* ** P=0.002)

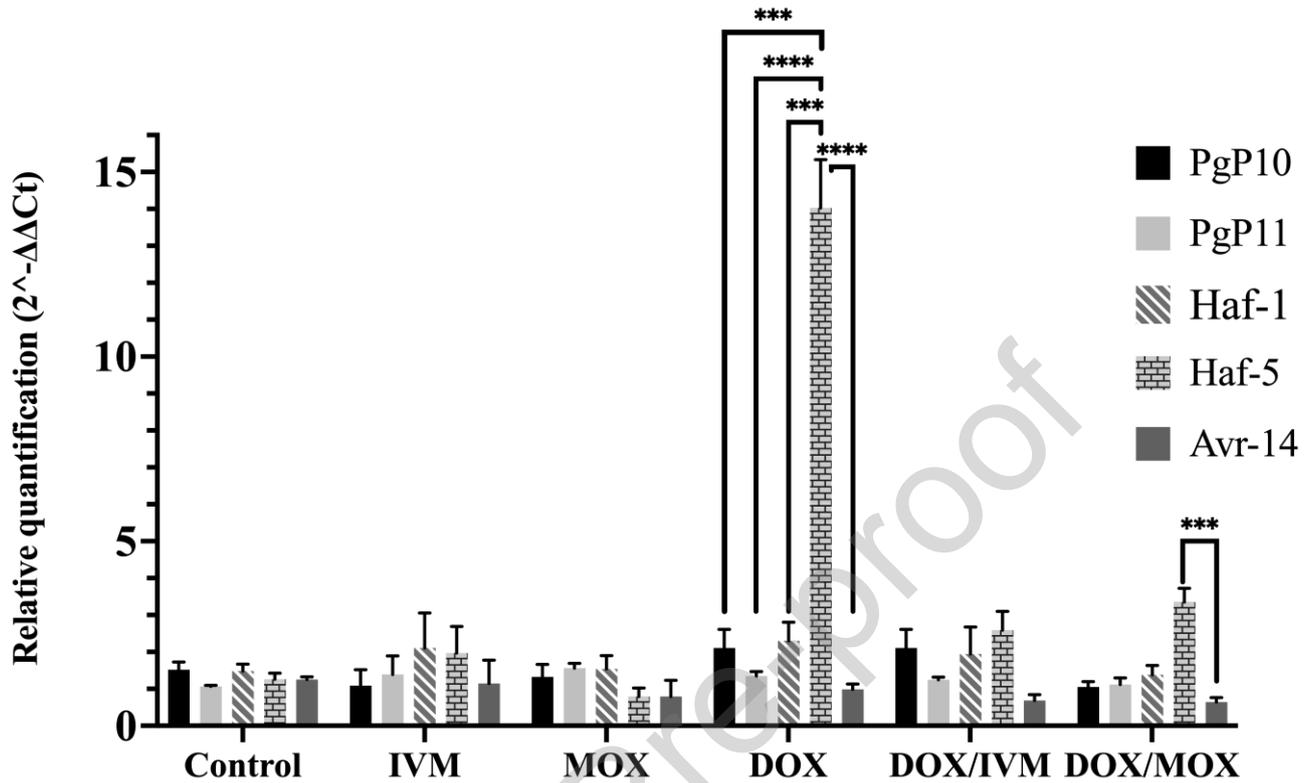


Fig. 3 Values of $2^{-\Delta\Delta C_t}$ recorded in male worms following 12 h incubation with the different drugs and combinations. (P values for DOX/IVM treatment: Dim-*pgp-10* vs. Di-*avr-14* **** P=0.00025; Dim-*pgp-11* vs. Di-*avr-14* **** P=0.0002; Dim-*haf-1* vs. Di-*avr-14* **** P=0.0003; Dim-*haf-5* vs. Di-*avr-14* **** P=0.00013).

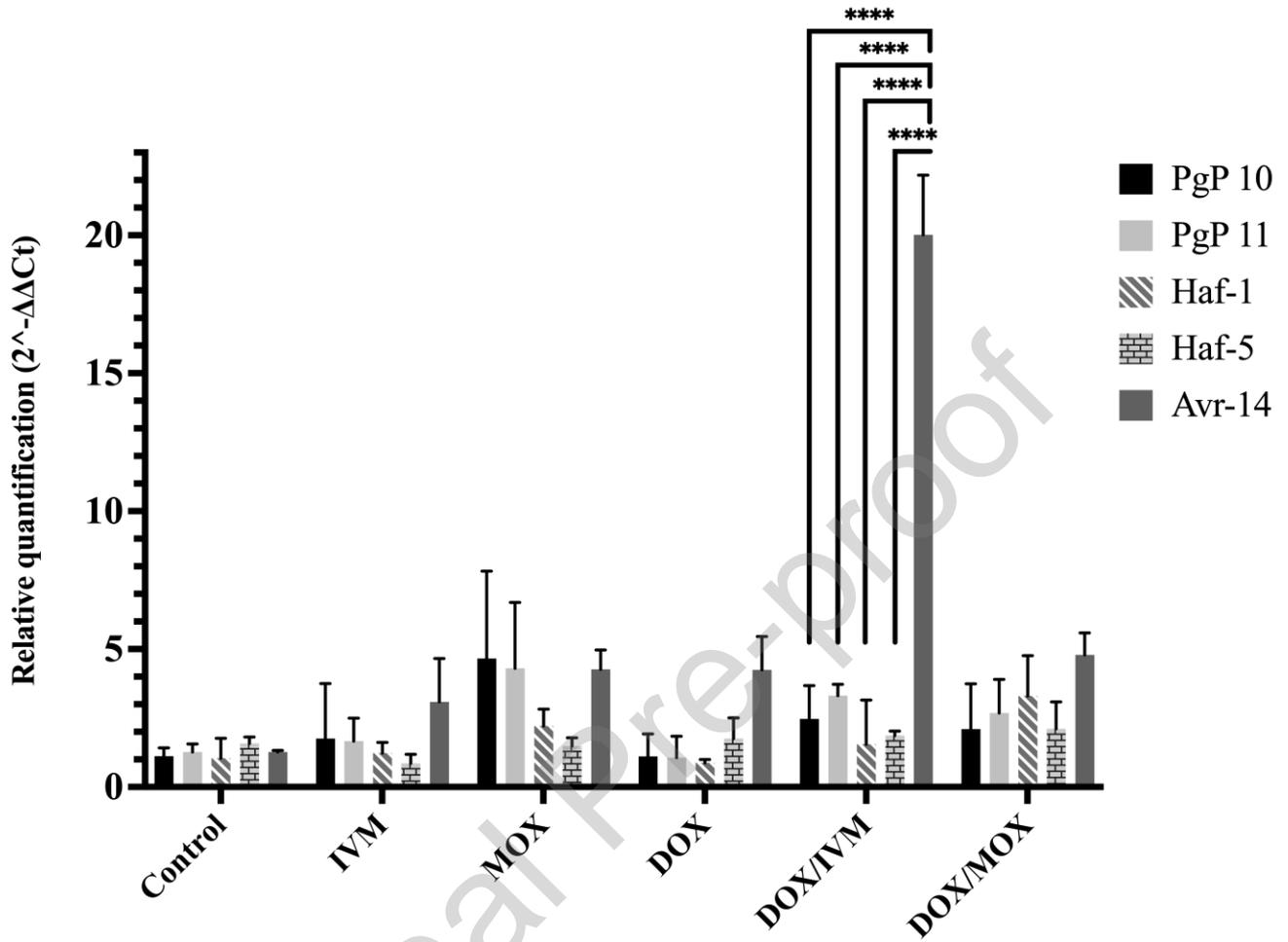


Fig. 4 Results of quantitative Real-time PCR reported as the ratio value between the number of gene copies of *Wolbachia* (*ftsZ* gene) and the relative adult worm (18S rDNA) x 1000.

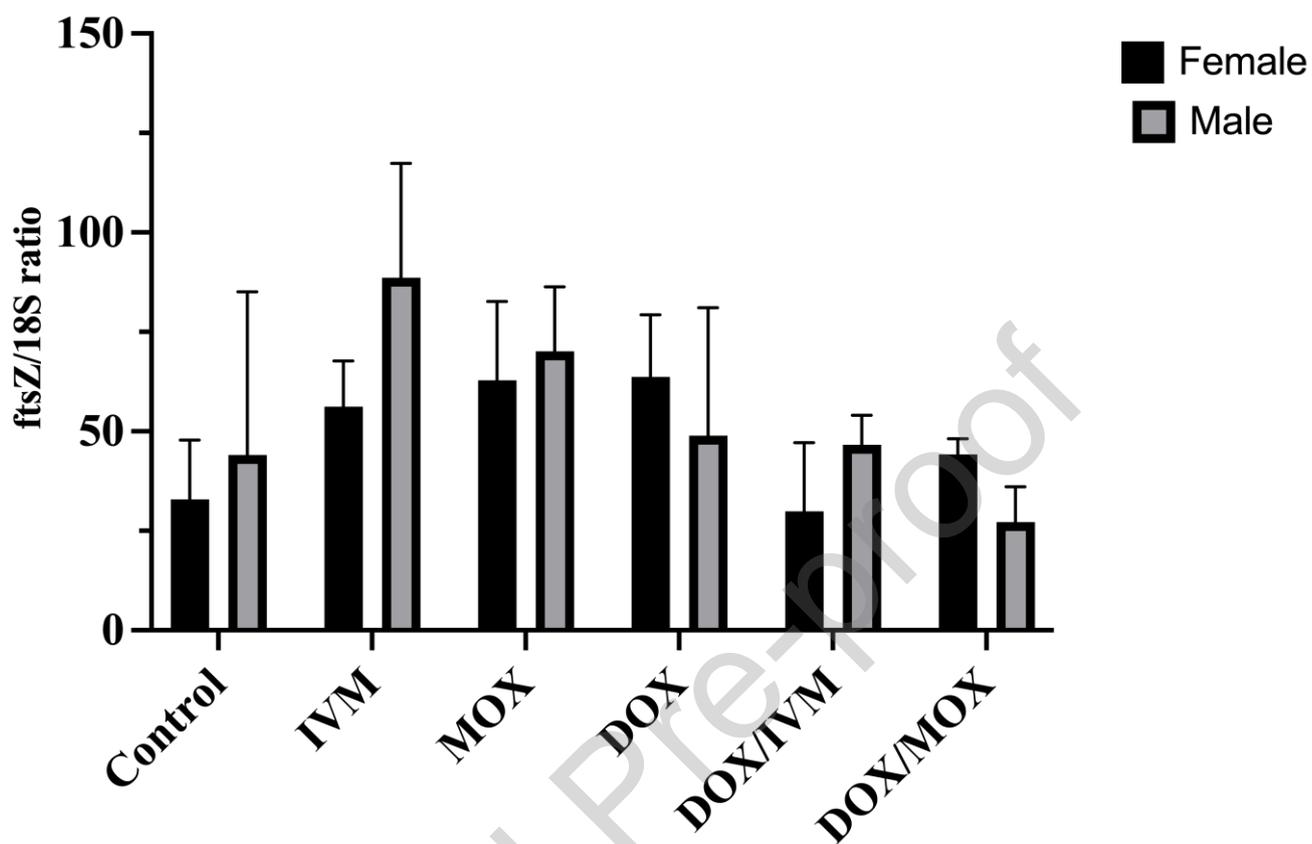


Table 1. Treatment protocols

Treatment	Definition
C	NI media + 1% DMSO
IVM	Ivermectin (3.54 nM) + 1% DMSO
MOX	Moxidectin (31nM) + 1% DMSO
DOX	Doxycycline (56.5 μ M) + 1% DMSO
DOX/IVM	Doxycycline (56.5 μ M) + ivermectin (3.54 nM) + 1%

	DMSO
DOX/MOX	Doxycycline (56.5 μ M) + moxidectin (31 nM) + 1% DMSO

Table 2. Characteristics of oligonucleotides used for the study of ABC transporters genes studied

Primer name	Forward primer	Reverse primer	Reference
Dim-<i>pgp-10</i>	5'-GCCATCGTAGGTCCATCAGGTTCTGGT-3'	5'-TGTTCAACTGAAACGACCACACGTC-3'	
Dim-<i>pgp-11</i>	5'-TTAACAGTGTTGATGAAGGATCAAATCC-3'	5'-ATATTCGCTGCGGTCTTGTGG-3'	Bourguinat et al., 2016
Dim-<i>haf-1</i>	5'-AGCACAGGAACCCATTCTAT-3'	5'-AGTTCCGTGTTCAACCAACAA-3'	
Dim-<i>haf-5</i>	5'-TCCAAGTGCATCCAAGGAAGAGG-3'	5'-TCGAATCACCGAATCGTGCAA-3'	
Di-<i>avr-14</i>	5'-GCAAGAAGACATCTTATTGACAAG-3'	5'-CGGGCAAGATAATACCAATGAAAG-3'	Yates and Wolstonholme, 2004

HIGHLIGHTS

- Gene expression following drug exposure is gender-dependent in *Dirofilaria immitis*.
- Expression of Macrocyclic Lactone receptor gene *Di-avr-14* is higher in males than females.
- Expression of ML receptor gene *Di-avr-14* is higher following combination treatment of worms with MLs plus doxycycline

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