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Jarrett Hines-Kay
Candidate

Biology
Department

This thesis is approved, and it is acceptable in quality and form for publication:

Approved by the Thesis Committee:

, Chairperson

Transcriptional analysis of *Schistosoma mansoni* treated with sub-lethal doses of the anthelmintic drug praziquantel

BY

Jarrett Hines-Kay

B.A., University of New Mexico, 2004
M.B.A., University of Arizona, 2007

THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science

Biology

The University of New Mexico
Albuquerque, New Mexico

07, 2011

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Jarrett Hines-Kay

M.S., Biology, University of New Mexico, 2011

ABSTRACT

Schistosoma mansoni is one of the most common etiological agents responsible for the disease schistosomiasis. More than 200 million people suffer from this disease making it the most severe tropical disease after malaria in terms of morbidity. Praziquantel (PZQ) is the treatment of choice for schistosomiasis and has been used almost exclusively to treat the disease since the 1970s. However, while the drug is lethal for sexually mature schistosomes, it is ineffective against juveniles. Thus, while morbidity can be eased, a cure is difficult to achieve. As a result there is an urgent need to develop a new generation of anti-schistosomal drugs, a task that will be made easier by understanding the mechanism of action of PZQ. As yet, neither the molecule to which PZQ binds nor the means by which it kills mature schistosomes is known. The overarching aim of this study was to understand the molecular basis of PZQ sensitivity in *S. mansoni.* We believe that juvenile worms survive PZQ treatment *in vivo* due to the induction of, as yet, unidentified protective molecular pathways. To address this hypothesis juvenile and adult PR1 *S. mansoni* were treated *in vitro* with sub-lethal concentrations of PZQ. mRNA was extracted from replicate samples, cRNA prepared and labeled with cyanine dyes for analysis using a 44K *S. mansoni* microarray. The data was then analyzed using Genespring. Our findings suggest that a number of genes associated with drug transport, iron homeostasis and apoptosis are induced in juvenile but not adult schistosomes and that this allows the juvenile worms to protect themselves against the lethal effects of PZQ long enough for the drug to be metabolized by the human host.

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Chapter 1

Background

More than 207 million people in over 70 countries suffer from the parasitic disease schistosomiasis (Steinmann *et al.*, 2006; Hotez and Fenwick, 2009). These individuals are largely infected with one of three species of schistosome (*Schistosoma haematobium*, *S. japonicum*, or *S. mansoni*) and the disease is implicated in over 200,000 deaths annually (WHO Schistosomiasis Fact Sheet) as well as the loss of approximately 24 – 56 million disability-adjusted life years (DALYs) (King, 2010). In fact, schistosomiasis is estimated to be the most severe tropical disease after malaria in terms of morbidity (Chitsulo *et al.*, 2004).

Schistosomes have a digenetic trematode life cycle that relies on only two specific hosts. In the case of *S. mansoni* the intermediate host is an aquatic snail of the genus *Biomphalaria* while the definitive host is a mammal, often humans. Free-swimming miracidia are hatched from eggs excreted in the definitive host's feces when the eggs come into contact with freshwater. These miracidia then seek their intermediate snail host, which they penetrate and then transform into sporocysts before undergoing successive rounds of asexual reproduction prior to leaving the snail as cercariae to infect their mammalian host. On penetrating the definitive host the worms sexually mature over a period of 4 – 6 weeks. Males and females pair and the female begins to release fertilized eggs. On average, an adult schistosome lives 3-5 years and an adult pair can hypothetically produce as many as 600 million schistosomes in their lifetime (Gryseels *et al.*, 2006). The most severe disease symptoms in the definitive host are caused when

schistosome eggs accumulate in the liver and invoke significant immune and inflammatory responses.

Praziquantel, an acylated quinoline-pyrazine, has been used almost exclusively to treat schistosomiasis since the 1970s. It is sold in 600 mg tablets costing around $ US 0.12 per tablet. A single oral dose of 60 mg/kg is considered sufficient to achieve cure rates of 60-90% (Caffrey, 2007). Praziquantel acts within one hour of ingestion, has low toxicity and no long-term safety difficulties have been noted (Gryseels *et al.,* 2006). One significant problem with PZQ is that it is largely ineffective at killing juvenile parasites (up to 4 weeks post infection), both *in vivo* (Andrews *et al*., 1977; Sabah *et al*., 1986) and *in vitro* (Pica-Mattoccia and Cioli, 2004; Aragon *et al*., 2009). Thus, the treatment regime must be repeated to kill worms that might have been at the juvenile stage of development when the initial treatment was administered.

In addition, though less relevant clinically, it has been shown that both *S. mansoni* miracidia and cercariae experience high levels of mortality when exposed to PZQ concentrations ranging from 1 µg/mL (~3.2 µM) to 313 µg/mL (~1.0 mM) (Andrews, 1978; Liang *et al.,* 2001). Conversely, *S. mansoni* sporocysts show little signs of death at 10 µg/mL (~32 µM) PZQ, but shedding interference occurs when 20 – 30 µg/mL (~64 – 96 µM) PZQ is added to the diet of *Biomphalaria glabrata* (Mattos *et al.,* 2006). Finally, Pica-Mattoccia and Cioli (2004) showed PZQ's ED_{50} *in vivo* was 30 times greater in 42 DPE than 28 DPE schistosomes. In addition, they demonstrated a large decrease in the mortality of schistosomes derived from single-sex female infections and a moderate decrease in single-sex male infections. The molecular basis of resistance to PZQ of

juvenile worms and those raised as single sex infections has yet to be elucidated. However, it is known that upon *in vitro* exposure to PZQ concentrations as low as 0.1 µg/mL (320 nM), *S. mansoni* worms exhibit muscular contraction (Pica-Mattoccia and Cioli, 2004). After being allowed to recover in drug-free medium, some worms recover to normal appearance and movement, but some remain contracted. These contracted worms show no signs of movement and often appear opaque. Pica-Mattoccia *et al.* (2004, 2007) defined this lack of movement, opaque appearance and permanent state of contraction as the criteria necessary to determine death. Using these criteria, it has been shown that adult male worms from bisexual infections begin to experience significant levels of mortality (approximately 50% dead 8 days after PZQ exposure) when exposed to as little as 0.5 µg/mL (~1.6 µM) PZQ overnight and complete mortality when exposed to 2 µg/mL (~6.4 µM) PZQ overnight (Pica-Mattoccia and Cioli, 2004). In comparison, the estimated effective therapeutic plasma concentration of PZQ is approximately 0.3 µg/mL (~960 nM) (Andrews, 1981), which is sufficient to cause muscular contraction and paralysis in schistosomes. However, it has been difficult to relate *in vitro* mortality to *in vivo* worm death because it is not readily apparent whether or not PZQ works *in vivo* by directly killing adult schistosomes or if the muscular contraction caused by PZQ simply dislodges the worms from the mesenteric and hepatic portal blood vessels allowing them to be swept to the liver where they are recognized by the host immune system and destroyed (Fetterer *et al.,* 1980).

Although the binding target and mode-of-action of PZQ remain to be discovered, it has been observed that in addition to the immediate muscular contraction and paralysis that *S. mansoni* undergo on exposure to PZQ, there is also an immediate influx of calcium ions

accompanied by a slower influx of sodium ions and a decreased influx of potassium ions (Pax *et al.,* 1978), which suggests PZQ may interfere with inorganic ion transport. It has also been observed that PZQ might alter the function of a schistosome voltage-operated calcium channel ($Ca_v\beta$) by acting on a subunit of this channel (Kohn *et al.,* 2001 and reviewed by Greenberg, 2005). It has been shown, however, that there is no difference in expression between adult and juvenile schistosomes in the genes that encode the $Ca_v\beta$ subunits (Valle *et al*., 2003; Aragon *et al*., 2009). This suggests that if PZQ binds to, or acts indirectly on, the $Ca_v\beta$ channel it is unlinked to resistance in juveniles. Complicating this story further, Pica-Mattoccia *et al.* (2008), showed that pre-incubation of adult male worms from single-sex infections with cytochalasin D caused increased calcium ion uptake despite the fact that cytochalasin D has previously been shown to suppress schistosomicidal effects (Pica-Mattoccia *et al.,* 2007), suggesting the calcium influx worms experience when exposed to PZQ does not fully explain PZQ's mechanism of action.

In addition to the interesting observations surrounding the calcium influx PZQ causes in schistosomes, another notable effect is the blebbing of worm tegumental and subtegumental structures caused by *in vitro* PZQ exposure (Shaw and Erasmus, 1984; Xiao *et al.,* 2009). This damage to the tegument is thought to expose surface antigens (Harnett and Kusel, 1986), which may contribute to the influx of calcium ions (Redman *et al.,* 1996) and possibly recognition and clearance of the parasite by the host immune system. Lending credence to this theory is the fact that tegumental damage and high rates of mortality in adult *S. japonicum* worms occurred with exposure to 10 – 30 µg/mL (~32 - 96 µM) PZQ while these same PZQ concentrations produced little to no blebbing and

no death in juvenile worms (Xiao *et al.*, 2009).

Another observation of PZQ's effect on schistosomes is that PZQ binds recombinant *S. mansoni* myosin light chain (rSmMLC) (Gnanasekar *et al.*, 2009). In addition, to these observations, Gnanasekar *et al.*, (2009) also showed that *S. mansoni* myosin light chain (SmMLC) was phosphorylated when exposed to PZQ *in vivo*, which is interesting because MLCs are generally calcium dependent proteins (Pfitzer, 2001) and their phosphorylation is thought to play a role in several biological processes including smooth muscle contraction (Satterwhite, 1992), thus providing a connection between PZQ, SmMLC, calcium and muscle contraction. Gnanasekar *et al.,* (2009) also noted that PZQ bound actin. This phenomenon was first observed by Tallima and El Ridi (2007); however, it and several other proposed PZQ modes of action such as the alteration of membrane fluidity (Harder *et al.,* 1987 and Lima *et al*., 1994), the inhibition of phosphoinositide turnover (Wiest *et al.*, 1992), the reduction of glutathione concentration (Ribeiro *et al.*, 1998) and adenosine antagonism (Angelucci *et al.*, 2007) were difficult to reconcile with the biology of PZQ and its differential action on juvenile and mature schistosomes.

More recently, researchers have started looking at epigenetic mechanisms that could be possible targets for future drug development. Epigenetic control refers to modifications of DNA and of DNA-associated proteins (i.e., histones) that affect chromatin structure. These modifications introduce heritable changes in gene expression caused by mechanisms other than changes in the underlying DNA sequence. Factors such as histone modification (acetylation, methylation and phosphorylation) and DNA

methylation play critical roles in chromatin compaction and thus control of transcription (Berger, 2007). *S. mansoni*, however, does not methylate its DNA, though histone-modifying proteins are present (Fantappie *et al.*, 2001). In *S. mansoni*, acetylation of histones plays an important role in epigenetic control, causing chromatin to be in its relaxed transcriptional state; for example, the transformation of miracidia to sporocysts can be blocked with histone deacetylase inhibitors (HDACi) (Azzi *et al.*, 2009). Histone deacetylases (HDAC) are a class of enzymes that remove acetyl groups from histones. Thus HDACi inhibit this activity, causing hyperacetylation of histones, which can in turn cause an increase in transcriptional activity. Histone deacetylase inhibitors have been shown to cause mortality; specifically, an increase in caspases 3 and 7 activities has been observed in adult *S. mansoni* treated with HDACi (Dubois *et al.*, 2009). Since caspases are involved in the apoptosis cascade, it is possible that PZQ acts through hyperacetylation of histones, which then leads to transcription of caspase genes and eventually worm death via apoptosis.

Due to the large number of people infected with schistosomiasis, massive public health efforts have been launched in the past several years, such as The Bill and Melinda Gates Foundation's Schistosomiasis Control Initiative, that have put millions of dollars into control through chemotherapy. Data from this initiative show the number of people treated in programs in Uganda, Burkina Faso, Niger, Tanzania and Zambia have increased from approximately 500,000 in 2003 to nearly 11 million in 2008. This increase in usage of PZQ to treat schistosomiasis raises fears of possible increases in parasitic resistance to the drug (Caffrey, 2007). With nearly $1/6^{th}$ of the world's global population either infected with schistosomiasis or at risk of infection (Chitsulo *et al.*,

2000), if widespread schistosome resistance to PZQ were to occur, it would likely cause a massive public health disaster. Thus, the need to discover the mechanism of action of PZQ as well as the underlying reasons for its ineffectiveness against juvenile worms is essential in order to inform the development of a new generation of anti-schistosomal drugs.

Treating schistosomes with PZQ *in vitro* is obviously different than treating an infected patient. For example, it is not known if PZQ directly kills *in vivo* or if the paralyzing effect observed upon *in vitro* exposure to PZQ allows the worms to be recognized by the host immune system, which then mounts a response resulting in death. Regardless, PZQ does kill adults *in vitro* while juvenile worms are able to recover. In order to understand the role of differential gene expression in the mechanism of PZQ killing in adult worms and the ability of juveniles to survive PZQ treatment, the first aim of my thesis was to identify sub-lethal exposures of PZQ. The second aim of my thesis was to conduct a transcriptional analysis of those identified sub-lethal PZQ exposures in order to measure the transcriptional differences between juvenile and adult worms. Finally, the third aim of my thesis was to identify molecular pathways and interactions that might be involved in schistosome protection and death when worms are exposed to PZQ.

Chapter 2

Methods

2.1 Schistosoma mansoni

Schistosome infected mice and snails were supplied by Dr. Fred A. Lewis, NIAID Schistosomiasis Resource Center at the Biomedical Research Institute (Rockville, MD). The abdomens of female Swiss Webster mice were exposed to 125 PR-1 *S. mansoni* cercariae. Mice were anesthetized and worms harvested by perfusion (Lewis, 1998) with RPMI 1640 (always containing L-Glutamine; Invitrogen) at 28 and 42 days post exposure (DPE). Mice were subsequently euthanized by cervical dislocation. All animal experimentation complied with the policies, regulations and guidelines mandated by the Institutional Animal Care and Use Committee (IACUC), University of New Mexico (UNM).

Adult worms (42 DPE) were separated into males and females by visual inspection. Juvenile worms (28 DPE) used were collected and maintained as mixtures of males and females as they are impossible to sex visually with any confidence at this time. Prior to all experiments, worms were allowed to recover overnight in enriched RPMI (RPMI 1640 containing 20 % heat inactivated fetal bovine serum (Sigma) and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin) (GIBCO) at 37 °C. This and all subsequent procedures that required worms to be maintained at 37 °C were performed using a water-jacketed incubator with 5% CO_2.

2.2 Mortality Study

Individual groups of 15 adult male, adult female or juvenile schistosomes derived from different mice were placed in separate Petri dishes containing 5 mL enriched RPMI 1640 with 1 % (v/v) dimethyl sulphoxide (DMSO; Sigma) and maintained at 37 °C for 1 h. After this time worms were exposed to 0, 0.1, 1.0, 10, 50 or 100 µg/mL racemic PZQ (Sigma) solubilized in 1% (v/v) DMSO in enriched RPMI 1640 for 0.25, 0.5, 1.0 or 20 h at 37 ^{o}C. After exposure, the worms were washed twice with RPMI 1640 and then allowed to recover in enriched RPMI 1640 at 37 ^{o}C. Each of these experiments was performed in triplicate.

Schistosomes were viewed at 4X magnification prior to PZQ exposure to ensure the worms being tested were alive, several minutes after PZQ exposure to observe if the worms contracted in response to the drug and again after 48 and 96 h recovery to determine the lethality of the various combinations of PZQ concentration and exposure time. At the end of the observation period, worms were defined 'dead' using the criteria of Pica-Mattoccia and Cioli (2004); i.e., if they remained contracted, did not resume movement during the observation period and acquired an opaque appearance.

Results were analyzed using a three-factor ANOVA (R version 2.12.1, cran.r-project.org) in order to determine which variables in the mortality study were significant as well as which variables were the most significant. The variables tested were DPE/sex, PZQ concentration and PZQ exposure time. Age and sex were combined in order to provide enough degrees of freedom to make the test significant for those variables. In addition, two-tailed homoscedastic student t-tests were performed in Excel (Microsoft) between

the control and experimental exposures in each group as well as between the groups at the same exposures in order to determine which treatments had significant levels of mortality and if the significant levels of mortality varied between the groups.

2.3 RNA Collection and Extraction

Adult male, adult female and juvenile worms were collected and exposed to PZQ in the same manner as described in section 2.2; however, the worms were collected in groups of 30 and exposed only to 0, 1.0 and 10 µg/mL PZQ for 1 or 20 h. In addition, the worms were harvested immediately after the exposure period by placing them in separate 1.5mL tubes with 600 µL of RLT Buffer (Qiagen) containing 1% (v/v) β-mercaptoethanol (Sigma) (RLT/β-ME buffer) and homogenizing them using a RNase-Free Pellet Pestle (VWR International; Aragon *et al.*, 2009). The homogenate was then frozen at –80 °C until RNA was extracted as described below. Each of these experiments was performed in triplicate thus providing three biological replicates for subsequent microarray and real-time PCR (RT-PCR) studies.

Total RNA was isolated from thawed worm homogenates using an RNeasy® Mini kit (Qiagen) including a DNase step according to the manufacturer's specifications, eluted with 100 µL of RNase free distilled water (dH_2O) and quantified using a NanoDrop® ND-1000 spectrophotometer with ND-1000 3.3 software (NanoDrop Technologies). In addition, RNA integrity was evaluated using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano LabChip® Kit (Agilent Technologies).

A total of 14 different samples of RNA were isolated and stored at -80 ^{o}C (Table 1) for further use in microarray and RT-PCR studies.

2.4 Reference RNA

To assist in the normalization of our microarray data (see sections 2.5 and 2.6) we employed a universal reference RNA sample (Novoradovskaya *et al.* 2004). This sample was derived mostly from 100 mixed sex adult schistosomes collected at 6 weeks after infection as previously described in section 2.2. These worms were allowed to recover for at least 1 h at 37 ^{o}C in enriched RPMI then homogenized, stored at -80 ^{o}C and had their RNA extracted in the same manner as described in section 2.3. To provide a positive hybridization signal at as many elements on the array as possible this RNA sample was spiked with 0.5 % (v/v) RNA isolated from adult mixed sex worms exposed to 10 µg/mL PZQ, 0.5 % (v/v) RNA isolated from juvenile worms exposed to 10 µg/mL PZQ and 0.5 % (v/v) RNA isolated from juvenile worms unexposed to PZQ.

2.5 Microarray Experiment

The oligonucleotide microarrays used in these studies were designed and described by Gobert *et al.* (2006) and obtained from Agilent Technologies. They have 12,166 *S. mansoni* features per array with four arrays per slide. Each *S. mansoni* gene featured on the array is represented by two independent oligonucleotides. These experiments were done in biological triplicates with no replicates on the same slide. All microarray experiments were performed according to Agilent's Two-Color Low Input Quick Amp Labeling kit protocol.

2.5.1 Spike- In Controls

Spike-in controls (Agilent Technologies) were prepared for signal standardization according to manufacturer's specifications. Two microliters of spike-in controls were added to approximately 100 ng of total RNA for each experimental sample as well as the reference sample. Spike B was added to each experimental sample and Spike A was added to each reference sample.

2.5.2 cDNA synthesis

cDNA was reverse transcribed from mRNA according to the kit protocol by adding a T7 Promoter Primer to the spiked samples and denaturing them at 65^{o}C for 10 min. A master mix containing a 5X first strand buffer, dithiothreitol (DTT), dNTPs and Affinity Script RNase Block Mix containing Affinity Script reverse transcriptase (Agilent Technologies) was then added to the samples and the mixture incubated at 40 ^{o}C for 2 h. The enzyme was then inactivated by incubation at 70 ^{o}C for 15 min.

2.5.3 cRNA synthesis, amplification and labeling

cDNA was transcribed to cRNA and amplified according to manufacturers instructions by adding a transcription master mix containing 5X transcription buffer, DTTs, NTPs and a blend of enzymes (Agilent Technologies) to the sample. The cyanine dye Cy-3 (Invitrogen) was then added to the reference samples and Cy-5 (Invitrogen) to the experimental samples and incubated at 40 ^{o}C for 2 h.

After the incubation period, the cRNA was purified using Qiagen's RNeasy Mini Kit and the RNA quantified. Calculations for labeling efficiency were performed using

manufacturer recommendations and only samples which met the manufacturer's suggested minimum activity for hybridization were processed further (Agilent Technologies).

2.5.4 Hybridization

A fragmentation mix was made by adding 825 ng total Cy-3 and Cy-5 labeled cRNA to 10X blocking agent and a 25X Fragmentation Buffer. This was then incubated at 60 ^{o}C for 30 min. The reaction was then placed on ice and a stop buffer was added to halt the reaction. Each sample was then pipetted onto a gasket slide sitting in a hybridization chamber and an array slide was then placed over the gasket slide sandwiching the samples into four separate chambers. The top of the hybridization chamber was then fitted to the samples and the whole apparatus was placed in an oven and rotated at 10 rpm and 65 ^{o}C for 17 h.

2.5.5 Wash and scan

After hybridization, the slides were washed in Agilent wash buffers and stabilized against ozone deterioration in Agilent's Stabilization Buffer. The slides were then scanned with Agilent's Microarray G2505C scanner and extracted using Agilent's Feature Extraction software by the University of California San Francisco's Viral Diagnostics and Discovery Center, China Basin Landing Facility.

2.6 Microarray Analysis in Genespring

The array data was analyzed using GeneSpring GX version 11 by first creating five separate experimental groupings: 28 DPE mixed sex 1 h PZQ exposures, 42 DPE male 1

h PZQ exposures, 28 DPE mixed sex 20 h PZQ exposures, 42 DPE male 20 h PZQ exposures and 42 DPE female 20 h PZQ exposures. A baseline transformation to the median of the control for each grouping (Table 1) was then performed. Features that were not positive, not significant, not above background noise, not uniform, saturated or a population outlier were not considered for analysis. Replicates in each grouping were then grouped based on PZQ concentration and only features with signal in all three replicates were retained. The signal from the replicates was averaged in each of the 14 treatment groups. The signal from these groups was $\log_2$ transformed. Differential analysis evaluated features that had fold change values greater than a magnitude of two. Analyses of significance compared the PZQ exposed groups to the control in each grouping with an unpaired, unequal variance t-test. Significant features had p-values < 0.05. In addition, Genespring was used to create a heatmap (Fig. 10).

2.7 Transcript Identification

The arrays used were custom arrays designed by Gobert *et al.* (2006) and have 12,166 *S. mansoni* features per array. Each feature has a probe sequence associated with it that is approximately 60 base pairs (60mer) long and represents a specific *S. mansoni* expressed sequence tag (EST). The EST's the oligonucleotides were designed to represent were accessed through the *S. mansoni* Genome Index (compbio.dfci.harvard.edu/tgi/tgipage.html) and blasted using NCBI's BlastX with both the Swissprot Protein Sequence Database and the Non-Redundant Protein Sequence Database (www.ncbi.nlm.nih.gov) as well as KEGG's BlastX database (www.genome.jp) and AmiGO's BlastX database (www.geneontology.org). In addition, GO term associations for each oligonucleotide were generated with AmiGO. Blast settings were

set so that only results with an Expect value less than 1E-05 were accepted. The four blast results for each feature along with the GO terms and Entrez Gene ID numbers were placed into Excel spreadsheets (Microsoft) where they could be searched for specific terms.

2.8 Real-Time PCR

Real-Time PCR (RT-PCR) was performed in two-steps by first creating complimentary DNA (cDNA) from the RNA isolated in section 2.3 and then using that cDNA as the template for RT- PCR.

2.8.1 cDNA Synthesis

Complimentary DNA was synthesized in a 20 µL reaction according to manufacturer's specifications using Transcriptor First Strand cDNA Synthesis Kit (Roche) with the kit's anchored-oligo(dT) primer and 100 ng of RNA template. The primer and template were combined and denatured at 65 °C for 10 min. Transcriptor RT 5X Reaction Buffer, RNase Inhibitor, dNTPs and Transcriptor Reverse Transcriptase were then added and the reaction was incubated at 50 °C for 60 min. Finally, the reverse transcriptase was inactivated at 85 °C for 5 min and the product quantified using a NanoDrop® ND-1000 spectrophotometer with ND-1000 3.3 software (NanoDrop Technologies).

2.8.2 Real-Time PCR

Subsequently, Real-Time PCR was performed to manufacturer's specifications using approximately 25 ng of cDNA (section 2.8.1) and the FastStart Universal Syber Green Master (ROX) kit (Roche) with 200 ng/µL of each of the forward and reverse primers

(Table 2) in a 25 µL reaction. Following an initial incubation at 50 °C for 2 min and a denaturation at 95 °C for 10 min, the reactions were amplified for 40 cycles of 95 °C for 15 s and 53 °C for 60 s on an ABI Prism 7000 Sequence Detection System. Each assay was performed in technical duplicates of each biological triplicates for all genes tested (Table 2).

The primer sequences shown in Table 2 were generated using both Primer3Plus (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and Integrated DNA Technologies OligoAnalyzer tool (www.idtdna.com). In addition, the OligoAnalyzer tool was used to help predict which primers would have the least chance of forming hairpins, self-dimerizing or hetero-dimerizing.

2.8.3 Analysis of Real-Time PCR data

Real-Time PCR data was first standardized with ABI Prism 7000 SDS software by manual adjustment of the threshold and baseline. Cycle threshold (C_t) values were then exported to Excel (Microsoft) where the technical duplicates were averaged and the values of the three biological replicates analyzed using the $2^{-\Delta\Delta}C_t$ method (Schmittgen and Livak, 2008) to obtain the relative expression ratio between the target genes (Table 2) and the endogenous control, *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

The housekeeping gene GAPDH (TC16678) was used for primary normalization as the endogenous control in all RT-PCR experiments. GAPDH was selected because it is a common reference housekeeping gene and was consistently expressed and unchanged in

all experimental and control groups in the microarray data, albeit at a low and non-significant level with a mean fold change of 0.44.

2.9 Creation of Additional Graphics

The Venn diagram in Figure 7 was created using the 4-way Venn diagram tool at www.pangloss.com/seidel/Protocols/venn4.cgi.

The map of significantly expressed gene overlap between groups (Fig. 6) was created with the data from the distance matrix in Table 10 and the Computational Biochemistry Research Group's TreeGen tool (www.cbrg.ethz.ch/services/TreeGen) using the 2DPlacement function. The distance matrix that was used as the input source into the 2DPlacement map was created by taking the amount of significantly expressed features that overlapped between each group in the same direction (i.e., either both upregulated or both downregulated in the treatment groups being compared) and then dividing that number by the sum of the total amount of significantly expressed features from the two groups being compared. This allowed for the similarity between two groups (amount of same direction gene overlap) to be displayed as a percentage of total significant gene expression. In addition, the area of each circle in Figure 6 correlates to the number of genes expressed in that group, so the 28 DPE 1 µg/mL PZQ 20 h group has the circle with the greatest area (3482 significantly expressed features) and the circle representing that group is approximately 20 time greater than the area of the circle representing the 42 DPE male 10 µg/mL PZQ 1 h group (170 significantly expressed features).

Chapter 3

Results and Discussion

3.1 *Mortality study of S. mansoni exposed to PZQ*

In order to conduct a microarray experiment that showed the transcriptional response of *S. mansoni* to *in vitro* exposures of PZQ, we first conducted a mortality study to identify sublethal PZQ concentrations over a variety of exposure times. We exposed adult male, adult female and juvenile mixed-sex worms to six different concentrations of PZQ (0, 0.1, 1.0. 10, 50 and 100 µg/mL) for four different time intervals (0.25, 0.5, 1.0 and 20 h). After the exposure period we allowed the worms to recover in drug-free media for 48 h before making mortality observations based on the criteria of Pica-Mattoccia and Cioli (2004), who defined a lack of movement, opaque appearance and permanent state of contraction as the definition of death in *S. mansoni.*

It can be seen in Table 3 that age/sex, concentration of PZQ and PZQ exposure time all had a highly significant effect on schistosome mortality (p-values<0.05). It can also be seen in the bottom four rows of Table 3 that the interactions between these variables are all highly significant as well. In other words, the concentration (p=8.27E-09) and exposure time (p=8.78E-05) have significantly different effects on adult male, adult female and juvenile worms. Finally, it can be seen from the f-values that the concentration of PZQ has the greatest effect on worm death (f=149.02) followed by PZQ exposure time (f=69.21).

Upon addition of PZQ to a final concentration of 0.1 µg/mL and greater, muscular contraction was observed immediately in worms in all groups (Fig. 1). Adult male worms were most sensitive to PZQ, reaching levels of mortality greater than 80% (Fig. 2A) when exposed to 10 µg/mL PZQ for 20 h (Table 4A) and there was significant mortality in all adult male groups exposed to 50 µg/mL PZQ irrespective of the exposure time (Tables 4A and 4D).

These results can be contrasted with the juvenile and adult female PZQ exposures that never achieved more than 40% mortality (Figs 3B and 3C). Adult females showed significant levels of mortality compared to untreated controls when exposed to 10 µg/mL PZQ for 20 h and 50 µg/mL PZQ for 1 or 20 h; however, the levels of mortality reached in these groups were all significantly less than the levels male worms reached in these same groupings (Tables 4B and 4E). This is consistent with other findings of variability in PZQ sensitivity between male and female schistosomes (Pica-Mattoccia and Cioli, 2004; Fitzpatrick *et al.*, 2005).

Juveniles reached significant levels of mortality only when exposed to 50 µg/mL PZQ for 20 h; however the levels of mortality reached were significantly less than the levels reached by males in this group (Tables 4C and 4F). The observation that juvenile worms are less sensitive to PZQ than adults has been described repeatedly in the literature (Xiao *et al*., 1985; Sabah *et al.,* 1986; Pica-Mattoccia and Cioli, 2004). In addition to visual observations of this phenomena, Aragon *et al.* (2009) were able to confirm juvenile worm insensitivity with a Real-Time PCR assay that utilized expression of specific heat shock related genes. Finally, the only significant difference between juveniles and females was

in the 50 µg/mL PZQ 1 h exposure group, where the juveniles experienced significantly less mortality than the females (Table 4F).

Overall, these experiments suggest that adult male worms are more sensitive to PZQ than the adult female or juvenile worms. One point of note was that these mortality experiments were also conducted using 100 µg/mL PZQ exposures for 1 and 20 h, however, these results were unreliable and are not shown. This was probably due to the fact that PZQ did not appear to become fully dissolved in solution at this concentration. In addition, the results from the 0.25, 0.5 and 1.0 h exposure times for adult and juvenile worms showed no significant difference between each other (Tables 5A, 5B and 5C), so only the 1 and 20 h exposure groups are compared in Fig. 1 andTable 4.

Thus in order to evaluate the transcriptional response of schistosomes to PZQ with the microarray, we chose nine sublethal exposure groups and five controls to the sublethal exposure groups (Table 1). The decision to use 1 as opposed to 0.25 or 0.5 h as the short-term PZQ exposure time with the microarray experiments was to provide sufficient time to ensure we saw a significant transcriptional response to the drug. Likewise, although the worms contracted when exposed to 0.1 µg/mL PZQ, we chose 1.0 µg/mL PZQ for the lower dosage of the drug to maximize the chance of detecting a transcriptional response. Conversely, we chose 10.0 µg/mL PZQ as the high-end exposure because it was this dose that we began to observe significant levels of death in adult schistosomes exposed for 20 h (Tables 3A and 3B); however, we did not evaluate the adult males at the 20 h 10 µg/mL PZQ exposure because the majority of them died at this exposure (Fig. 2) and this would not have provided for a robust transcriptional response to the drug.

3.2 *Transcriptional profile of S. mansoni treated with PZQ*

Since the mechanism of action of PZQ remains unknown, but it is known that juvenile worms are less sensitive to the lethal effects of the drug *in vitro* than adult worms, we performed a microarray analysis to better understand the transcriptional response of juvenile and adult *S. mansoni* to PZQ. Based on the findings from the mortality study, which showed not only a difference in PZQ sensitivity between juvenile and adult worms, but also a difference between adult female and adult male worms, we decided to also evaluate the transcriptional response of adult male and adult female schistosomes separately.

Upon treatment with PZQ, all groups expressed an increased amount of significantly expressed features (p-value<0.05 compared to group control; Table 6). The average number of genes differentially expressed (Fold Change > +/- 2.0) amongst the five control groups was only six, however, the average number of genes differentially expressed amongst the nine treated groups was 244 (Table 7). The groups with the greatest number of differentially expressed genes were the two 20 h juvenile PZQ treated groups, which averaged 741 differentially expressed genes. This was followed by the two 20 h adult female PZQ treated groups that averaged 182 differentially expressed genes. In contrast, the adult male treated groups showed differential expression of only 53 genes. Thus, the transcriptome of juvenile schistosomes appears to be more responsive to PZQ treatment than that of adult female and especially adult male worms (Table 7).

Overall, a signal was detected associated with array features representing 8582 unique genes expressed from all 14 different exposure groups with an average of 7855 expressed

genes per group (Table 8). The group with the most expressed genes was the males exposed to 1 µg/mL PZQ for 20 h (8332 genes). The least expressed genes were in the juvenile 20 h control group (7458 genes). On average half the genes were upregulated and half downregulated compared to the reference sample. The overall magnitude of the change in signal associated with these genes features was approximately neutral with a fold change of +0.01. Individual groups whose transcript profile showed a reduction in overall signal compared to the reference sample also had negative magnitudes of fold change with the exception of the juvenile 20 h exposure groups, which had approximately 52% of the transcript downregulated but a positive fold change in signal magnitude overall. The inference from this snapshot of the data is that juveniles exposed to PZQ for 20 h up-regulate a portion of their transcriptome to a greater extent than any other group.

This observation is supported when filtering the raw transcriptome data for array features reporting differential expression of at least a 2-fold change in signal. Using this criterion, 1323 genes are differentially expressed across all groups (column 8, Table 8). This represents approximately 15% of the different genes expressed; however, there is only an average of 159 genes differentially expressed per group, representing an average of only 2% of the expressed genes per group.

Juveniles exposed to PZQ for 20 h have the greatest number of differentially expressed genes with 868 and 614 differentially expressed genes in the groups treated with 1 and 10 µg/mL PZQ respectively (Fig. 3). Of a similar magnitude, females exposed to 10 µg/mL PZQ for 20 h have 322 differentially expressed genes. In comparison, all other PZQ treated groups of adult worms of either sex have far fewer differentially regulated genes.

Females exposed to 1 µg/mL PZQ for 20 h have only 41 genes differentially expressed while males exposed to 1 and 10 µg/mL PZQ for 1 h have only 42 and 50 genes differentially expressed respectively. Similarly, males exposed to 1 µg/mL PZQ for 20 h have only 66 genes differentially expressed. Finally, when filtering for differential expression there is a clear bias towards gene induction with an average of 63% of the differentially expressed genes being up-regulated.

To further determine if expression was significantly different ($p<0.05$) between the experimental and control treatments, unpaired, unequal variance T-Tests were performed on the microarray data for each feature. Overall there were 6161 different significantly expressed features (Table 6); however, there was only an average of 1526 significantly expressed features per group.

This large difference between the number of overall expressed features and the average amount of features expressed in each group indicates that there is a lot of variance in the genes that are being differentially expressed between the treatment groups. In other words, the transcriptional response to PZQ exposure is different amongst the groups we studied.

The group with the most significantly expressed features was the juveniles exposed to 1 µg/mL PZQ for 20 h (3482 genes; Fig 4). That with the least significantly expressed genes were those of males exposed to PZQ for 1 h (average of 189 genes). Thus, it appears that upon treatment with PZQ the adult male worms do not respond with a large transcriptional response, particularly when compared to the response of juvenile worms.

This trend of having greater transcriptional activity in both 20 h juvenile exposures as well as the 10 µg/mL PZQ 20 h female exposure is further highlighted when looking at the genes that are both significantly ($p<0.05$) and differentially (Fold Change > +/- 2.0) regulated (Fig 5 and Table 6) suggesting that these particular groups are not only transcribing more genes, but they are doing so with greater intensity, whether that be upregulation or downregulation. Probably the most significant fact to arise from these data, however, is that the juvenile schistosome response is different than that of the adult worms.

3.3 Comparing the transcriptome profiles of PZQ treated schistosomes

After evaluating the individual transcriptional responses of each grouping, we evaluated the transcriptional relationships between the treatment groups. In order to determine the degree by which the transcriptomes of the different PZQ groups overlapped, we created a distance matrix showing the number of significantly expressed genes ($p<0.05$ compared to control) that overlap in the same direction (i.e., significantly upregulated or downregulated) between each group (Table 9). We then took the overlap of the significantly expressed genes that overlapped in the same direction from Table 9 and made a second matrix showing the overlap as a percentage of the total amount of significantly expressed genes in the groups being compared (Table 10). Much like a matrix showing the relationships between different locations based on distance, the matrix depicted in Tables 9 and 10 shows the relationships of the different treatment groups from the microarray study based on the amount of gene overlap between the groups. The data from Table 10 then allowed us to map out these relationships in two dimensions, much like locations on a map, providing a visual representation of the

transcriptional similarity between all nine treatment groups (Fig. 6). Figure 6 displays the similarity between groups much like a Venn diagram might; however, we are able to evaluate the relationships between all nine groups in this manner as opposed to only being able to relate three or four groups at a time as is typical with Venn diagrams. In Figure 6, the percentage of overlap between each group is represented by the distance between the centers of each circle; hence, the closer the circles the greater the similarity in expression is between groups. In addition, the area of each circle correlates to the number of genes expressed in that group, so the 28 DPE 1 µg/mL PZQ 20 h group has the circle with the greatest area (3482 significantly expressed features) and this is approximately 20 time greater than the area of the circle representing the 42 DPE male 10 µg/mL PZQ 1 h group (170 significantly expressed features). From Figure 6 it can be seen that the greatest outliers from the main cluster are the 42 DPE male 1 and 10 µg/mL PZQ 1 h treatment groups. This is probably due to the fact that these groups have such a small transcriptional response to the affects of PZQ, significantly expressing an average of only 189 genes compared to an average of 1526 significantly expressed genes from all groups (Table 6). Other apparent outliers are the 1 and 10 µg/mL 28 DPE 1 h PZQ exposures. These two groups are highly related to each other, but it appears they are expressing a suite of genes that is largely different than any other group. The most closely related groups are the 1 and 10 µg/mL PZQ 28 DPE 20 h exposures together with the 1 and 10 µg/mL PZQ 42 DPE female 20 h PZQ exposures suggesting that these females are expressing a fairly similar suite of genes to the 28 DPE 20 h PZQ exposures. Sitting as an outlier from this main cluster is the 42 DPE male 1 µg/mL PZQ 20 h exposure. This is the group from the microarray study with the highest level of death at approximately 10% (Fig. 2). Thus, it can be inferred from Figure 6 that when *S. mansoni*

are exposed to PZQ for relatively long lengths of time (i.e., 20 h in this study) there is probably a fairly similar transcriptional response in terms of the actual genes being differentially regulated though the degree of that regulation may differ. Conversely, the suite of genes the 28 DPE 1 h PZQ groups are transcribing appears to be largely unique and may be indicative of a response to PZQ that allows them to survive the lethal effects of the drug long enough for the host to metabolize or excrete it. In contrast to the juvenile 1 h exposures, the 42 DPE male 1 h exposures are outliers not because they express a unique set of genes, but because they fail to mount a significant response to PZQ. It is this failure to respond adequately to PZQ that may ultimately lead to the high rates of mortality seen in adult male schistosomes (Fig. 2A).

Table 11 also displays a distance matrix relating overlap to total expression; however, the overlap is for all features, not just the features overlapped in the same direction. In Table 12 we then divided the features overlapped in the same direction (Table 10) by the total features overlapped (Table 11). From this we can see that ~100% of the overlap between the 28 DPE 1 µg/mL PZQ 20 h group and the 28 DPE 10 µg/mL PZQ 20 h group is in the same direction. Conversely, we can see that only about 12 % of the overlap between the 28 DPE 1 µg/mL PZQ 20 h group and the 28 DPE 1 µg/mL PZQ 1 h group is in the same direction. From Tables 10 and 12 and Fig. 6 we can also see that the overlap between the 28 DPE 1 h PZQ exposed groups is the greatest with each other and that overlap is expressed in the same direction. We can also see that this holds true for the 42 DPE male 1 h PZQ exposed groups, the 28 DPE 20 h PZQ exposed groups and the 42 DPE female 20 h PZQ exposed groups. The 42 DPE male 20 h PZQ exposed group does not share a majority overlap in the same direction with any other specific group. This lack

of relation to other groups can also be seen at the bottom of Table 9 (which shows the average overlap a group has to all the other groups) where the 42 DPE male 20 h PZQ exposed group has the smallest average relation to any other group (17%). The fact the 42 DPE male 20 h PZQ exposed group shows some relation to other groups, particularly the four other 20 h PZQ exposed groups, but not strong overlap with any of them is most likely due to the fact that this group is the most sensitive to PZQ of the groups tested in the microarray study (Fig. 2A). In addition, it can be seen in Figure 2A that the majority of worms are dead in the 42 DPE male 20 h PZQ exposed groups with concentrations greater than 1 µg/mL. Thus it is possible the reason for high mortality rates in adult male schistosomes is due to the fact that they are unable to express genes that allow juvenile and adult female worms to better survive the lethal effects of PZQ.

Another point of interest worth considering that arises from the data in Table 12 is the relationships of the overlapped regions to each other. For example, by taking the overlap between the two 42 DPE female 20 h PZQ exposed groups (93%) and the overlap between the two 28 DPE 20 h PZQ exposed groups (100%) and overlapping those overlaps, we find that ~89% of that overlap is in the same direction, so it can be implied that adult female schistosomes and juvenile worms have a broadly similar transcriptional response when treated to PZQ for 20 h. Similarly, if we examine the overlap between the two 28 DPE 20 h PZQ exposed groups (100%) and the 42 DPE male 20 h PZQ exposed group, we find ~79% is accounted for by shared genes being differentially expressed in the same direction. If, however, we take the overlap between the two 28 DPE 20 h PZQ exposed groups (100%) and the overlap between the two 28 DPE 1 hour PZQ exposed groups (99%) and compare those overlaps, we find that only about 10 % of the genes in

this category are regulated in the same direction. Figure 7 is a Venn diagram depicting this relationship between the up-regulated and down-regulated features from the 28 DPE 20 h PZQ exposed groups and the upregulated and down-regulated features from the 28 DPE 1 h PZQ exposed groups. It can be seen in Figure 7 that of the 1289 genes that are downregulated in both 28 DPE 20 h PZQ exposed groups, only six are also downregulated in the 28 DPE 1 h PZQ exposed overlap. It can also be seen that of the 1107 genes that are upregulated in both 28 DPE 20 h PZQ exposed groups, only 14 are also upregulated in the 28 DPE 1 h PZQ exposed overlap. Conversely, 176 genes show conflicting patterns of regulation between the 28 DPE 20 h PZQ exposed overlap and the 28 DPE 1 h PZQ exposed overlap and 2464 genes are not shared between these overlaps at all. Together this data suggests that the transcriptomic changes associated with exposure to PZQ in 28 DPE 1 h exposed schistosomes is very different than that of the 28 DPE 20 h PZQ exposed groups, suggesting that juvenile worms are able to mount a response to PZQ upon exposure that allows them to survive for a time, but that amount of time might not be indefinite as the mortality data in Figure 2C shows.

To reiterate, we believe that this analysis of the transcriptomic changes associated with each treatment group suggests that 42 DPE male *S. mansoni* do not mount a sufficiently robust response on exposure of PZQ and thus are the most sensitive to the lethal effects of the drug. In contrast, 28 DPE *S. mansoni* are able to mount a significant transcriptional response on initial exposure of PZQ, which may allow them to survive for some period of time.

3.4 Feature Annotation and Identification

To help identify and better understand the interesting transcriptional patterns we found in sections 3.2 and 3.3, we blasted the EST sequences the microarray probes represented with NCBI's Swissprot and Non-Redundant (NR) databases as well as KEGG and GO databases. Eighty seven percent of the 8582 expressed features had annotation from at least one of these databases (Table 13). Likewise, we found that 89% of the 6161 significantly expressed features had annotation from at least one of these databases. Finally, we found that approximately 97% of the 1052 significantly and differentially expressed features were annotated. This suggests a bias towards conserved genes within the cohort of the more highly differentially regulated genes in PZQ treated worms, which implies that PZQ's mode probably acts through previously understood mechanisms.

3.5 Confirmation of Transcriptional Profile

Real-Time PCR (RT-PCR) was employed to validate the gene expression data obtained from the microarray analyses and was performed on nine genes from the 28 DPE 1 µg/mL PZQ 20 h exposure group and four genes from the 28 DPE 10 µg/mL PZQ 20 h exposure group. Of the initial group of nine genes, three were chosen because they were significantly and differentially upregulated on the array (TC11991, TC16575 and TC7600), three because they were significantly and differentially downregulated (TC7830, TC14697 and TC14236) and three genes because they were not differentially expressed (TC10825, TC9859 and TC9950) (Fig. 8). The four genes from the juvenile 10 µg/mL PZQ 20 h group (Fig 9) consisted of two genes chosen because they were significantly and differentially upregulated (TC16575 and TC7600) and two genes that were significantly and differentially downregulated (TC14236 and TC14697). Figures 8

and 9 demonstrate that the RT-PCR study showed the same pattern of expression all the genes as was seen on the microarray. Tables 14 and 15 depict the expression results of both the microarray and the Real-Time PCR for the tested features.

3.6 Extreme Gene Regulation

To help with identification of genes that PZQ might be acting on, we generated a list of those genes undergoing the most extreme degrees of differential regulation compared to untreated controls. These were genes that not only had high fold-change values within individual treatment groups, but they also varied extensively between groups. For example, array feature TC13647, for which there was no annotation, was not only up-regulated over 20-fold in the 28 DPE 20 h PZQ exposed groups, but it was down-regulated approximately 3-fold in the 28 DPE 1 h PZQ exposed groups. There were 74 gene features that met these criteria and of these 13 were hypothetically expressed schistosome proteins and 20 had no annotation whatsoever, leaving only 55% of these extremely regulated genes annotated. Some interesting genes from this annotated group include calmodulin (TC18546), ferritin 1 (TC7601), ferritin 2 (TC7600), two egg proteins (TC9623 and TC7919), several heat shock proteins (TC16575, TC17408 and TC16535) and a drug transporter (TC14236). A list of all 74 features and the respective fold change values for each group can be found in Table 16. In addition, Figure 10 is a heat map depicting the expression of these 74 features. It can be seen from this figure that the 28 DPE 20 h PZQ exposures are upregulating the vast majority of these genes, the 28 DPE 1 h PZQ exposures are downregulating more of these genes than any other group and that the 42 DPE male 1 h PZQ exposures only significantly express two of these genes between them. This again lends credence to the idea that short-term PZQ exposed

juvenile worms are mounting a unique transcriptional response to the drug and that the short-term exposed adult male worms are relatively transcriptionally inactive.

3.7 Gene expression analysis

Clearly it would be overly time-consuming and perhaps relatively unproductive to examine each of the differentially expressed genes individually. Rather, we chose to select groups of genes for further investigation based on what is known about schistosome biology and the worm's physiological response to PZQ. Thus, we used the annotations described in section 3.4 to further investigate expression of four groups of genes; those involved in:

(i) calcium regulation

(ii) apoptosis

(iii) iron homeostasis

(iv) drug binding and detoxification

3.7.1 Calcium Regulation

Since a large influx of calcium ions is one of the most prominent observations in schistosome exposure to PZQ, we decided to look at how many calcium related genes were significantly expressed in the treatment groups. There were 186 different significantly expressed genes with a calcium related function and an average of 50 expressed in each treatment group (Table 17). The 28 DPE 20 h PZQ exposed groups expressed the most calcium related genes with an average of 105 being expressed and the 42 DPE male 1 h PZQ exposed groups expressed the least with an average of 4 being

significantly expressed. However, when accounting for calcium related expression as a percentage of total significant gene expression, the 28 DPE 10 µg/mL PZQ 1 h group expressed the most calcium related genes compared to the other groups (~4.31%) and the 42 DPE male 1 µg/mL PZQ 1 h exposure group the least (~1.44%) (Table 18). In addition, it can be seen that on average half of the significantly expressed calcium related genes were up-regulated. Though it appears the 42 DPE male 1 h PZQ exposed groups are upregulating the most calcium related genes (Table 19), they only are significantly expressing an average of 4 calcium related genes (Table 17) between them. Thus, it is truly the 42 DPE male 1 µg/mL PZQ 1 h group that is significantly upregulating the most calcium related genes (56%; Table 19).

Furthermore, when evaluating the overlap in genes related to calcium expression, it can be seen when comparing the two 28 DPE 1 h exposed groups, 20 significantly expressed genes overlapped in the same direction (i.e., up- or down-regulated). Included in the 11 upregulated genes were two calcium binding atopy-related autoantigens (TC15469 and TC8640), which are involved in binding calcium when mitochondrial calcium concentrations increase, calpain (TC17049), a mitochondrial solute carrier (TC19570) and an Na+/K+ transporting ATPase subunit alpha (TC7518). There were no genes from this cohort that had conflicting expression patterns (i.e., were up-regulated in one group but down regulated in the other), thus leaving 41 genes individually expressed in one or the other group. Of the 28 DPE 20 h exposed groups, 78 significantly expressed genes overlapped in the same direction. There were no genes from this cohort that had conflicting expression patterns, leaving 53 genes individually expressed. In the 42 DPE female 20 h exposed groups, 11 significantly expressed genes overlapped in the same

direction and three genes from this cohort had conflicting expression patterns, thus leaving 85 genes individually expressed. None of the 42 DPE male 1 h exposed group had genes that significantly overlapped with each other.

In addition, of the two 28 DPE 1 h PZQ exposed overlaps, 10 genes overlapped, but with conflicting expression. Among these were TC8640 and TC17049 as well as an annexin (TC15390). The full list and significant expression values can be found in Table 20. Of the 28 DPE 20 h overlap, four significantly expressed genes overlapped in the same direction with the 42 DPE female 20 h overlap and two genes overlapped with conflicting expression patterns. These genes and their respective significant expression values can all be found in Table 20.

3.7.2 Apoptosis

Calcium can be a trigger for apoptosis, or programmed cell death, through several mechanisms; one of them being that an influx of calcium can lead to activation of caspases (Li *et al.*, 2003; Mattson, 2007 and He *et al.*, 2000). Caspases, also known as cysteine proteases, are necessary for the cascade of events that eventually lead to programmed cell death (Fan *et al.*, 2005). In addition, blebbing, an outward bulge caused by destruction of a cell's cytoskeleton during apoptosis (Deschesnes *et al.*, 2001), as a result of *in vitro* PZQ exposure has been shown to occur in schistosome tegumental and subtegumental structures (Shaw and Erasmus, 1984 and Xiao *et al.*, 2009). With these observations in mind, we thought it would be prudent to further investigate the transcription of genes related to programmed cell death in our treatment groups.

We found 137 different significantly expressed genes with an apoptosis related function (Table 17). The 28 DPE 20 h PZQ exposed groups expressed the most apoptosis related genes with an average of 66 and the 42 DPE male 1 h PZQ exposed groups expressed the least with an average of 5 being significantly expressed.

When evaluating the overlap in genes related to apoptosis, it can be seen that of the 28 DPE 1 h exposed groups, 14 significantly expressed genes overlapped in the same direction and one gene, p30 DBC (TC7500), had conflicting expression between the two exposure groups. This leaves 26 apoptosis related genes individually expressed in the 28 DPE 1 h exposed groups. Of the 28 DPE 20 h exposed groups, 55 significantly expressed genes overlapped in the same direction. There was one gene, an annexin (TC16153), which had a conflicting expression between the two groups and left 19 genes individually expressed in this cohort. In the 42 DPE female 20 h exposed groups, 11 significantly expressed apoptosis related genes overlapped in the same direction and one gene from this cohort had a conflicting expression pattern, thus leaving 59 genes individually expressed. Finally, only one gene overlapped between the 42 DPE male 1 h exposed group, a member of the sno and ski oncogene family (TC14224) leaving 9 genes independently expressed in this cohort.

In addition, of the two 28 DPE overlaps, 7 genes overlapped, but with conflicting expression (Table 21). Six of these genes were downregulated in the 28 DPE 1 h exposed overlap and upregulated in the 28 DPE 20 h exposed overlap. Of these six, three were programmed cell death proteins (TC10907, TC11294 and TC11843), one had similar homology to death associated protein 5 (TC18668), one was an annexin (TC15390) and

one was a protein kinase (TC9163). This suggests that PZQ might inducing the apoptosis cascade via some unknown path or mechanism and that the juvenile schistosomes exposed to PZQ for 1 h are able to sustain this damage while the juveniles exposed to PZQ for 20 h are not and this may be the reason that we start seeing significant mortality in the 28 DPE 10 µg/mL PZQ 20 h group (Fig. 2). The 28 DPE 1 h exposed overlap also overlapped with five genes related to apoptosis from the 42 DPE male 1 µg/mL PZQ 20 h group. These were all regulated in the same direction and can be found in Table 21. The overlap from the 28 DPE 20 h exposed schistosomes overlapped with seven apoptosis related genes from the 42 DPE female 20 h exposed overlap all in the same direction. Included in the three upregulated genes were a programmed cell death protein (TC17324) and a homologue to death effector domain-associated factor A (TC9795). Finally, the overlap from the 28 DPE 20 h exposed overlapped with ten apoptosis related genes to the 42 DPE male 1 µg/mL PZQ 20 h group. Of these ten, two were upregulated in the 28 DPE 20 h exposed overlap and downregulated in the 42 DPE male 1 µg/mL PZQ 20 h group. These were an annexin (TC15390) and a serine/threonine protein kinase (TC17560). The other eight genes that overlapped between these groups were all regulated in the same direction. Included in the three of these eight that were upregulated was a gene that shared homology to death-associated protein kinase 3 (TC12214). Of the five that were downregulated was an inhibitor of apoptosis (TC19191) and a programmed cell death protein (TC13976). These genes all suggest that susceptible groups to PZQ such as the 42 DPE male 1 µg/mL PZQ 20 h group are undergoing some form of damaging apoptosis, perhaps to cells of the tegument, which again lends support to the idea that PZQ somehow induces apoptosis. These genes and their respective significant expression values can be found in Table 21.

3.7.3 Iron Homeostasis

Another interesting aspect of schistosome biology revolves around ferritin. Iron homeostasis is regulated by ferritin and the transferrin receptor (TfR) (Leibold and Guo, 1992). Typically, iron is brought into the cell via TfR mediated endocytosis of diferric transferrin. After being delivered to the endosome the iron is released and transferred to ferritin, a multisubunit molecule that helps sequester iron in the cytoplasm (Theil, 1987). Ferritin keeps iron in an available form for biological use in processes such as oxygen transfer, electron transfer, nitrogen fixation and DNA synthesis (Theil, 1990). In most eukaryotes ferritin is regulated posttranscriptionally. The reason for this is thought to be because of the toxic nature of free intracellular iron (Leibold and Guo, 1992), that can form reactive oxygen species (ROS) which can in turn cause the degradation of cellular constituents such as lipid membranes. Ferritin synthesis is controlled by cellular iron levels (Rouault *et al.,* 1992). Translational regulation of ferritin mRNA depends on a highly conserved regulatory region called the iron-response element (IRE) (von Darl *et al*., 1994 and Dunkov *et al.,* 1995). This IRE is found in all known ferritin mRNAs of higher eukaryotes (Theil, 1994) except for the yolk ferritin of *Lymnaea* (von Darl *et al*., 1994) and the ferritin of *S. mansoni* (Schussler *et al*., 1996). In the majority of higher animals when iron levels are low, the iron-response element binding protein (IRE-BP) binds IREs and this represses translation of ferritin and stabilizes TfR mRNA resulting in an increase in iron uptake and a decrease in iron stores (Leibold and Guo, 1992). Conversely, when there are high levels of iron, IRE-BP does not bind IRE causing the degradation of TfR mRNA and an increase in ferritin synthesis. This results in a decrease of iron uptake and an increase in iron stores. This, however, does not appear to be the case for *S. mansoni*. Schussler *et al*. (1995) demonstrated that *S. mansoni* posses two

isoforms of ferritin, soma (SmFER2) and yolk (SmFER1) ferritin. Soma ferritin is expressed at constant levels in most cells of both sexes perhaps because schistosomes are able to obtain constant supplies of iron from their host. One proposed way the worms achieve this is by digesting host erythrocytes in the gastrodermis by cytolytic and proteolytic enzymes that catabolize hemoglobin to free amino acids or dipeptides for gastrodermal syncytium absorption (Lawrence, 1973; Kasschau and Dresden, 1986; Brindley *et al.,* 2001). More recently, however, it has been proposed that trans-tegumental transport of host ferritin is the likely means of iron acquisition (Smyth *et al.*, 2006). Conversely, yolk ferritin is female-specific and expressed in high levels in the vitellarium. The roles for SmFER2 include early embryogenesis (Schussler *et al*. 1995) and the formation of cross-linked proteins in eggshell formation (Jones *et al.,* 2007). All schistosomes have a high demand for iron, but female worms have been shown to ingest as many as 330,000 host erythrocytes per hour, the equivalent of 0.88 µL of blood daily, whereas male worms ingest 39,000 erythrocytes per hour (Lawrence, 1973). In addition to having two types of ferritin, both types of *S. mansoni* ferritin lack an IRE sequence and no IRE-BP has been identified in the worms (Schussler *et al*., 1996). This may be related to the fact that schistosomes are thought to permanently ingest bound iron as erythrocytes or serum ferritin at constant levels so gene expression doesn't need to be adapted to iron concentrations. Finally, Schussler *et al*. (1996) concluded that they cannot rule out the possibility of non-canonical schistosome IRE or IRE-BP; however, they do not think it likely because they believe both SmFERs are controlled transcriptionally and independent of iron as is the case for ferritin in mammalian glioma cells (Yokomori *et al.,* 1991), thyroid cells (Chazenbalk *et al.,* 1990), pancreatic insulin cells (MacDonald *et*

al., 1994) and monocytes (Fahmy and Young, 1993) where ferritin is regulated by proteins other than iron.

Outside of these peculiar observation, little is known about the iron transport and regulation mechanisms that schistosomes possess; however, along with previously known iron storage molecules such as ferritin, schistosomes must possess sufficient machinery to survive for many years in their hosts' without fatal damage from iron-generated ROSs. Smyth *et al.* (2006) identified two divalent metal transporters, SmDMT1A and SmDMT1B, in *S. mansoni*. Through iron uptake assays with these DMT1s, Smyth and colleagues were able to rescue iron-transport deficient yeast, showing they were involved in schistosome iron transport. SmDMT1A was expressed in *S. mansoni* eggs, miracidia, cercariae, schistosomula and adult worms; whereas SmDMT1B was expressed primarily in schistosomula and adult worms, suggesting it is probably related to parasitism of the mammalian host. The cDNA of the two transmembrane DMT1s are almost identical except for a short N-terminal sequence that is predicted to be intracellular. Smyth *et al.* (2006) hypothesize that the "variable region (of DMT1) targets either a transporter to distinct membrane domains with distinct sets or partner regulatory proteins." The mechanism by which transferrin delivers its iron load remains elusive, but it has been shown that schistosomes bind host transferrin on their surface (Clemens and Basch, 1989). Not surprisingly these schistosome DMT1s are localized primarily to the worm tegument, which suggests "trans-tegumental transport as one means of iron acquisition" (Smyth *et al.*, 2006). However, the tegument is not involved in the degradation of erythrocytes (Xiao *et al.*, 2000) so ferric iron from the host's transferrin must be released through some surface-associated molecular pathway. This is a likely scenario since

endocytosis of host transferrin has been shown to exist in trypanosome (Fast *et al*., 1999) and *Plasmodium* (Rodriguez and Jungery, 1986). Smyth *et al.* (2006) conclude that it is possible a hypothetical schistosome transferrin receptor provides ferric ions for uptake by SmDMT1. In addition to the need to identify how host transferrin is endocytosed, Glanfield *et al.* (2007) believe that further investigation into heme utilization mechanisms is critical to understanding schistosome physiology.

Since so little is understood about schistosome iron homeostasis and because it is hypothesized that much of this process takes place in the worm tegument, an organ known to be differentially affected by PZQ, we decided to look more in depth at how genes related to iron homeostasis were being significantly expressed in our treatment groups. There were 68 different significantly expressed genes with an iron related function and an average of 18 expressed in each treatment group (Table 17). The 28 DPE 20 h PZQ exposed groups expressed the most iron related genes with an average of 38 being expressed and the 42 DPE male 1 h PZQ exposed groups expressed the least with an average of 4 being significantly expressed. It can also be seen that the 28 DPE 1 h PZQ exposed groups are expressing an average amount of genes related to iron (Table 18), but the percent of these genes that are upregulated is higher than any of the other groups with an average of 64% of the iron related genes being upregulated between the two treatment groups (Table 19). In addition, it is again the adult 20 h exposures that are downregulating the greatest number of iron related genes with an average of only 36% being upregulated and the obvious outlier from these is the 42 DPE male 1 µg/mL PZQ 20 h group with only 14% of iron related genes being upregulated. Furthermore, when evaluating the overlap in genes related to iron homeostasis, it can be seen that of the 28

DPE 1 h exposed groups, 7 significantly expressed genes overlapped in the same direction. Three of these seven are upregulated and include *S. mansoni* yolk ferritin (TC7601) and *S. mansoni* soma ferritn (TC7600). There were no genes from this cohort that had conflicting expression patterns, thus leaving 13 genes individually expressed. Of the 28 DPE 20 h exposed groups, 33 significantly expressed genes overlapped in the same direction. Again, no genes from this cohort had conflicting expression patterns, leaving only 8 genes individually expressed. In the 42 DPE female 20 h exposed groups, 5 significantly expressed genes overlapped in the same direction and none from this cohort had conflicting expression patterns either, thus leaving 33 genes individually expressed. None of the 42 DPE male 1 h exposed group had iron related genes that significantly overlapped with each other.

In addition, of the two 28 DPE overlaps (i.e., the significantly expressed gene overlap that overlaps between the 28 DPE 1 h PZQ exposed overlap and the 28 DPE 20 h PZQ exposed overlap), 4 genes overlapped and they were all expressed in the same direction. The two upregulated genes were SmFerritin1 (TC7601), yolk ferritin, and SmFerritin2 (TC7600), soma ferritin. The two downregulated genes were a mitochondrial intermediate peptidase (TC10450) and DNA primase large subunit (TC15620). Of the 28 DPE 20 h overlap, six significantly expressed genes overlapped in the same direction with the 42 DPE male 20 h overlap and one gene overlapped with conflicting expression patterns. The six genes in the same direction were all downregulated and included in them was a histone acetyltransferase-related gene (TC19229) and two HIRA interacting proteins (TC18476 and TC9147), which interact with (HIRA), a histone chaperone. This might suggest that free iron could be entering the schistosomes in these 20 h exposures

and creating damaging oxygen radicals (ROS). However, there was not a clear bias towards regulation of enzymes that protect against ROS damage. For example, there were two superoxide dismutase precursor proteins significantly expressed on the microarray (TC16777 and TC16781). They were both significantly upregulated in the 42DPE female 20 h PZQ exposed groups and TC16777 was significantly upregulated in the 42 DPE male 10 µg/mL PZQ 1 h exposed group. In addition, there were three superoxide dismutases significantly expressed on the microarray (TC10844, TC12708 and TC14056). TC10844, a copper/zinc superoxide dismutase was significantly upregulated in the 42 DPE male 10 µg/mL PZQ 1 h exposed group. TC12708, also a copper/zinc superoxide dismutase was significantly upregulated in the two 28 DPE 20 h PZQ exposed groups. Finally, TC14056, a manganese superoxide dismutase was significantly upregulated in the 42 DPE male 1 µg/mL PZQ 20 h exposed group and the 28 DPE 1 µg/mL PZQ 1 h exposed group. In addition TC8031, glutathione peroxidase-2, was significantly upregulated in the 42 DPE male 1 µg/mL PZQ 20 h group and downregulated in the 42 DPE female 10 µg/mL PZQ 20 h group. Finally TC10653, phospholipid glutathione peroxidase, was significantly upregulated in both the 42 DPE female 20 h PZQ exposed groups as well as the 28 DPE 20 h PZQ exposed groups. There is clearly a bias towards upregulation of these enzymes, which help protect against ROS damage, however, there is no clear pattern of regulation between the groups.

Iron metabolism in schistosomes is shrouded in mystery. Questions as to how ferritin is regulated, since it has been shown that ferritin lacks an IRE and no IRE-BP exists, and to why there are two ferritin isoforms remain largely unanswered. It is thought however that a receptor for host ferritin exists on the schistosome tegument and that the receptor is

most likely regulated transcriptionally by some unknown cue. Thus it is possible that disruption to the tegument, either through blebbing due to apoptosis or some other means, occurs in adult schistosomes allowing free iron to enter cells. The juvenile worms remain unaffected because their tegument is not as damaged and not as much iron enters. The adult female schistosomes are naturally prepared to handle much larger iron levels than males due to the fact that they possess a second ferritin isoforms, yolk ferritin. Thus adult male schistosomes become the most susceptible to damage from ROS and experience the highest levels of mortality (Fig. 2).

3.7.4 Drug Binding and Detoxification

The previous observations, particularly with iron related genes, suggest the 28 DPE 1 h PZQ exposed worms are able to do something none of the other groups can manage. However, the most striking example of this observation might be in the genes associated with detoxification. Obviously these genes have not evolved to excrete PZQ from *S. mansoni* in the evolutionarily brief period of time since PZQ has been used to treat schistosomiasis, but many drug transporters are not specific for clearance of one substance. Instead they are capable of clearing multiple toxins from an organism as is the case with MDR1 in *Plasmodium falciparum*, which can confer the parasite with resistance to mefloquine, lumefantrine, halofantrine, quinine and artemisinin (Sidhu *et al.*, 2006). In our study we found 31 different significantly expressed genes with a drug related function and an average of 12 expressed in each treatment group (Table 17). Of these, three had a drug binding function, two were involved in drug metabolism, nine were involved in a response to drugs and most of the others had some sort of role in drug transport. The 28 DPE 20 h PZQ exposed groups expressed the most drug related genes

with an average of 19 and the 42 DPE male 1 h PZQ exposed groups expressed no drug related genes. However, when accounting for drug related expression as a percentage of total significant gene expression, it can be seen that both 28 DPE 1 h PZQ exposed groups are dedicating a greater percentage of their transcriptome to expression of drug related genes than any of the other treatment groups (Table 18). In fact, the 28 DPE 10 µg/mL PZQ 1 h group is significantly expressing almost three times the amount of drug related genes as the 42 DPE male 1 µg/mL PZQ 20 h group, which was downregulating the five drug related genes that were being expressed in that group. In addition, the average amount of upregulated drug related genes from all the 20 h PZQ exposed groups is approximately 17%; whereas, the average upregulated genes from the 28 DPE 1 h PZQ exposed groups is ~67% (Table 19).

Furthermore, when evaluating the overlap in genes related to drug expression, it can be seen that of the 28 DPE 1 h exposed groups, 4 significantly expressed genes overlapped in the same direction. Three of these were upregulated and include ABCG2 (TC10285), which is known to transport drugs out of cells (Koenderink *et al.,* 2010), dihydropyrimidinase related protein-2 (TC13625) and cyclin B (TC17205), both of which have been shown to be involved in responses to drugs (AmiGo). The downregulated gene from this cohort was ABCF3 (TC15933), which encodes a protein of unknown function (Dean *et al.,* 2001). There were no genes from this cohort that had conflicting expression patterns, thus leaving 10 genes individually expressed. Of the 28 DPE 20 h exposed groups, 14 significantly expressed genes overlapped in the same direction. Two were upregulated, a prohibitin homologue (TC10689) and a HlyD family secretion protein homologue (TC12637), both involved in response to drugs. The 12 genes that

were downregulated in both 28 DPE 20 h PZQ exposed groups included an ABCB1 member (TC14236), SMDR2 (TC14689), ABCB7 (TC17965 and TC8655), ABCG2 (TC8681) and cAMP-specific 35-cyclic phosphodiesterase (TC11920), which is known to bind drugs. There were no genes from this cohort that had conflicting expression patterns, leaving 10 genes individually expressed. In the 42 DPE female 20 h exposed groups, 6 significantly expressed genes overlapped in the same direction and they were all downregulated leaving 8 genes individually expressed. Included in the six downregulated genes were an ABCB1 member (TC14236), SMDR2 (TC14689) and an ABCG2 (TC8681) member. None of the 42 DPE male 1 h exposed group had genes any significantly expressed drug related genes.

From this drug related expression data, the ATP-binding cassette transporters (ABC transporters) are probably the most interesting group of genes. ABC transporters that actively transport drugs have been shown to confer drug insensitivity to the organism expressing them. Recent work by Kasinathan *et al.* (2010a) showed SMDR2 (TC14689) was expressed at higher levels in *S. mansoni* with reduced susceptibility to PZQ and that SMDR2 was a substrate for PZQ. Kasinathan *et al.* (2010b) went on to show that SmMRP1 and SMDR2 were expressed at higher levels in the PZQ insensitive juvenile worms than adults and they concluded that, “increases in levels of schistosome multidrug transporters may be involved in development or maintenance of reduced susceptibility to PZQ.” However, in the study performed by Kasinathan *et al.* (2010b) they compared RT-PCR data for unexposed juvenile worms, choosing just to look at normal expression of the drug transporters. In our study, we examined the response to genes encoding *S. mansoni* MDRs when exposed to PZQ. Like Kasinathan *et al.* (2010a and 2010b), our

data indicated that there probably was a response amongst drug transporters to the effect of PZQ and that juvenile worms exposed to PZQ for 1 h were expressing drug transporters at higher levels than adults, but at the 20 h exposure the juvenile worms were downregulating the majority of drug transporters much like the adults. This phenomenon might explain why juvenile schistosomes start to experience significant levels of mortality when exposed to PZQ for 20 h *in vitro* (Fig. 2).

ABC transporters are part of a large and ancient superfamily that are involved in transporting a wide variety of substances from metals to lipids. There are seven ABC transporter gene subfamilies in mammals, named ABCA through ABCG. These subfamilies can have one or several representatives (Dean *et al.,* 2001). Some ABC transporters are involved in pumping out drugs from cells, which decreases intracellular drug concentrations and ultimately confers organismal resistance to the drug (Borges-Walmsley *et al.,* 2003). These ABC transporters are known as multiple drug resistant proteins (MDRs) and include classes of p-glycoproteins (PGPs), multidrug resistant proteins (MRPs) and breast cancer resistant protein (BCRP). ABC subfamilies that are known to actively transport drugs include the B, C and G subfamilies (Koenderink *et al.,* 2010). The typical structure of ABC transporters is two transmembrane domains (TMDs), with six transmembrane helices (TM) in each domain, as well as two cytosolic nucleotide-binding domains (NBDs) that bind ATP (Koenderink *et al.,* 2010). The rearrangement of the TM helices is the mechanism ATP transporters utilize to translocate substrate across the membrane (Jones *et al.,* 2009). This mechanism is induced by the dimerization of the NBDs after ATP binds, hydrolyzes and flips them. Despite this knowledge, there still is very little understanding of the drug binding sites or how many

sites a transporter might posses (Borges-Walmsley *et al.*, 2003). For example, it has been shown that *P. falciparum* MDR1 conferred parasite resistance to mefloquine, lumefantrine, halofantrine, quinine and artemisinin (Sidhu *et al.*, 2006), but not to chloroquine (Koenderink *et al.*, 2010). ABC transporters can be encoded as full transporters (TMD-NBD-TMD-NBD) or as half transporters (TMD-NBD) that combine to form a full functional unit when they are translated into proteins (Koenderink *et al.*, 2010).

ABC transporter members are typically placed in their subfamilies based on phylogenetic analysis of the NBDs (Sauvage *et al*., 2009). The publication of the *S. mansoni* genome (Berriman *et al.*, 2009) allowed for the prediction of 24 genes that encode ABC transporters in *S. mansoni*; 8 B subfamily members, 9 C subfamily members and 4 G subfamily members were among the 24 transporters. Of these 21 ABC transporter genes in the *S. mansoni* genome related to drug transport, seven were identified as significantly expressed in our microarray data; ABCG1 (TC9950), two members of ABCG2 (TC8681 and TC10285), two members of ABCB1 (TC14236 and TC14689 also described as SMDR2), an ABCC1 member (TC9859) and an ABCB7 member (represented by both TC17965 and TC8655). In addition, we obtained expression values for two more drug transporters that were not on the array with our RT-PCR study. Those included a member of ABCB8 (SmDR1) and another ABCC1 member (SmMRP1). Expression data for these transporters can be found in Table 22.

SMDR1, an ABCB8 member, has been shown to encode a half transporter (NBD-TMD) (Bosch *et al*., 1994), which is typical of all subfamily B members with the exception of

the B1 members (Koenderink *et al.,* 2010). In support of this finding in other organisms, SMDR2 (TC14689), an ABCB1 member, was shown by Bosch and colleagues to encode a full transporter consisting of two NBDs and two TMDs, or twelve total TMs. Thus, it is likely that the ABCB7 (TC17965 and TC8655) member we found in our data encodes a half transporter and the other ABCB1 (TC14236) member encodes a full transporter. The protein encoded by the ABCB1 gene is known both as PGP and SMDR2 (Kasinathan *et al*., 2010a), so two PGPs are encoded by TC14236 and TC14689; the protein encoded by ABCB8 is SMDR1 (Dean *et al.,* 2001); and the protein encoded by ABCB7 (TC17965 and TC8655) has been shown to be a mitochondrial transporter involved in biogenesis of iron-sulfur clusters (Zutz *et al*., 2009). ABC transporter subfamily C transporters are known to encode full transporters (Koenderink *et al.,* 2010), thus, both TC9859 and SmMRP1 probably both encode two full transporters called MRP1s. Finally, ABC transporter subfamily G members typically encode half transporters (Koenderink *et al.,* 2010), making this the likely scenario for TC10285 (ABCG2), TC8681 (ABCG2) and TC9950 (ABCG1). In mammals, ABCG2 encodes BCRP (Dean *et al.,* 2001) and can transport many various substrates across membranes (Vlaming *et al.,* 2009).

Interestingly, only one ABC C subfamily member was present on the array (TC9859). In *Plasmodium falciparum* the ABC C subfamily has been shown to only be present in asexual stages of the parasite (Koenderink *et al.,* 2010). Similarly, TC9859 was present in juvenile schistosomes; however, it was upregulated in the 1 µg/mL PZQ 1 h exposure and down regulated in the 1 µg/mL PZQ 20 h exposure, possibly offering some insight into why the 1 h exposed juveniles do not experience any significant levels of mortality, but the 20 h exposures do (Fig. 2). In addition, as with 20 h PZQ exposed juveniles,

TC9859 was shown to be downregulated in adult female worms. *Schistosoma mansoni* adults lay numerous amounts of eggs, which are mostly responsible for the pathology of schistosomiasis. This egg production requires a lot of energy. To meet these high energy demands, schistosomes essentially transport a constant stream of glucose from the host's blood via diffusion across their tegument (Skelly and Shoemaker, 1996). These demands are so high that adult worms consume their weight in glucose every 5 h (Bueding, 1950). In addition, drug transport via ABC transporters is energetically expensive, requiring two molecules of ATP to successfully transport its substrate across a membrane (Dean, 2001). Thus, it is possible that one of the reasons female schistosomes might be less sensitive to the lethal effects of PZQ than the adult male worms is because they posses this ABCC drug transporter, but after extended exposure to the drug (i.e., 20 h), the energy demands of the transporter cause its downregulation and the parasite begins to experience significant levels of mortality. Finally, we studied another member of the ABC C family, SmMRP1, with RT-PCR and found similar results as to TC9859 expression (Table 22). We targeted SmMRP1 in the juvenile exposure groups and found significant up regulation in the 1 h exposed groups and significant downregulation in the 1 µg/mL 20 h exposure group, again lending support to the idea that ABC C subfamily members might be involved in protecting juvenile schistosomes. Although juvenile worms do not lay eggs, and as a consequence have less energy demands than sexually mature adults, the downregulation after 20 h exposure suggests that like the adult female worms, juvenile schistosomes are unable to sustain the ATP demands of the transporters indefinitely.

In addition to testing SMDR1 and SmMRP1 with RT-PCR, since they weren't on the array, we also chose to confirm expression of five other drug transporters from the array

by RT-PCR: TC10285, TC14236, TC14689, TC9859 and TC9950. In this study, there was gene expression conflict between the RT-PCR and microarray results for three data points. It can be seen in Figure 11 that SMDR1 is significantly upregulated ($p<0.05$ compared to the endogenous control SmGAPDH) in the 28 DPE 1 µg/mL PZQ 1 h group, but not significantly expressed in the 28 DPE 10 µg/mL PZQ 1 h group or the 28 DPE 1 µg/mL PZQ 20 h group. It can also be seen that SmMRP1 is significantly upregulated in both 28 DPE 1 h PZQ exposed groups, but downregulated in the 28 DPE 1 µg/mL PZQ 20 h group. The rest of the data matched the significant expression from the microarray with the exception the 28 DPE 1 µg/mL PZQ 1 h group's genes TC10285, which was significantly upregulated on the array and upregulated in the RT-PCR data though not significantly, and TC9950, which was significantly downregulated on the array and not significantly expressed with RT-PCR. As well as TC 14689 in the 28 DPE 1 µg/mL PZQ 20 h, which was significantly downregulated on the array and downregulated in the RT-PCR data though not significantly (Fig. 11).

In all, there was significant expression of nine ABC drug transporter genes in the two 28 DPE 1 h PZQ exposed groups and seven of them were upregulated (Table 22). This can be contrasted with the two 28 DPE 20 h PZQ exposed groups which significantly expressed 13 ABC drug transporter genes that were all being downregulated compared to the group control. The 42 DPE male 1 h PZQ exposed groups didn't significantly express any ABC drug transporters and the 42 DPE male 20 h PZQ exposed group only expressed an ABCB7 member, which was downregulated. Finally, the two 42 DPE female 20 h PZQ exposed groups significantly expressed 10 ABC drug transporter genes, three of which were upregulated compared to the group control.

This data suggests that juvenile worms are transcriptionally able to upregulate drug transport related genes in the short-term that allow them to survive the lethal effects of PZQ until the host has metabolized the drug. However, it appears that this response is not perpetual as is made evident by the fact that the 28 DPE 20 h PZQ exposed groups are downregulated their drug transport related genes and they are beginning to experience significant levels of mortality (Fig. 2). The reason that juvenile worms might not be able to sustain prolonged drug transport might be related to the fact that these transporters require ATP to excrete the drugs. In addition, this might explain why adult worms die from PZQ exposure as well. It might not only be due to the fact that they, for some reason, are unable to express as many, or the correct, drug transporters, but the high ATP demands of the transporters coupled with the stage in their life when they are mating, which likely has high energy demands of its own, is too much of a burden for the schistosomes and they begin to suffer significant levels of mortality.

Chapter 4

Conclusion

The schistosome tegument is a critical organ as it is the principle interface with the host, including the blood. Hence, there are many important proteins expressed in the worm tegument related to nutrient uptake, maintenance of solute balance (Braschi *et al*., 2006a), transport, structure, enzyme activity (Braschi *et al*., 2006b) and immunity (Castro-Borges *et al.*, 2011). Though the means by which PZQ damages the tegument of schistosomes is unknown, the observation that blebbing occurs in mature (but not juvenile) worms **(**Xiao *et al.*, 2009) suggests a possible role for apoptotic pathways (Deschesnes *et al.*, 2001)**.** Since juvenile worms contract upon PZQ exposure in much the same way as adult worms (Pica-Mattoccia and Cioli 2004) but are much less sensitive to PZQ (Xiao *et al*., 1985; Sabah *et al.*, 1986; Pica-Mattoccia and Cioli, 2004), it is perhaps unlikely that the observed calcium ion influx that occurs on exposure to PZQ is actually related to *in vitro* worm mortality. In addition, if the blebbing that occurs to the mature worm tegument is caused by apoptosis, calcium is probably not the trigger. However, identifying what might be triggering programmed death in cells on the surface of schistosomes, where they come into contact with PZQ, is difficult to ascertain from complex transcriptional data alone as many of the proteins involved in apoptosis are preformed and the process is complicated, involving many genes that have multiple roles (Hengartner, 2000). Thus combining the microarray analysis and a study of the molecules that PZQ binds to directly, might provide insight into how PZQ initiates a cascade leading to apoptosis. Furthermore, since significant levels of mortality were found in juvenile worms exposed to PZQ for 20 h (Fig. 2), it is possible that whatever protects juvenile schistosomes from

the lethal effects of PZQ is not perpetual and they begin to lose the mechanism that allows them to weather PZQs lethality at some point and this leads to death. Thus, this makes genes that show different expression patterns between the 28 DPE 1 h exposed groups and the 28 DPE 20 h exposed groups particularly interesting. From our analysis of apoptosis related genes we found three programmed cell death proteins (TC10907, TC11294 and TC11843), two death associated protein kinases (TC18668 and TC12214) and death effector domain-associated factor A (TC9795) which were all downregulated compared to the group control in the 28 DPE 1 h exposed groups, but upregulated in the 28 DPE 20 h exposed groups and some of the other adult 20 h exposed groups (Table 21). These six genes are all interesting targets for RNAi knockdown in juvenile worms that will be exposed to PZQ for 20 h to see if the decreased expression in these genes leads to a reduction in sensitivity to PZQ.

Since PZQ has been shown to cause tegumental disruption, particularly in adult male worms (Shaw and Erasmus, 1984; Xiao *et al.*, 2009), this likely interferes with many critical schistosome biological processes in adult worms. One of these processes is likely iron homeostasis, since the worms bind host ferritin on their surface (Clemens and Basch, 1989) and likely endocytose it through a hypothetical receptor on the worm tegument (Smyth *et al.*, 2006). However, since adult female worms also possess a second ferritin isoform, yolk ferritin, they are capable of binding much more intracellular iron than adult male worms. In fact, adult female schistosomes have such a high demand for iron, they can ingest almost 10 times the number of erythrocytes per hour as adult male worms (Lawrence, 1973). Thus, if tegumental damage caused by exposure to PZQ increases intracellular iron levels, it is possible adult male worms will experience fatal damage

from oxygen radicals; however, the adult females will experience these effects much less since they are able to bind greater amounts of iron due to the fact they possess yolk ferritin. In addition, the effects will be much less for the juveniles since they do not experience the same degree of tegumental damage as the adults. Our array data show that 28 DPE 1 h PZQ exposed worms are significantly downregulating both yolk ferritin (TC7601 and TC7940) and soma ferritin (TC7600 and TC17896), however, when they are exposed to PZQ for 20 h, significant expression of both ferritin isoforms dramatically increases (Table 16) even though they are sexually immature and not producing eggs. The 42 DPE female 20 h exposed schistosomes are also upregulating both ferritin isoforms; however, there is no significant regulation of ferritin in any of the adult male treatment groups. This again suggests that the adult male tegument is disrupted, which allows exposure to toxic compounds such as free iron while at the same time destroying the unknown regulatory cue to produce more ferritin to bind the free iron. This effect is not experienced by the juvenile worms which undergo very little tegumental damage and thus they have no need to produce more ferritin. After long-term exposure to PZQ, however, the small amount of damage to the juvenile tegument begins to take its toll and the worms begin to express more ferritin to bind the free iron. The females are better able to cope as they naturally produce more ferritin, but at the 20 h PZQ exposure we begin to see significant levels of mortality in both the adult female and juvenile worms (Fig. 2). To explore this theory further, work toward identifying whether or not there is increased tegumental damage to juvenile worms exposed to PZQ for long periods of time (i.e., 20 h) should be undertaken. In addition, it is important to identify the proposed transferrin receptor and the mechanism of iron store regulation as this might provide novel insight into possible new therapeutic targets to combat schistosomiasis. Finally, RNAi

knockdown of the two ferritin isoforms in adult female schistosomes in order to see if they become more sensitive to the fatal effects of PZQ might provide insight into whether or not mortality in adult males is linked to iron influx.

The possibility that PZQ damages the worm tegument through some mechanism like apoptosis, which then allows entry of damaging compounds like iron to enter the schistosome seems feasible and even likely given the results of our data; however, it doesn't explain why the tegument of adult worms is disrupted more than the tegument of juveniles. One possibility for why juvenile worms are less sensitive than adults is related to expression of drug transporters. There are approximately 24 *S. mansoni* ABC transporters (Berriman *et al.*, 2009) and 21 of them are in the B, C and G subfamilies, whose members are known to actively excrete drugs (Koenderink *et al.*, 2010). Of these 21 drug transporters, nine were significantly expressed in the treatment groups we studied. In all, there was significant expression of nine ABC drug transporter genes in the two 28 DPE 1 h PZQ exposed groups and seven of them were upregulated (Table 22). This can be contrasted with the two 28 DPE 20 h PZQ exposed groups which significantly expressed 13 ABC drug transporter genes that were all downregulated compared to the group control. The 42 DPE male 1 h PZQ exposed groups didn't significantly express any ABC drug transporters and the 42 DPE male 20 h PZQ exposed group only expressed one downregulated drug transporter. Finally, the two 42 DPE female 20 h PZQ exposed groups significantly expressed 10 ABC drug transporter genes, three of which were upregulated. The two ABC C subfamily members (TC9859 and SmMRP1) were upregulated in the 28 DPE 1 h PZQ exposed groups and down regulated in the 28 DPE 1 µg/mL PZQ 20 h group. This is of particular interest because ABC

subfamily C members are only expressed in asexual stages of *P. falciparum* (Koenderink *et al.*, 2010). Thus it is possible that some *S. mansoni* ABCC transporters are involved in excretion of PZQ but not expressed in adult male schistosomes. This possibility makes these genes ideal targets for RNAi knockdown in juvenile worms exposed to PZQ for 1 h to see if they become susceptible to the drug. In addition, further studies into age-specific expression of drug transporters might provide key insights into other ABC transporters that are expressed only in sexually immature worms and thus might be playing a role in PZQ excretion. Other possible ABC transporter targets for RNAi knockdown in 28 DPE 1 h PZQ exposed schistosomes might be those that are upregulated; i.e., ABCB1 (TC14236), ABCB8 (SMDR1) and ABCG2 (TC10285). TC10285 is of particular interest because it is not only upregulated in the 28 DPE 1 h exposures, but the 42 DPE female schistosomes exposed to 10 µg/mL PZQ for 20 h are also upregulating this transporter as well.

This data suggests that juvenile worms are transcriptionally able to upregulate drug transport related genes in the short-term that allow them to survive the lethal effects of PZQ until the host has metabolized the drug. However, this response does not appear to be long term as is made evident by the fact that the 28 DPE 20 h PZQ exposed groups are downregulating their drug transport related genes the same time as they are beginning to experience significant levels of mortality (Fig. 2). The reason that juvenile worms might not be able to sustain prolonged drug transport could be related to the fact that these transporters require ATP to excrete the drugs. In addition, this might explain why adult worms die from PZQ exposure as well. It might not only be due to the fact that they, for some reason, are unable to express as many, or the correct, drug transporters, but the high

ATP demands of the transporters coupled with the stage in their life when they are mating, which has high energy demands of its own (Khayath *et al*., 2006), is too much of a burden for the schistosomes and they begin to suffer significant levels of mortality.

With almost total reliance on one drug, PZQ, to treat schistosomiasis and over 200 million people infected as well as another 779 people inhabiting areas where they are at risk of contracting the disease (King, 2009), it is pertinent that we develop new therapeutic strategies for treating schistosomiasis. Hopefully the work we have done in this study will contribute to new ideas and theories as to what PZQ's mechanism of action is and what genes we might be able to target with novel drugs.

Figures

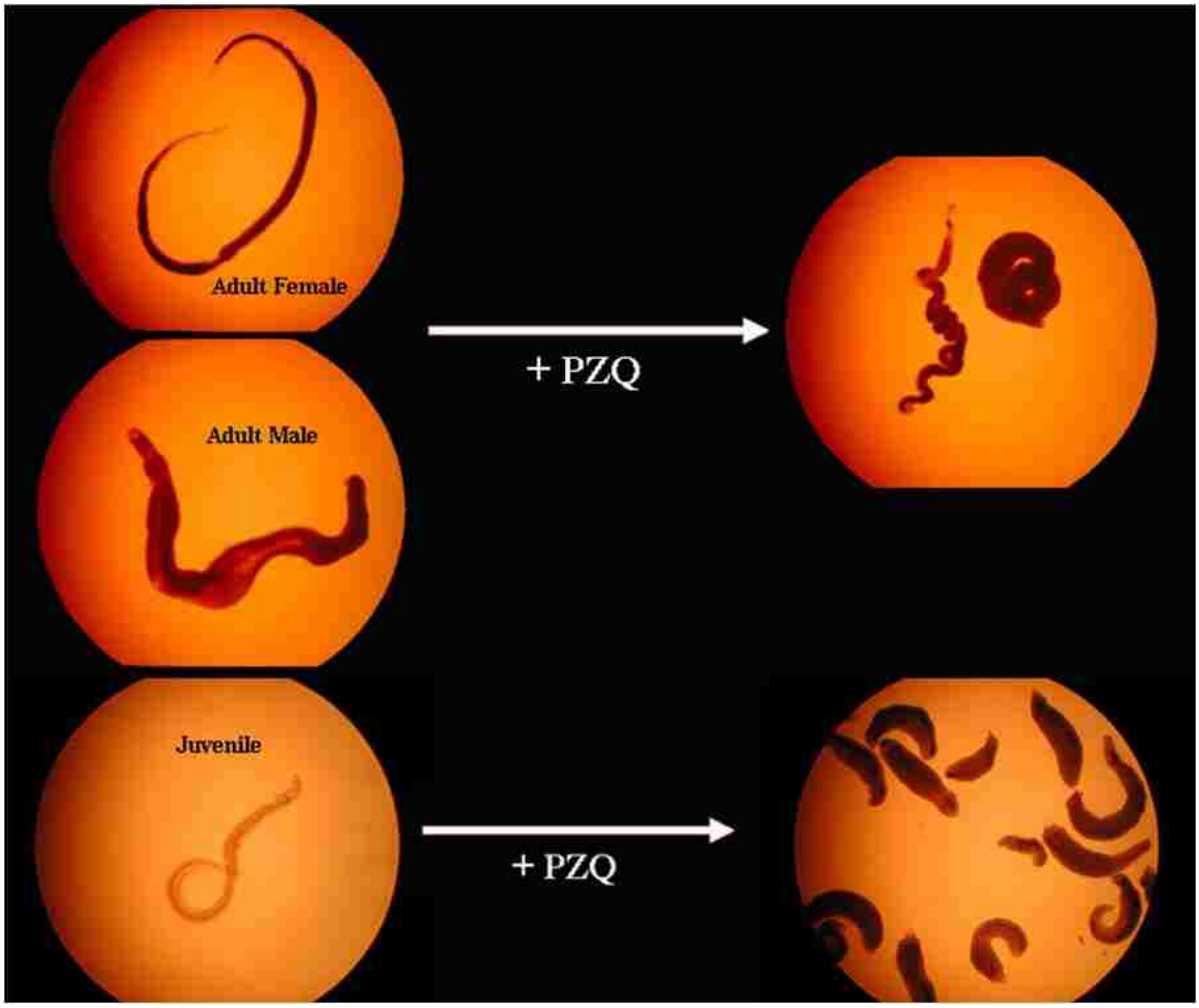
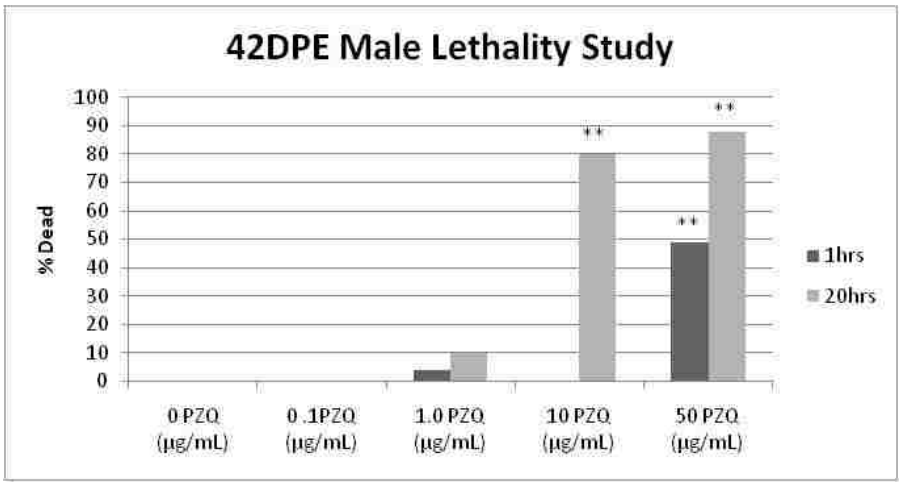


Figure 1. Muscular contraction of adult and juvenile schistosomes several minutes after exposure to 10 µg/mL PZQ.

A



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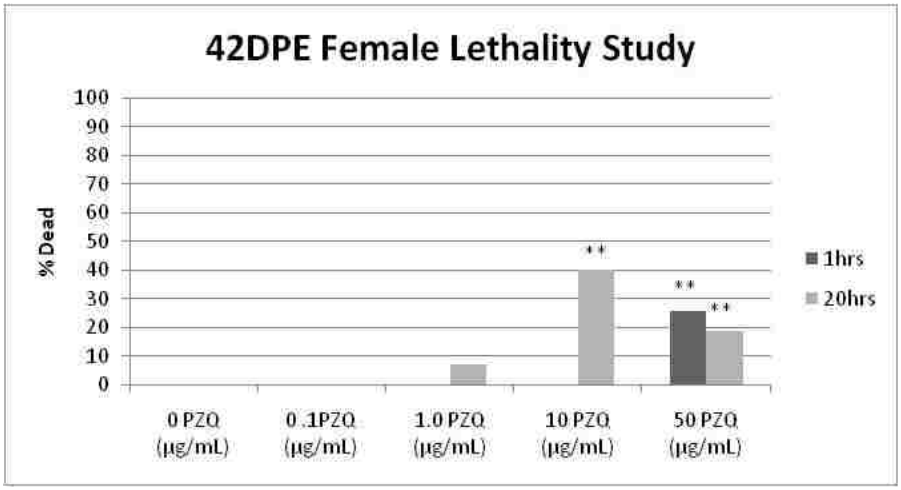

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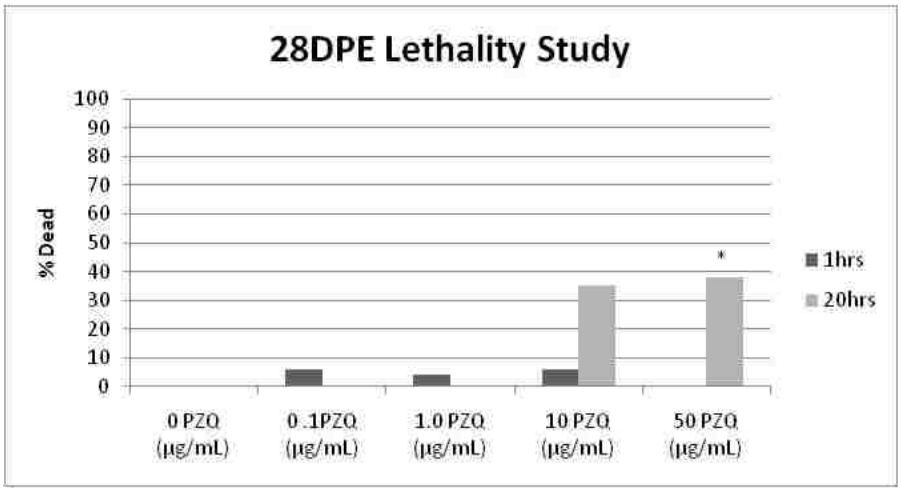

Figure 2. The percentage of schistosome found dead at varying PZQ concentrations at either a 1 h exposure time (dark grey) or 20 h exposure time (light grey) in A) adult males, B) adult females or C) juvenile worms. Asterisks indicate a significant percent of worm death compared to the group control (0 µg/mL PZQ). One asterisks represents $P<0.05$ and two asterisks represents $P<0.01$.

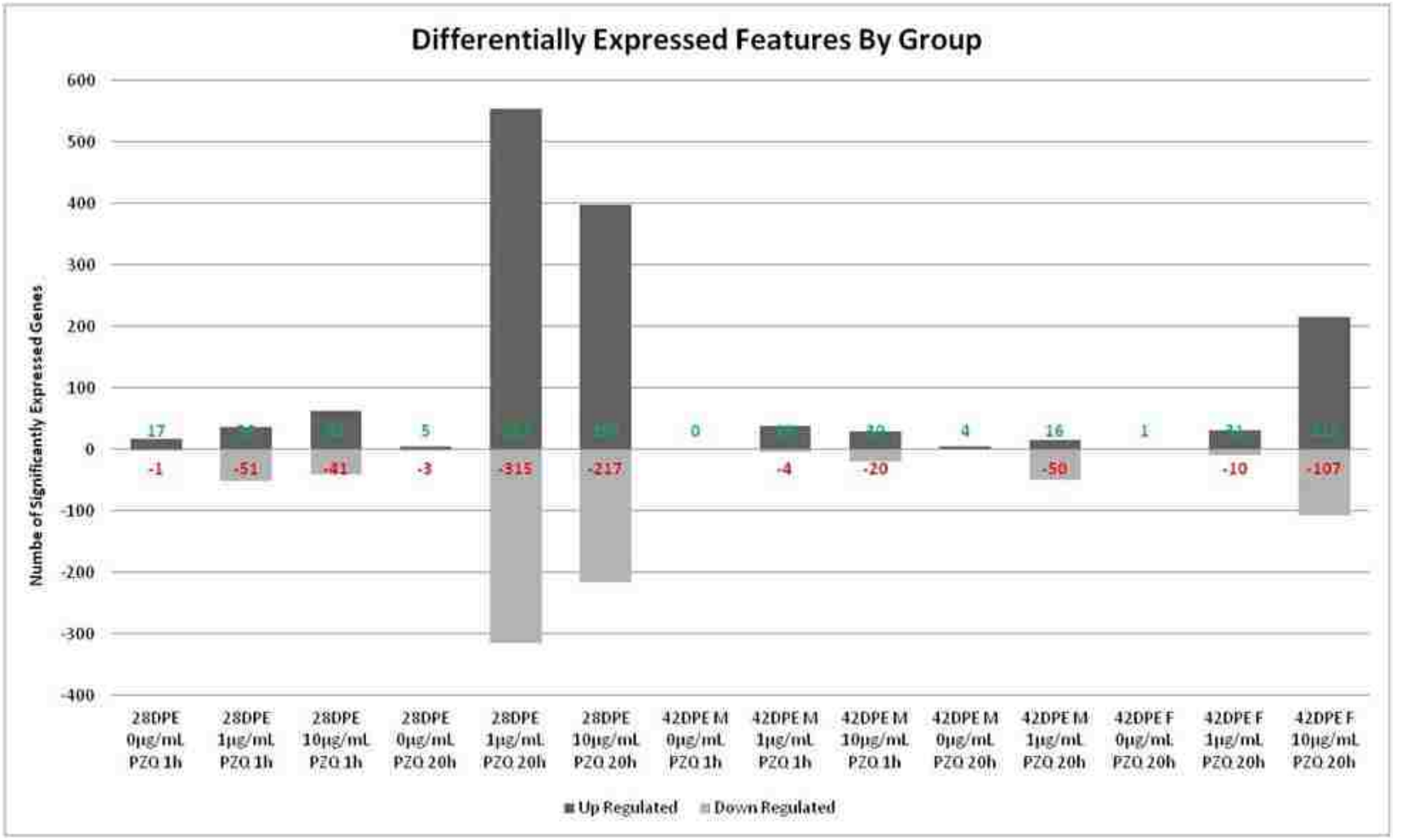

Figure 3. The number of upregulated and downregulated differentially expressed features in each treatment group.

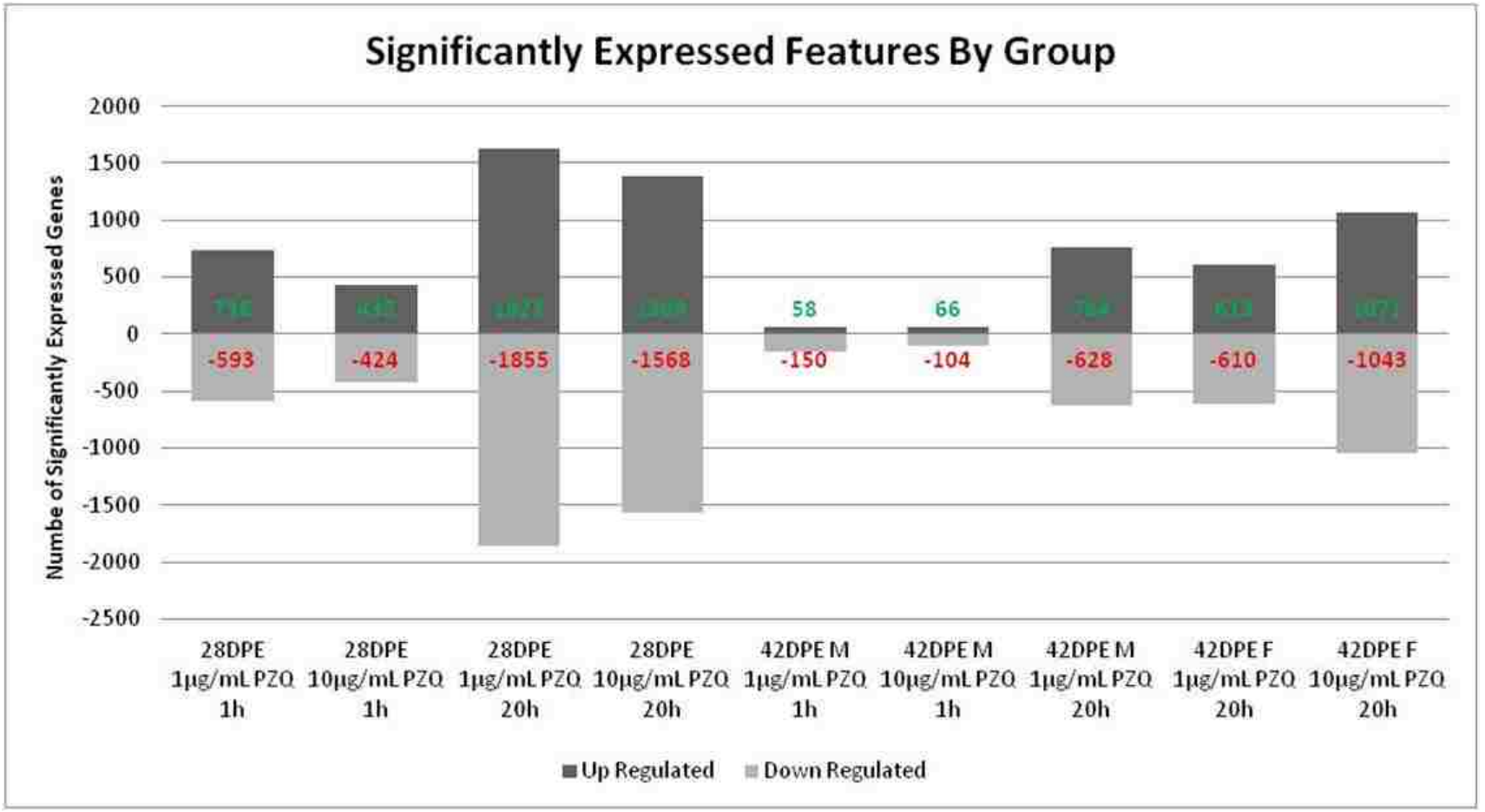

Figure 4. The number of upregulated and downregulated significantly expressed features in each treatment group.

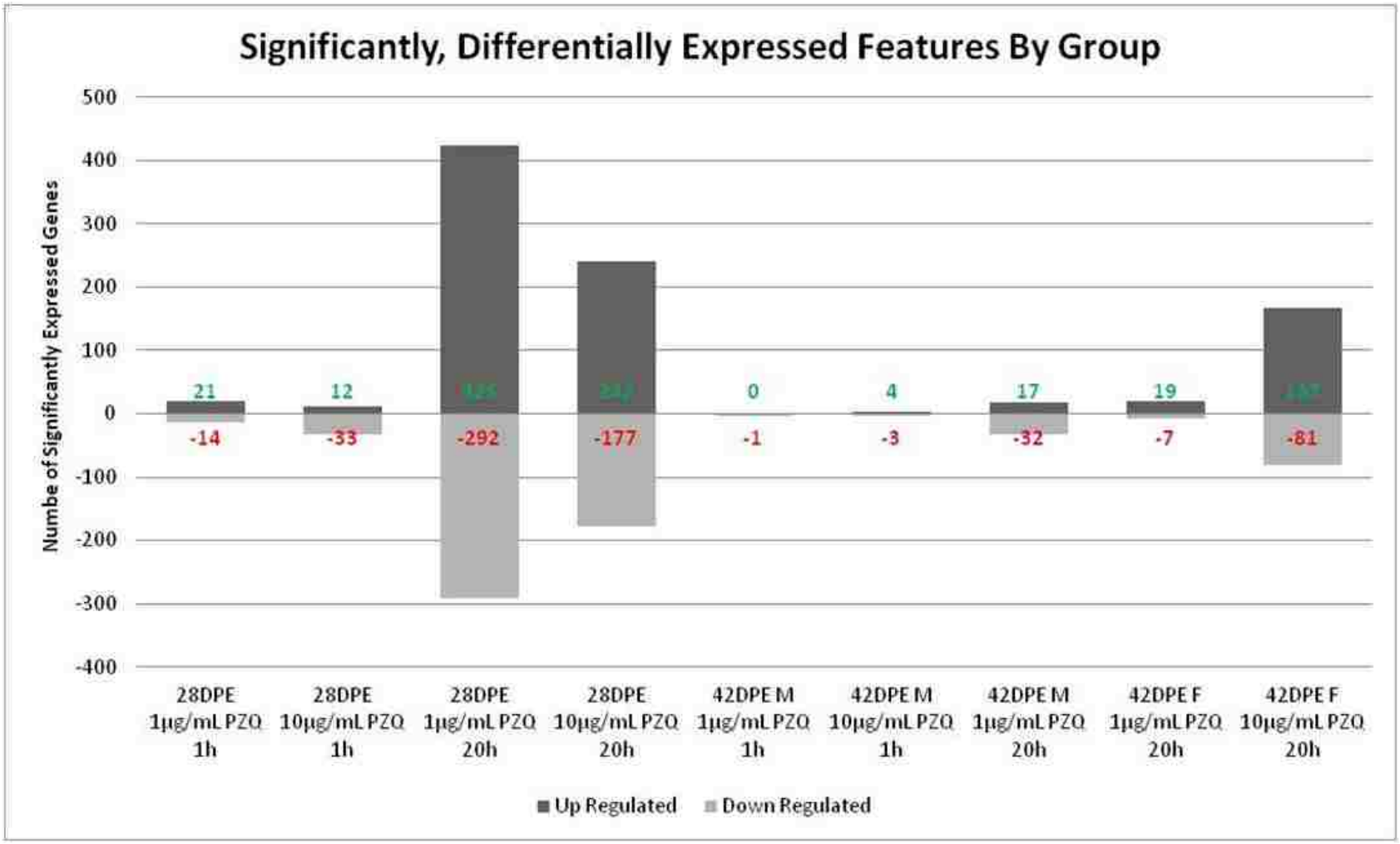

Figure 5. The number of up and downregulated significantly and differentially expressed features in each treatment group.

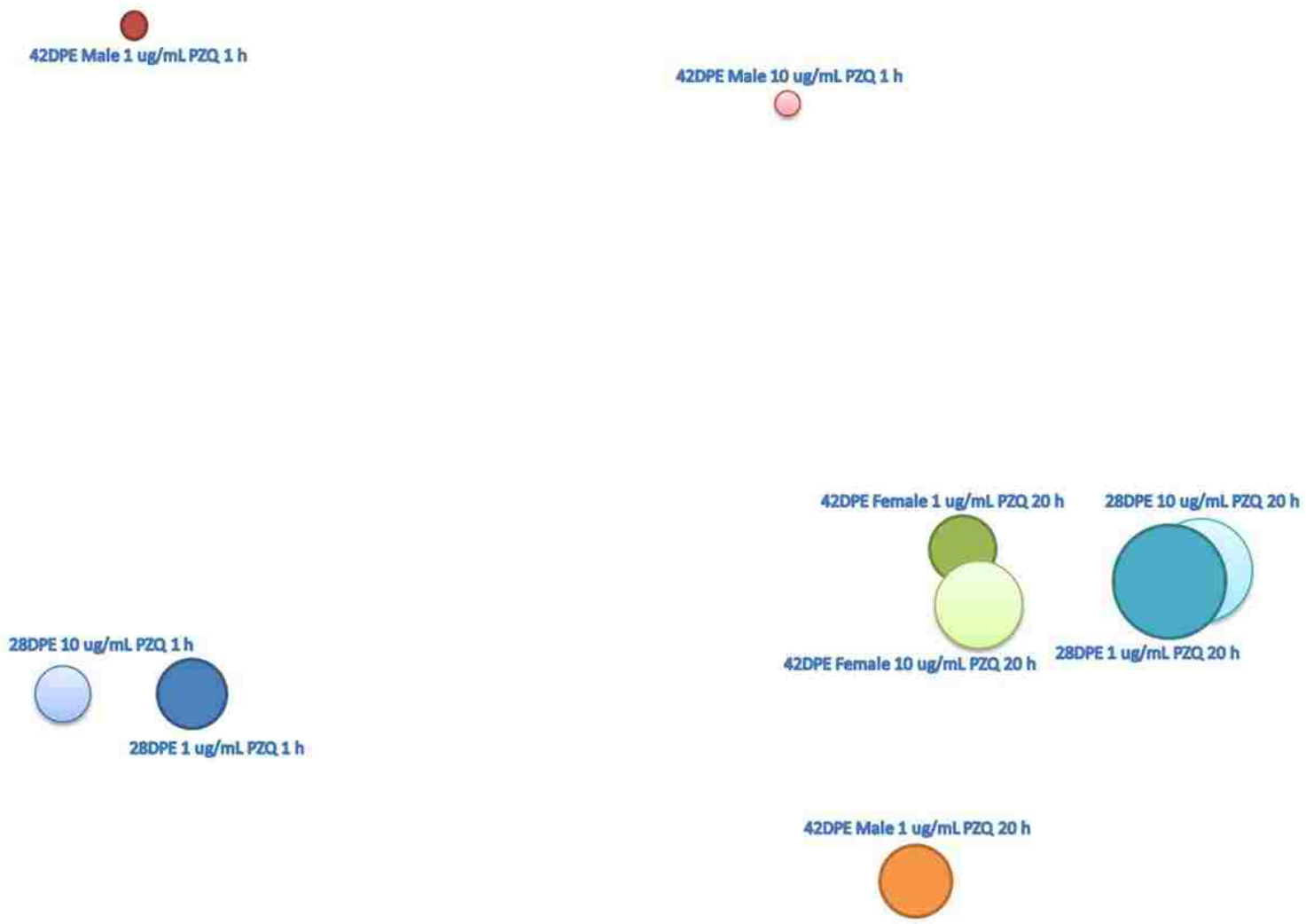

Figure 6. Map depicting the overlap between significantly expressed genes regulated in the same direction (i.e., either both upregulated or both downregulated) by the distance between the centers of the circles. The distance between the groups was calculated with an equation to express the amount of directional overlap as a percentage of total expression in the groups (2 x (Number of significantly expressed feature overlap in same regulatory direction between groups A & B)) / (Sum of significantly expressed features in groups A & B). The area of each circle is correlated to the amount of significantly expressed genes in each group.

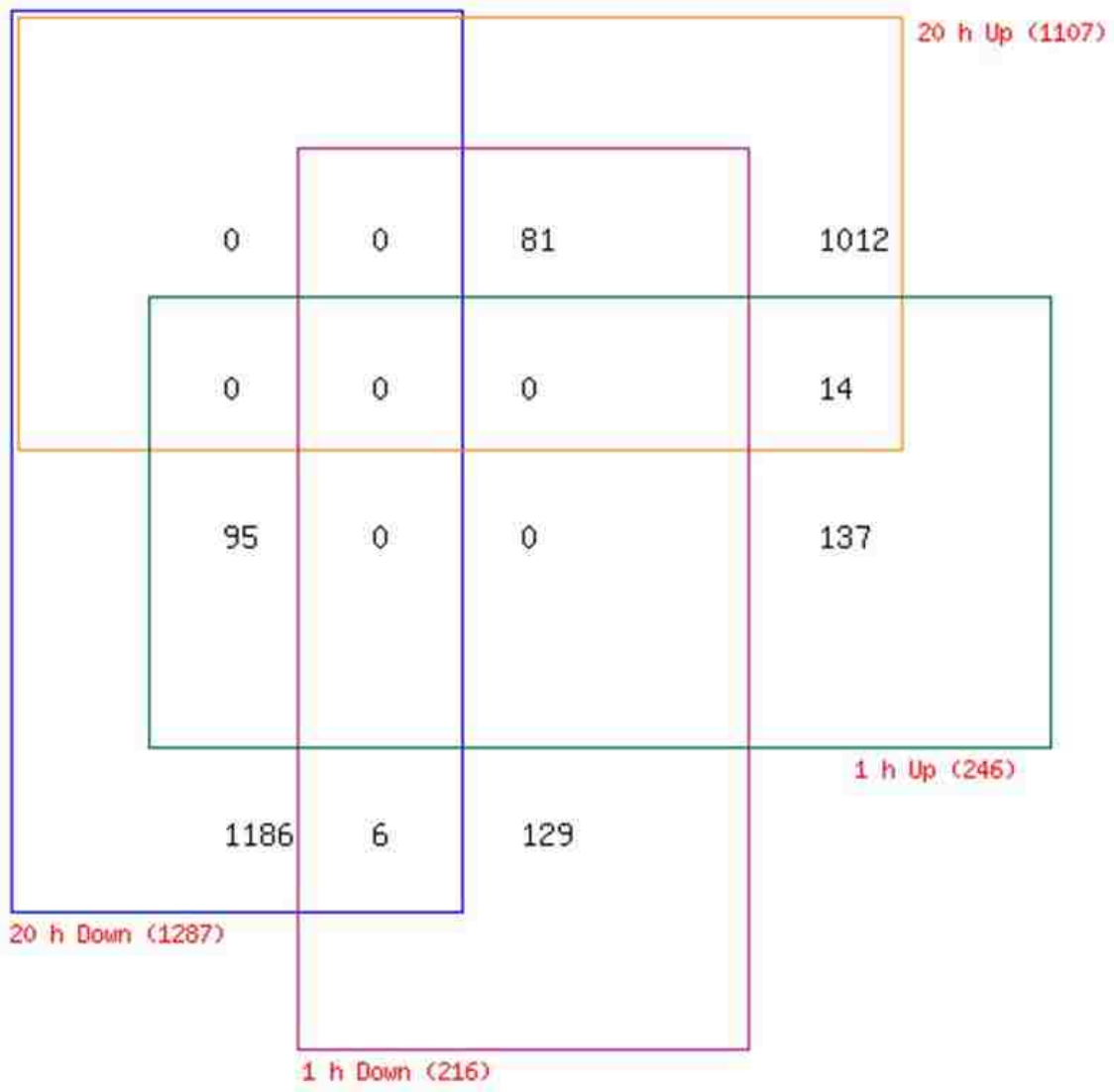

Figure 7. Venn diagram comparing the upregulated and downregulated significantly expressed genes from the 28 DPE 20 h overlap and the 28 DPE 1 h overlap in Table 6.

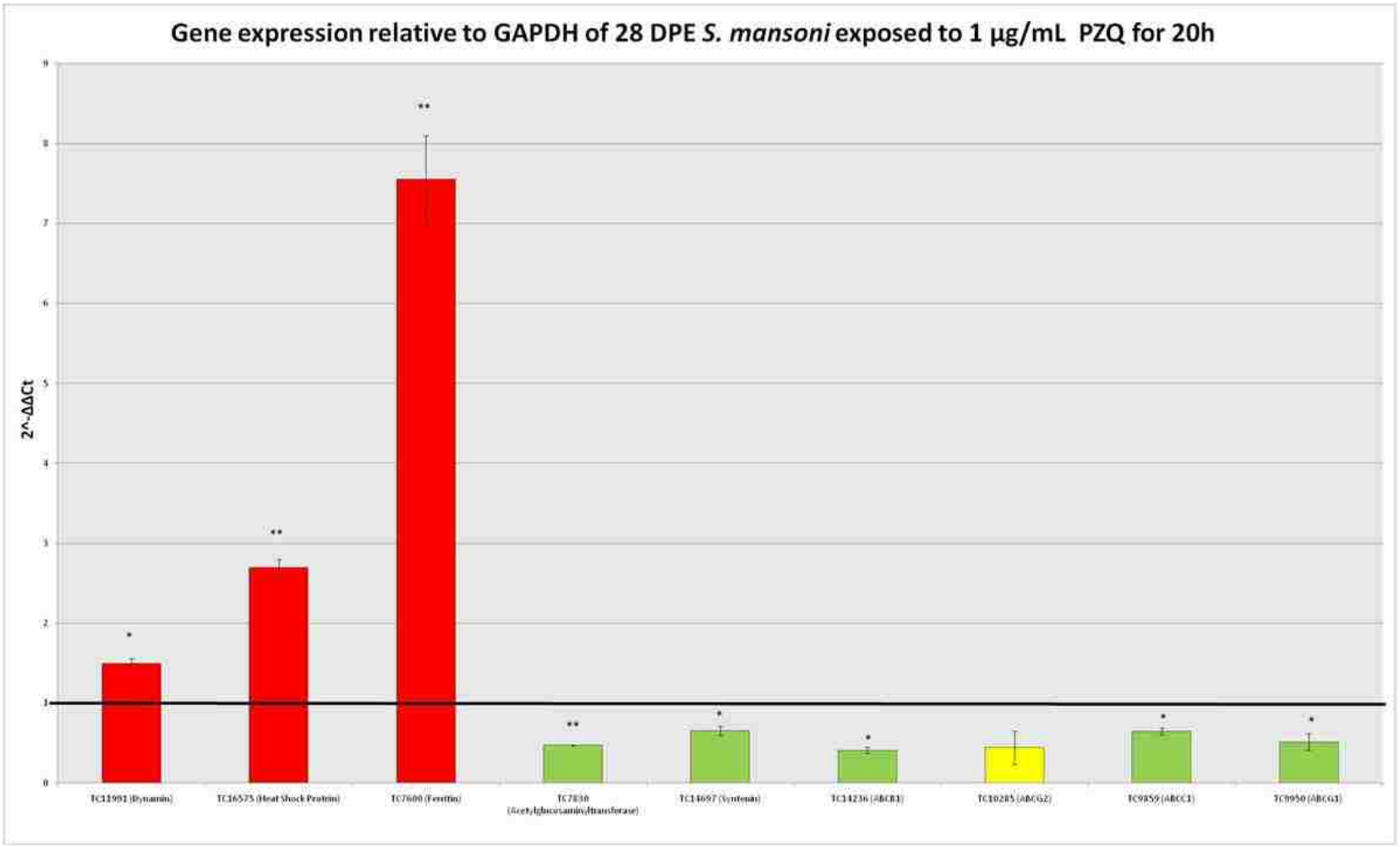

Figure 8. RT-PCR expression data for 28 DPE 1 µg/mL PZQ 1 h exposed *S. mansoni*. The colors of the bars in this figure correlate with the microarray expression where red represents upregulated features, green represents downregulated features and yellow represents features that weren't significantly expressed on the microarray. The bar heights represent the $2^{-\Delta\Delta}C_t$ values the Real-Time PCR data. This method compares expression of a single feature in an experimental group to the expression of that same feature in a control group and normalizes it to an endogenous control, which in this case was SmGAPDH. Bars above a $2^{-\Delta\Delta}C_t$ value of 1 are upregulated and bars below a $2^{-\Delta\Delta}C_t$ of 1 are downregulated. In addition, bars with a single asterisk above them passed a significance test with a p-value<0.05 and bars with a double asterisk above them were significant with a p-value<0.01.

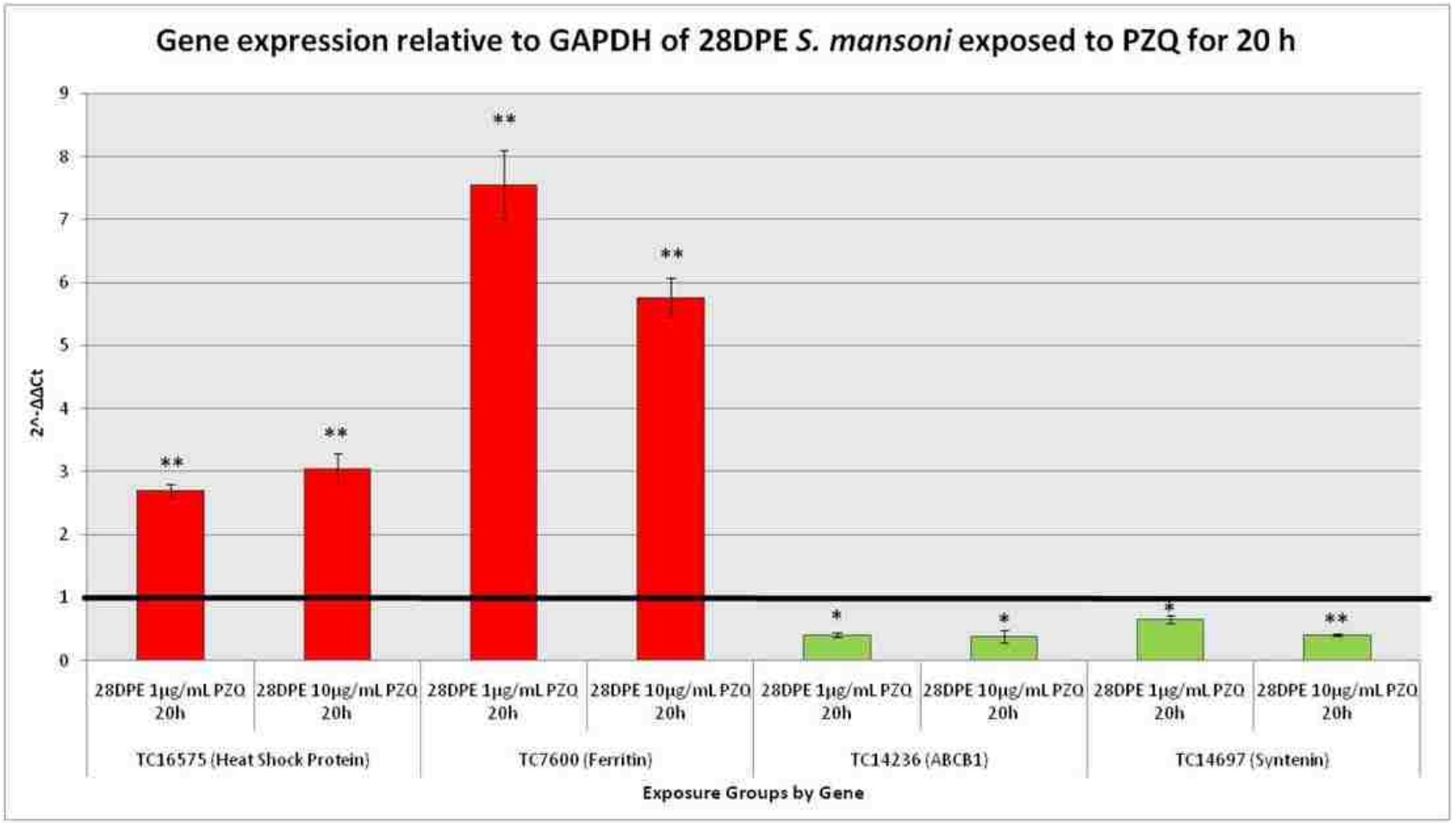

Figure 9. RT-PCR expression data for 28 DPE 10 µg/mL PZQ 1 h exposed *S. mansoni*. The colors of the bars in this figure correlate with the microarray expression where red represents upregulated features, green represents downregulated features and yellow represents features that weren't significantly expressed on the microarray. The bar heights represent the $2^{-\Delta\Delta}C_t$ values the Real-Time PCR data. This method compares expression of a single feature in an experimental group to the expression of that same feature in a control group and normalizes it to an endogenous control, which in this case was SmGAPDH. Bars above a $2^{-\Delta\Delta}C_t$ value of 1 are upregulated and bars below a $2^{-\Delta\Delta}C_t$ of 1 are downregulated. In addition, bars with a single asterisk above them passed a significance test with a p-value<0.05 and bars with a double asterisk above them were significant with a p-value<0.01.

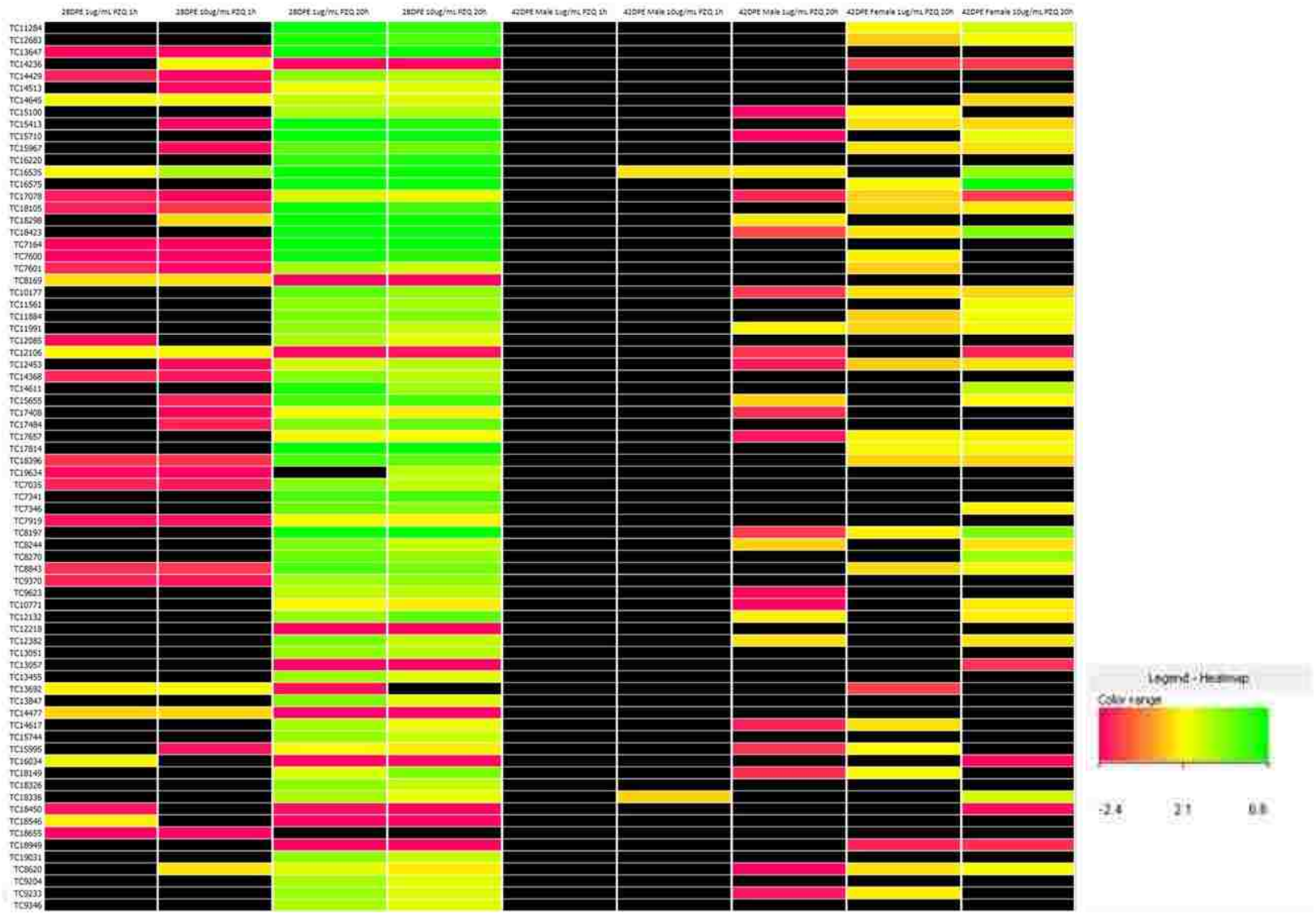

Figure 10. Heat map of the 74 most extremely regulated genes from the microarray study with green representing upregulation, red representing downregulation, yellow representing mild regulation and black representing no significant expression.

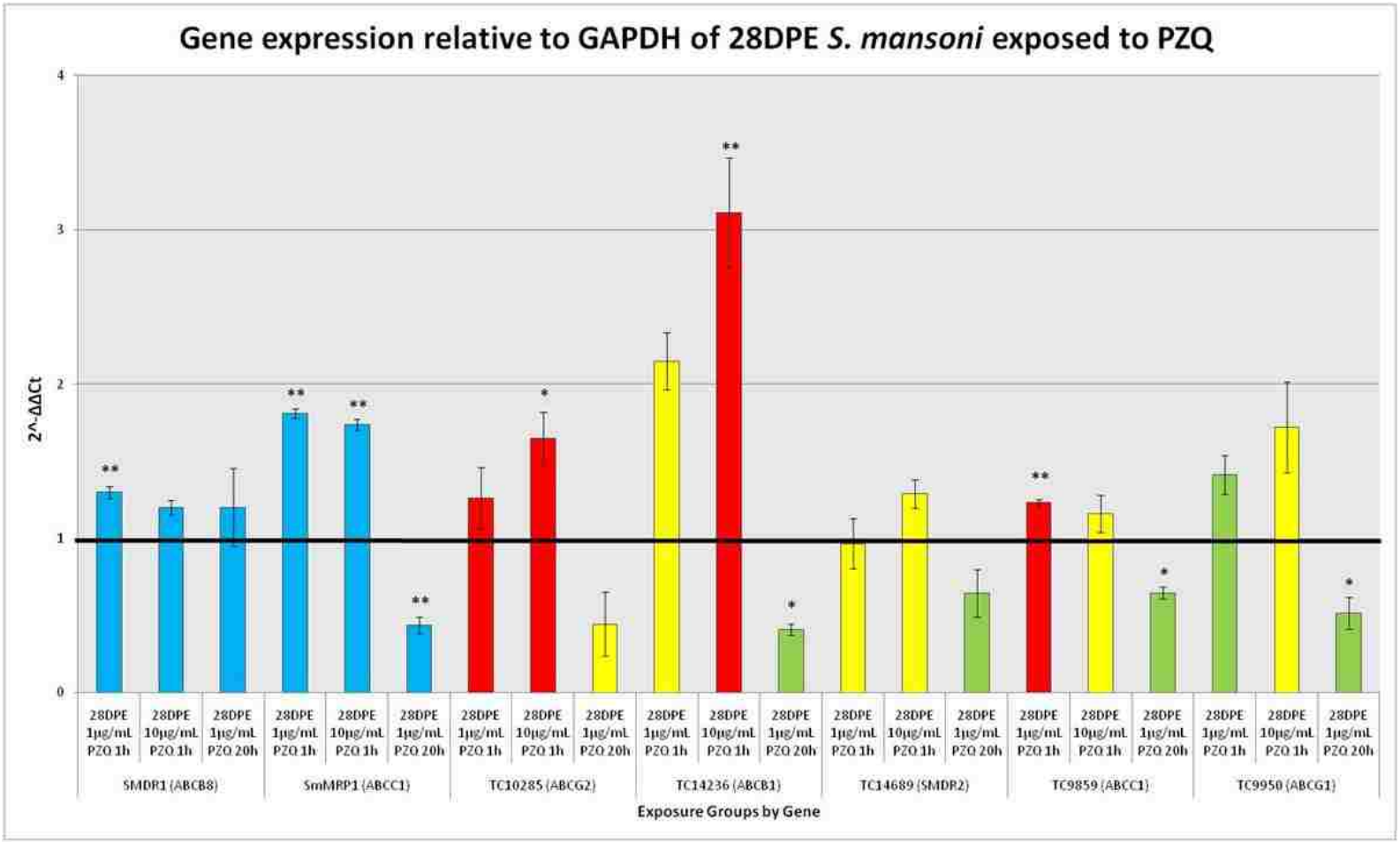

Figure 11. RT-PCR expression of ABC drug transporters. The colors of the bars in these figures correlate to the microarray expression where red represents upregulated features, green represents downregulated features, yellow represents features that weren't significantly expressed on the microarray and blue represents genes that were not included on the microarray at all. The bar heights represent the $2^{-\Delta\Delta}C_t$ values the Real-Time PCR data. Bars above a $2^{-\Delta\Delta}C_t$ value of 1 are upregulated and bars below a $2^{-\Delta\Delta}C_t$ of 1 are downregulated. In addition, bars with a single asterisk above them passed a significance test with a p-value<0.05 and bars with a double asterisk above them were significant with a p-value<0.01.

Tables

Table 1: PZQ treatment regimes employed in the microarray study on *S. mansoni*

Groups	Type
28 DPE exposed to 0 µg/mL PZQ for one hour	Group Control
28 DPE exposed to 1 µg/mL PZQ for one hour	Experimental
28 DPE exposed to 10 µg/mL PZQ for one hour	Experimental
28 DPE exposed to 0 µg/mL PZQ for 20 hours	Group Control
28 DPE exposed to 1 µg/mL PZQ for 20 hours	Experimental
28 DPE exposed to 10 µg/mL PZQ for 20 hours	Experimental
42 DPE male exposed to 0 µg/mL PZQ for one hour	Group Control
42 DPE male exposed to 1 µg/mL PZQ for one hour	Experimental
42 DPE male exposed to 10 µg/mL PZQ for one hour	Experimental
42 DPE male exposed to 0 µg/mL PZQ for 20 hours	Group Control
42 DPE male exposed to 1 µg/mL PZQ for 20 hours	Experimental
42 DPE female exposed to 0 µg/mL PZQ for 20 hours	Group Control
42 DPE female exposed to 1 µg/mL PZQ for 20 hours	Experimental
42 DPE female exposed to 10 µg/mL PZQ for 20 hours	Experimental

Note, 28 DPE worms were mixed sex.

Table 2: *S. mansoni* gene expression tested with Real-Time PCR

Feature	Gene	Forward Primer	Reverse Primer
TC16678	GAPDH	GTCATTCCAGCACTAAACGG	CCTTCCCTAACCTACATGTCAG
TC11991	Dynamin	TGAAGGAACAGATGCACAGG	TCTACGTTCGGCTTCCAAAG
TC16575	Heat Shock Protein	ACTTAATCGGACCATACACGG	GCAAACATTCCACGTCTCAAAC
TC7600	Ferritin	TGTCAATGGAGAAGGCAGTG	TGACCAAGTCCACTTCCAAC
TC10285	ATP-binding cassette sub-family g2	GCAGCCCTACGAATGACTATG	CCAACCTTACTATCCGCTACAG
TC14689	SmMDR2	CTAGTCGGTTCTAGTGGTTCTG	GAGCATTAGCTTTGATGGCAG
TC9859	ATP-binding cassette C1	TTATGGCGTGCAATAGAATCTG	AGATTTGCTCCACCTTCACC
TC9950	ABC transporter	GTTTAGTCCCTTACCTCGCATC	ATTCACCACAAGCCACTTCC
Not on array	SmMRP1	GCTATTTGGCACGCACTTG	AGCCACCTTCTGAGATGTTC
Not on array	SMDR1	CTTATTCGGTGCCATTCTGG	TTCGTGAGTCACAACGCATC
TC14236	P glycoprotein 1, 2, 3	GGAGTTGAAACGATACCGAGAG	GCAGTAGAACAAAACACAGTCAG
TC7830	Acetylglucosaminyltransferase	TGGATTGGGATTTCCTTACGAAG	TGATTGCTTCTGCTGTGAGG
TC14697	Syntenin	CCACTTCCAGCACCATTCG	GTGAAAACCCCTCCACAAATG

All primers are in the 5’ to 3’ direction.

Table 3: Three-factor ANOVA comparing variables from the mortality study

Variable	Degrees of Freedom	F-Statistic	F-Value	P-Value
DPE and Sex	2	9.55	10.66	4.39E-05
Concentration PZQ	2	9.55	149.02	2.20E-16
PZQ Exposure Time	3	9.28	69.21	2.92E-14
DPE and Sex : Concentration PZQ	2	9.55	20.83	8.27E-09
DPE and Sex : PZQ Exposure Time	2	9.55	9.88	8.78E-05
Concentration PZQ : PZQ Exposure Time	1	10.13	22.49	4.49E-06
DPE and Sex : Concentration : Exposure Time	2	9.55	13.66	3.17E-06

Table 4: Statistical comparison of mortality of *S. mansoni* treated with varying concentration of PZQ for 20 or 1 h

A 20 h PZQ Exposures

42DPE Male	Test Against:		
PZQ (µg/mL)	Control	42DPE Male	42DPE Female
0	-	-	-
0.1	p = 1	-	-
1	p = 0.37	-	-
10	p = 1.6E-04	-	-
50	p = 8.3E-06	-	-

B

42DPE Female	Test Against:		
PZQ (µg/mL)	Control	42DPE Male	42DPE Female
0	-	p = 1	-
0.1	p = 1	p = 1	-
1	p = 0.37	p = 0.80	-
10	p = 3.5E-04	p = 4.3E-03	-
50	p = 8.1E-03	p = 1.6E-04	-

C

28DPE	Test Against:		
PZQ (µg/mL)	Control	42DPE Male	42DPE Female
0	-	p = 1	p = 1
0.1	p = 1	p = 1	p = 1
1	p = 1	p = 0.37	p = 0.37
10	p = 0.06	p = 0.04	p = 0.75
50	p = 0.03	p = 0.02	p = 0.18

D 1 h PZQ Exposures

42DPE Male	Test Against:		
PZQ (µg/mL)	Control	42DPE Male	42DPE Female
0	-	-	-
0.1	p = 1	-	-
1	p = 0.37	-	-
10	p = 1	-	-
50	p = 9.6E-07	-	-

E

42DPE Female	Test Against:		
PZQ (µg/mL)	Control	42DPE Male	42DPE Female
0	-	p = 1	-
0.1	p = 1	p = 1	-
1	p = 1	p = 0.37	-
10	p = 1	p = 1	-
50	p = 1.5E-03	p = 2.3E-03	-

F

28DPE	Test Against:		
PZQ (µg/mL)	Control	42DPE Male	42DPE Female
0	-	p = 1	p = 1
0.1	p = 0.37	p = 0.37	p = 0.37
1	p = 0.37	p = 0.94	p = 0.37
10	p = 0.12	p = 0.12	p = 0.12
50	p = 1	p = 9.6E-07	p = 1.5E-03

P-values are from t-tests comparing the 20 h mortality results by PZQ concentration to the control for A) adult male worms, B) adult female worms and C) juvenile worms in column 2. Column 3 employs the same significance test to compare adult male worm mortality to B) adult female and C) juvenile worm mortality at the same PZQ concentrations for 20 h. Column 4 uses the same significance test to compare adult female worm mortality to C) juvenile worm mortality at the same PZQ concentrations for 20 h.

Table 5: Statistical comparison of mortality of *S. mansoni* treated with varying concentration of PZQ for 0.25, 0.5 or 1 h

A

42DPE Male	PZQ Time Exposure Comparison (Hours)		
PZQ (µg/mL)	0.25 to 0.5	0.25 to 1.0	0.5 to 1.0
0	p = 1	p = 1	p = 1
0.1	p = 0.37	p = 1	p = 0.37
1	p = 1	p = 0.37	p = 0.37
10	p = 1	p = 1	p = 1
50	p = 0.71	p = 0.29	p = 0.09

B

42DPE Female	PZQ Time Exposure Comparison (Hours)		
PZQ (µg/mL)	0.25 to 0.5	0.25 to 1.0	0.5 to 1.0
0	p = 1	p = 1	p = 1
0.1	p = 1	p = 1	p = 1
1	p = 1	p = 1	p = 1
10	p = 0.37	p = 0.37	p = 1
50	p = 0.97	p = 0.70	p = 0.37

C

28DPE	PZQ Time Exposure Comparison (Hours)		
PZQ (µg/mL)	0.25 to 0.5	0.25 to 1.0	0.5 to 1.0
0	p = 1	p = 1	p = 1
0.1	p = 1	p = 0.37	p = 0.37
1	p = 0.37	p = 0.37	p = 0.89
10	p = 1	p = 0.12	p = 0.12
50	p = 0.94	p = 0.37	p = 0.37

P-values are from t-tests comparing the mortality between varying short-term exposures to PZQ (0.25, 0.5 and 1.0 h) at different PZQ concentration in A) adult males, B) adult females and C) juveniles. A p-value less than 0.05 is considered significant.

Table 6: Significantly expressed features by exposure group in *S. mansoni* treated with PZQ

DPE	Sex	PZQ (µg/mL)	Exposure Time (Hours)	Significantly Expressed Genes	Percent Genes Significantly Expressed	FC>+/-2 and T-Test	Percent of Significantly Expressed Genes
28	Mixed	1	1	1329	17%	35	3%
28	Mixed	10	1	859	10%	45	5%
28	Mixed	1	20	3482	47%	717	21%
28	Mixed	10	20	2957	38%	419	14%
42	Male	1	1	208	3%	1	0%
42	Male	10	1	170	2%	7	4%
42	Male	1	20	1392	17%	49	4%
42	Female	1	20	1223	16%	26	2%
42	Female	10	20	2114	27%	244	12%
	AVERAGE			**1526**	**20%**	**171**	**11%**
	Different			6161		1052	17%

Significantly expressed genes ($p < 0.05$) by exposure group compared to the group's control using an unpaired, unequal variance T-Test (column 5) and the percentage that are significantly expressed of the total genes expressed in that group (column 6) as well as the amount of those significantly expressed genes that are differentially expressed (Fold Change > +/- 2) (column 7) and the percentage those are of the significantly expressed genes (column 8).

Table 7: Comparison of differentially expressed features in PZQ treated and untreated *S. mansoni*

Group	Total Expressed Genes	Average
All FC > 2 Genes	2224	159
All PZQ Untreated Groups	31	6
All PZQ Treated Groups	2193	244
PZQ Untreated Adult Males	4	2
PZQ Treated Adult Males	158	53
PZQ Untreated Adult Females	1	1
PZQ Treated Adult Females	363	182
PZQ Untreated Juveniles	26	13
PZQ Treated Juveniles	1672	418
1 Hour PZQ Exposed Juveniles	190	95
20 Hour PZQ Exposed Juveniles	1482	741

Table 8: Expressed features by exposure group in *S. mansoni* treated with PZQ

DPE	Sex	PZQ (µg/mL)	Exposure Time (Hours)	Expressed Genes	Percent Up	Magnitude and Direction	FC>+/-2	Percent of Expressed Genes	Percent FC>+/-2 Up
28	Mixed	0	1	8033	50%	0.01	18	0.22%	94%
28	Mixed	1	1	7671	53%	0.06	87	1.13%	41%
28	Mixed	10	1	8212	51%	0.07	103	1.25%	60%
28	Mixed	0	20	7458	50%	-0.01	8	0.11%	63%
28	Mixed	1	20	7483	48%	0.06	868	11.60%	64%
28	Mixed	10	20	7714	48%	0.02	614	7.96%	65%
42	Male	0	1	7689	50%	0.01	0	0.00%	0%
42	Male	1	1	7685	45%	-0.11	42	0.55%	90%
42	Male	10	1	8055	48%	-0.03	50	0.62%	60%
42	Male	0	20	7900	49%	-0.01	4	0.05%	100%
42	Male	1	20	8332	51%	0.00	66	0.79%	24%
42	Female	0	20	8057	53%	0.07	1	0.01%	100%
42	Female	1	20	7769	49%	-0.02	41	0.53%	76%
42	Female	10	20	7915	48%	-0.01	322	4.07%	67%
	AVERAGE			**7855**	**50%**	**0.01**	**159**	**2.02%**	**63%**
	Different			8582			1323	15.42%	

Expressed genes by exposure group (column 5), the percentage that are upregulated (column 6) and the average expression value of all the expressed genes in each group (column 7); as well as the amount of those expressed genes that are differentially expressed (Fold Change > +/- 2) (column 8), the percentage those are of the expressed genes (column 9) and the percent of those differentially expressed genes that are upregulated.

Table 9: Distance matrix exhibiting the number of significantly expressed directional gene overlap between treatment groups

	28DPE 1µg/mL PZQ 1h	28DPE 10µg/mL PZQ 1h	42DPE Male 1µg/mL PZQ 1h	42DPE Male 10µg/mL PZQ 1h	28DPE 1µg/mL PZQ 20h	28DPE 10µg/mL PZQ 20h	42DPE Male 1µg/mL PZQ 20h	42DPE Female 1µg/mL PZQ 20h	42DPE Female 10µg/mL PZQ 20h
28DPE 1µg/mL PZQ 1h	**1329**	463	23	13	77	88	66	74	182
28DPE 10µg/mL PZQ 1h	463	**859**	11	15	63	55	51	48	102
42DPE Male 1µg/mL PZQ 1h	23	11	**208**	14	62	55	18	19	40
42DPE Male 10µg/mL PZQ 1h	13	15	14	**170**	37	20	13	32	29
28DPE 1µg/mL PZQ 20h	77	63	62	37	**3482**	2394	125	541	914
28DPE 10µg/mL PZQ 20h	88	55	55	20	2394	**2957**	56	441	898
42DPE Male 1µg/mL PZQ 20h	66	51	18	13	125	56	**1392**	69	133
42DPE Female 1µg/mL PZQ 20h	74	48	19	32	541	441	69	**1223**	541
42DPE Female 10µg/mL PZQ 20h	182	102	40	29	914	898	133	541	**2114**
Average as percent of expressed	22%	24%	27%	25%	28%	29%	17%	31%	29%

The number aligning each column and row is the number of significantly expressed genes that are either upregulated or downregulated between the respective groups. The bottom row displays the average amount of overlap each group has with the other groups as a percentage of the significantly expressed genes in that group.

Table 10: Distance matrix exhibiting the number of significantly expressed, same direction gene overlap between *S. mansoni* PZQ treatment groups as a percentage of the total significantly expressed genes in compared groups

	28DPE 1µg/mL PZQ 1h	28DPE 10µg/mL PZQ 1h	42DPE Male 1µg/mL PZQ 1h	42DPE Male 10µg/mL PZQ 1h	28DPE 1µg/mL PZQ 20h	28DPE 10µg/mL PZQ 20h	42DPE Male 1µg/mL PZQ 20h	42DPE Female 1µg/mL PZQ 20h	42DPE Female 10µg/mL PZQ 20h
28DPE 1µg/mL PZQ 1h	-	42%	3%	2%	3%	4%	5%	6%	11%
28DPE 10µg/mL PZQ 1h	42%	-	2%	3%	3%	3%	5%	5%	7%
42DPE Male 1µg/mL PZQ 1h	3%	2%	-	7%	3%	3%	2%	3%	3%
42DPE Male 10µg/mL PZQ 1h	2%	3%	7%	-	2%	1%	2%	5%	3%
28DPE 1µg/mL PZQ 20h	3%	3%	3%	2%	-	74%	5%	23%	33%
28DPE 10µg/mL PZQ 20h	4%	3%	3%	1%	74%	-	3%	21%	35%
42DPE Male 1µg/mL PZQ 20h	5%	5%	2%	2%	5%	3%	-	5%	8%
42DPE Female 1µg/mL PZQ 20h	6%	5%	3%	5%	23%	21%	5%	-	32%
42DPE Female 10µg/mL PZQ 20h	11%	7%	3%	3%	33%	35%	8%	32%	-

The number aligning each column and row is the number of significantly expressed genes that are either upregulated or down regulated between the respective groups expressed as a percentage of the total amount of significantly expressed genes in those two groups by the equation: (2 x (Number of significantly expressed feature overlap in same regulatory direction between groups A & B)) / (Sum of significantly expressed features in groups A & B).

Table 11: Distance matrix exhibiting the total number of significantly expressed gene overlap between *S. mansoni* PZQ treatment groups as a percentage of the total significantly expressed genes in compared groups

	28DPE 1µg/mL PZQ 1h	28DPE 10µg/mL PZQ 1h	42DPE Male 1µg/mL PZQ 1h	42DPE Male 10µg/mL PZQ 1h	28DPE 1µg/mL PZQ 20h	28DPE 10µg/mL PZQ 20h	42DPE Male 1µg/mL PZQ 20h	42DPE Female 1µg/mL PZQ 20h	42DPE Female 10µg/mL PZQ 20h
28DPE 1µg/mL PZQ 1h	-	43%	5%	3%	26%	24%	17%	13%	18%
28DPE 10µg/mL PZQ 1h	43%	-	5%	5%	21%	21%	15%	14%	17%
42DPE Male 1µg/mL PZQ 1h	5%	5%	-	8%	4%	5%	5%	4%	7%
42DPE Male 10µg/mL PZQ 1h	3%	5%	8%	-	3%	3%	4%	5%	5%
28DPE 1µg/mL PZQ 20h	26%	21%	4%	3%	-	75%	29%	27%	39%
28DPE 10µg/mL PZQ 20h	24%	21%	5%	3%	75%	-	28%	26%	40%
42DPE Male 1µg/mL PZQ 20h	17%	15%	5%	4%	29%	28%	-	21%	26%
42DPE Female 1µg/mL PZQ 20h	13%	14%	4%	5%	27%	26%	21%	-	35%
42DPE Female 10µg/mL PZQ 20h	18%	17%	7%	5%	39%	40%	26%	35%	-

The number aligning each column and row is the total number of significantly expressed genes that overlap between the respective groups expressed as a percentage of the total amount of significantly expressed genes in those two groups by the equation: (2 x (Number of significantly expressed feature overlap between groups A & B)) / (Sum of significantly expressed features in groups A & B).

Table 12: Distance matrix comparing the percentage of significantly expressed gene overlap in the same direction to the total significantly expressed gene overlap in *S. mansoni* PZQ treatment groups

	28DPE 1µg/mL PZQ 1h	28DPE 10µg/mL PZQ 1h	42DPE Male 1µg/mL PZQ 1h	42DPE Male 10µg/mL PZQ 1h	28DPE 1µg/mL PZQ 20h	28DPE 10µg/mL PZQ 20h	42DPE Male 1µg/mL PZQ 20h	42DPE Female 1µg/mL PZQ 20h	42DPE Female 10µg/mL PZQ 20h
28DPE 1µg/mL PZQ 1h	-	99%	53%	62%	12%	17%	28%	43%	58%
28DPE 10µg/mL PZQ 1h	99%	-	39%	60%	14%	14%	30%	34%	41%
42DPE Male 1µg/mL PZQ 1h	56%	39%	-	88%	75%	67%	43%	70%	51%
42DPE Male 10µg/mL PZQ 1h	65%	60%	88%	-	63%	39%	41%	84%	56%
28DPE 1µg/mL PZQ 20h	12%	14%	75%	63%	-	100%	18%	84%	84%
28DPE 10µg/mL PZQ 20h	17%	14%	67%	39%	100%	-	9%	80%	88%
42DPE Male 1µg/mL PZQ 20h	28%	30%	43%	41%	18%	9%	-	25%	29%
42DPE Female 1µg/mL PZQ 20h	44%	34%	70%	84%	84%	80%	25%	-	93%
42DPE Female 10µg/mL PZQ 20h	58%	41%	51%	56%	84%	88%	29%	93%	-

A comparison between the percentage of significantly expressed gene overlap in the same direction to the total significantly expressed gene overlap was obtained by dividing each cell in Table 10 by each cell in Table 11.

Table 13: Feature annotation by BLAST database

	Expressed Genes	Percent Annotated	Significantly Expressed Genes	Percent Annotated	Significantly & Differentially Expressed Genes	Percent Annotated
Total Genes	**8582**		**6161**		**1052**	
Swissprot	4981	58%	3749	61%	942	90%
Kegg Genes	7381	86%	5419	88%	1011	96%
Go Proteins	5086	59%	3820	62%	943	90%
NR	7493	87%	5492	89%	1017	97%
GO Terms	4784	56%	3604	58%	933	89%
Total Annotated	**7507**	**87%**	**5501**	**89%**	**1017**	**97%**

The number of expressed genes that were annotated in one of the five databases blasted (column 2) and the percent of the total expressed genes those represent (column 3); as well as the amount of significantly expressed genes that were annotated in one of the five databases blasted (column 4) and the percent of the total significantly expressed genes those represent (column 5). Finally, the number of significantly and differentially expressed genes that were annotated in one of the five databases blasted (column 6) and the percent of the total significantly and differentially expressed genes those represent (column 7).

Table 14: Microarray and RT-PCR expression data for 28 DPE 1 µg/mL PZQ 1 h exposed *S. mansoni*

Feature	Gene	Array Expression (Fold Change)	Real-Time PCR Expression (2−ΔΔCt)
TC11991	Dynamin	4.04	1.50
TC16575	Heat Shock Protein	28.45	2.70
TC7600	Ferritin	7.44	7.56
TC7830	Acetylglucosaminyltransferase	-3.25	0.47
TC14697	Syntenin	-2.12	0.66
TC14236	ABCB1	-2.86	0.41
TC10285	ABCG2	Not Significant	Not Significant
TC9859	ABCC1	-1.91	0.65
TC9950	ABCG1	-1.27	0.52

RT-PCR was performed to test expression results from the microarray data in 28 DPE *S. mansoni* exposed to 1 µg/mL PZQ for 1 h. These genes were chosen because they were either significantly and differentially upregulated on the microarray (TC11991, TC16575 and TC7600), significantly and differentially downregulated on the microarray (TC7830, TC14697 and TC14236) or because they were not differentially expressed on the microarray (TC10825, TC9859 and TC9950). The $2^{-\Delta\Delta}C_t$ method employed in the RT-PCR study compares expression of a single feature in an experimental group to the expression of that same feature in a control group and normalizes it to an endogenous control, which in this case was SmGAPDH. Values above $2^{-\Delta\Delta}C_t$ of 1 are upregulated and values below $2^{-\Delta\Delta}C_t$ of 1 are downregulated.

Table 15: Microarray and RT-PCR data for 28 DPE 10 µg/mL PZQ 1 h exposed *S. mansoni*

Feature	Gene	Array Expression (Fold Change)	Real-Time PCR Expression (2−ΔΔCt)
TC16575	Heat Shock Protein	16.19	3.05
TC7600	Ferritin	6.01	5.76
TC14697	Syntenin	-1.96	0.41
TC14236	ABCB1	-2.66	0.39

RT-PCR was performed to test expression results from the microarray data in 28 DPE *S. mansoni* exposed to 10 µg/mL PZQ for 1 h. These genes were chosen because they were either significantly and differentially upregulated on the microarray (TC16575 and TC7600) or significantly and differentially downregulated on the microarray (TC14697 and TC14236). The $2^{-\Delta\Delta}C_t$ method employed in the RT-PCR study compares expression of a single feature in an experimental group to the expression of that same feature in a control group and normalizes it to SmGAPDH. Values above $2^{-\Delta\Delta}C_t$ of 1 are upregulated and values below $2^{-\Delta\Delta}C_t$ of 1 are downregulated.

Table 16: List of 74 extremely regulated genes and their patterns of expression

Feature	Annotation	28DPE 1ug/mL PZQ 1h	28DPE 10ug/mL PZQ 1h	28DPE 1ug/mL PZQ 20h	28DPE 10ug/mL PZQ 20h	42DPE Male 1ug/mL PZQ 1h	42DPE Male 10ug/mL PZQ 1h	42DPE Male 1ug/mL PZQ 20h	42DPE Female 1ug/mL PZQ 20h	42DPE Female 10ug/mL PZQ 20h
TC11284	CAP domain-containing protein			7.16	5.71				1.95	2.76
TC14698	suppressor of Mek1			6.06	5.22				1.21	2.17
TC18947	No Hit	-2.80	-6.66	11.80	11.60					
TC14286	multidrug resistance protein 1 2 3		2.12	-2.06	-2.66				-1.80	-1.43
TC14429	Alpha-2-macroglobulin	-1.76	-2.18	4.12	3.40					
TC14518	hypothetical protein Schistosoma mansoni smp_CAZ82265.1		-2.21	2.86	2.55					
TC14645	RNA binding exportfactor binding protein	2.00	2.19	3.18	2.69					1.44
TC15100	Phenylalkylamine Ca2+ antagonist 1			3.45	3.40			-2.66	1.90	
TC15429	5 transmembrane domain similar to Ring receptor complex 2 U		-9.84	9.67	5.89				1.48	1.42
TC15710	hypothetical protein Schistosoma japonicum			14.20	9.42			-4.62		2.44
TC15967	Heat stress protein		-2.76	4.95	4.75				1.61	1.60
TC16220	cytohesin-related guanine nucleotide-exchange protein			5.52	4.01					
TC16355	Heat Shock Protein 70	2.24	3.62	9.70	7.69		1.60	4.74		3.54
TC16775	heat shock protein			20.45	18.19				2.14	18.55
TC17078	hypothetical protein Schistosoma mansoni smp_CAZ39908.1	-1.88	-9.89	1.76	2.89			-1.74	1.40	-1.17
TC17105	negative cofactor 2 transcriptional co-repressor	-1.88	-1.44	6.20	5.46				1.41	1.58
TC18298	No Hit		1.54	8.62	8.46			1.67		
TC18429	No Hit			7.89	6.82			-1.15	1.71	4.26
TC1784	hypothetical protein Schistosoma mansoni smp_CAA11769.1	-4.16	-10.16	67.54	18.81					
TC1800	Peritrophin-3 Domain	-2.47	-5.52	7.44	6.01				1.88	
TC1801	Ferritin 1 (Soma)	-1.87	-2.58	8.58	2.96				1.15	
TC2269	No Hit	1.55	1.57	-4.54	-9.66					
TC10177	hypothetical protein Schistosoma mansoni smp_CAZ86084.1			5.01	4.01			-1.66	1.64	1.80
TC11361	Retinoblastoma binding protein homologue			4.12	3.61					2.26
TC11884	serine/threonine protein kinase			4.93	4.91				1.25	2.27
TC12991	filamin			4.04	3.11			1.51	1.48	1.99
TC12065	No Hit	-2.14		3.42	3.48					
TC12106	proline oxidase	2.04	1.90	-2.26	-2.00			-1.80		-1.78
TC12458	Uff1 protein		-2.17	2.90	3.98			-1.91	1.17	1.62
TC14963	macroglobulin/complement Delta/Notch ligand	-1.61	-1.85	4.19	3.24					
TC14911	Rab6-interacting			6.08	3.66					3.86
TC15655	Ser/Thr protein phosphatase family protein		-1.62	5.84	5.46			1.24		2.07
TC17400	heat shock protein 38		-2.79	3.25	1.82			-1.54		
TC17464	No Hit		-1.72	4.24	4.90					
TC17637	beta-amyloid binding protein-related			3.25	2.18			-2.09	1.50	1.88
TC17814	merlin/moesin/ezrin-like protein			14.41	9.48				1.84	2.26
TC18396	No Hit	-1.58	-1.53	5.96	4.88				1.84	1.98
TC19684	No Hit	-2.14	-9.04		9.19					
TC7085	hypothetical protein Schistosoma mansoni smp_CAZ88022.1	-1.79	-1.84	4.25	3.11					
TC7841	Retroviral-related Pol polyprotein from transposon 17.6			5.24	5.29					
TC7846	Similar to orf			4.76	4.24					1.92
TC7918	egg protein CP422-like	-2.10	-2.06	2.04	1.91					
TC8197	acid phosphatase-related			11.67	9.89			-1.48	1.69	4.67
TC8244	ubiquitin 1			4.80	9.08			1.84		1.68
TC8270	ubiquitin 1			4.76	9.29					3.48
TC8648	No Hit	-1.60	-1.40	5.28	4.48				1.45	1.97
TC8670	hypothetical protein Schistosoma mansoni smp_CAZ85517.1	-1.78	-2.11	6.60	6.60					
TC8828	egg protein CP832-like			9.15	9.04			-2.04		
TC10771	hypothetical protein Schistosoma mansoni smp_CAZ86648.1			2.04	1.77			-2.44		1.62
TC12182	hypothetical protein Schistosoma mansoni smp_CAZ81444.1			3.96	4.09			1.78		1.77
TC12218	No Hit			-9.71	-10.64					
TC12882	Fibrocystin protein 82			4.87	8.15			1.56		1.64
TC18051	No Hit			9.85	9.81					
TC18057	hypothetical protein Schistosoma mansoni smp_CAZ89288.1			-2.76	-9.51					-1.45
TC18455	No Hit			9.81	1.78					
TC18692	aldehyde dehydrogenase	1.84	2.11	-2.18					-1.29	
TC18847	family ICTB non-peptidase homologue			4.82	2.69					
TC14477	cyclin-I	1.82	1.41	-4.75	-2.69					
TC14647	No Hit			9.62	3.48			-1.67	1.58	
TC15744	No Hit			5.82	2.98					
TC17995	No Hit		-2.07	3.18	1.88			-1.51	2.00	
TC18064	No Hit	1.91		-9.86	-2.89					-2.75
TC18149	hypothetical protein Schistosoma mansoni smp_CAZ87252.1			1.74	4.86			-1.79	2.20	
TC18926	multiple zinc ion domain protein			4.09	3.18					
TC18986	No Hit			3.62	2.41		1.29			2.85
TC18450	No Hit	-2.01		-2.07	-6.29					-2.14
TC18546	Calcium-calmodulin-dependent protein kinase	1.91		-9.81	-2.41					
TC18655	No Hit	-4.98	-6.86							
TC18948	Solute carrier family 12 member 7			-6.61	-2.91				-1.74	-1.55
TC19091	hypothetical protein Schistosoma mansoni smp_CAZ81502.1			4.06	9.00					
TC8420	Talin suture carrier family 17 member 3)		1.65	2.31	1.79			-2.64	1.88	2.18
TC8204	Tyrosine kinase			9.85	2.62					
TC8295	hypothetical protein Schistosoma mansoni smp_CAZ89014.1			5.80	2.62			-2.98	1.74	
TC8846	No Hit			8.34	2.70					

List of the 74 most extremely regulated genes from the microarray study and the significant expression value for each gene in the respective treatment groups.

Table 17: Significant gene expression related to categories of interest

	Significantly Expressed Features	Significantly Expressed Calcium	Significantly Expressed Apoptosis	Significantly Expressed Drug	Significantly Expressed Iron
28DPE 1µg/mL PZQ 1h	1329	44	27	10	17
28DPE 10µg/mL PZQ 1h	859	37	29	8	10
42DPE Male 1µg/mL PZQ 1h	208	3	4	0	5
42DPE Male 10µg/mL PZQ 1h	170	5	6	0	2
28DPE 1µg/mL PZQ 20h	3482	103	65	22	40
28DPE 10µg/mL PZQ 20h	2957	106	66	16	35
42DPE Male 1µg/mL PZQ 20h	1392	39	32	5	14
42DPE Female 1µg/mL PZQ 20h	1223	38	31	7	14
42DPE Female 10µg/mL PZQ 20h	2114	75	54	13	29
Average	**1526**	**50**	**35**	**12**	**18**
Different	6161	186	137	31	68

List of four areas of interest in regards to *S. mansoni's* response to PZQ and the amount of significantly expressed genes in each of the five interest areas.

Table 18: Percent significant gene expression related to categories of interest

	Significantly Expressed Features	Percent Calcium Related	Percent Apoptosis Related	Percent Drug Related	Percent Iron Related
28DPE 1µg/mL PZQ 1h	1329	3.31%	2.03%	0.75%	1.28%
28DPE 10µg/mL PZQ 1h	859	4.31%	3.38%	0.93%	1.16%
42DPE Male 1µg/mL PZQ 1h	208	1.44%	1.92%	0.00%	2.40%
42DPE Male 10µg/mL PZQ 1h	170	2.94%	3.53%	0.00%	1.18%
28DPE 1µg/mL PZQ 20h	3482	2.96%	1.87%	0.63%	1.15%
28DPE 10µg/mL PZQ 20h	2957	3.58%	2.23%	0.54%	1.18%
42DPE Male 1µg/mL PZQ 20h	1392	2.80%	2.30%	0.36%	1.01%
42DPE Female 1µg/mL PZQ 20h	1223	3.11%	2.53%	0.57%	1.14%
42DPE Female 10µg/mL PZQ 20h	2114	3.55%	2.55%	0.61%	1.37%
Average	**1526**	**3.28%**	**2.29%**	**0.79%**	**1.21%**
Different	6161	3.02%	2.22%	0.50%	1.10%

List of four areas of interest in regards to *S. mansoni's* response to PZQ and the percent of significantly expressed genes in each of the five interest areas of the total expressed genes in each treatment group.

Table 19: Percent of genes significantly upregulated in categories of interest

	Percent of Calcium Upregulated	Percent of Apoptosis Upregulated	Percent of Drug Upregulated	Percent Iron Upregulated
28DPE 1µg/mL PZQ 1h	55%	33%	60%	59%
28DPE 10µg/mL PZQ 1h	54%	52%	75%	70%
42DPE Male 1µg/mL PZQ 1h	67%	0%	0%	20%
42DPE Male 10µg/mL PZQ 1h	80%	67%	0%	50%
28DPE 1µg/mL PZQ 20h	44%	55%	23%	45%
28DPE 10µg/mL PZQ 20h	51%	56%	13%	46%
42DPE Male 1µg/mL PZQ 20h	56%	53%	0%	14%
42DPE Female 1µg/mL PZQ 20h	42%	32%	14%	36%
42DPE Female 10µg/mL PZQ 20h	52%	59%	23%	38%
Average	**50%**	**51%**	**25%**	**43%**

The percentage of the genes in each area of interest from Table 17 that are upregulated in each of the four areas of interest.

Table 20: Calcium related genes showing interesting significant expression patterns and overlap between treatment groups

Feature	Annotation	28DPE 1µg/mL PZQ 1h	28DPE 10µg/mL PZQ 1h	28DPE 1µg/mL PZQ 20h	28DPE 10µg/mL PZQ 20h	42DPE Male 1µg/mL PZQ 1h	42DPE Male 10µg/mL PZQ 1h	42DPE Male 1µg/mL PZQ 20h	42DPE Female 1µg/mL PZQ 20h	42DPE Female 10µg/mL PZQ 20h
TC13537	Spectrin beta chain, brain 4	-1.11	-1.15	1.26	1.36					
TC14368	macroglobulin/complement	-1.61	-1.95	4.19	3.24					
TC15390	annexin	-1.65	-1.85	1.93	1.48		1.58	-1.55	1.18	
TC17625	eh domain containing/past-1-related	-1.41	-1.36	2.18	1.79					
TC18516	alpha-actinin	-1.42	-1.25	1.44	1.40					1.64
TC10450	mitochondrial intermediate peptidase (M03 family)	1.46	1.43	-1.80	-1.65					
TC11511	phospholipase	1.45	1.46	-1.98	-2.09					-1.44
TC12106	proline oxidase	2.01	1.98	-2.26	-2.00			-1.38		-1.78
TC17049	Calpain	1.27	1.34	-1.47	-1.39					
TC8640	calcium binding atopy-related autoantigen	1.29	1.28	-1.36	-1.43					
TC7936	nucleobindin			1.49	1.17				1.22	1.41
TC9229	matrix metallopeptidase-9 (M10 family)			2.67	2.57			1.47	1.75	4.55
TC8058	Protein orai-1			1.25	1.43				-1.63	-1.26
TC16966	eukaryotic elongation factor 2 kinase			-1.67	-1.51			-1.69	1.40	1.13
TC14233	tyrosine kinase			-2.33	-1.86			1.37	-1.08	-1.47
TC16813	calmodulin			-1.96	-1.49				-1.50	-1.38
TC10206	hemicentin			2.17	1.86	1.24		1.51		
TC12133	neuronal calcium sensor			2.05	1.55			1.47		1.69
TC12214	Death-associated protein kinase 3		-1.39	2.07	1.47			1.35		
TC12680	titin			1.66	1.92			1.32		
TC13622	hemicentin			1.68	1.65	1.20		1.47		
TC9786	ryanodine receptor related			1.62	1.71			1.32		
TC17560	serine/threonine protein kinase			1.19	1.31			-1.26		-1.09
TC10092	serine/threonine protein kinase			-1.74	-1.83			1.43		
TC11311	feline leukemia virus subgroup C receptor-related			-1.77	-1.59			1.25		
TC18129	high voltage-activated calcium B channel S.U. 2			-1.52	-1.69			1.27		-1.31
TC10075	cadherin			-2.15	-2.04			-1.52		
TC13171	high voltage-activated calcium channel Cav2A			-2.47	-2.63			-1.59		
TC16113	cysteine-rich with egf-like domains protein			-2.37	-1.88			-1.52	-1.39	
TC18934	Plasma membrane calcium ATPase, isoform L	-1.17		-3.58	-3.25			-2.05		-2.04
TC19272	cadherin			-1.79	-2.02			-1.19		-1.65

Table 21: Apoptosis related genes showing interesting significant expression patterns and overlap between treatment groups

Feature	Annotation	28DPE 1µg/mL PZQ 1h	28DPE 10µg/mL PZQ 1h	28DPE 1µg/mL PZQ 20h	28DPE 10µg/mL PZQ 20h	42DPE Male 1µg/mL PZQ 1h	42DPE Male 10µg/mL PZQ 1h	42DPE Male 1µg/mL PZQ 20h	42DPE Female 1µg/mL PZQ 20h	42DPE Female 10µg/mL PZQ 20h
TC10455	app binding protein	1.30	1.27	-1.46	-1.33					
TC10907	programmed cell death protein	-1.75	-2.37	1.96	2.06					1.33
TC11294	programmed cell death protein	-1.17	-1.15	1.34	1.23					
TC11843	programmed cell death protein	-1.56	-1.37	1.83	1.76					
TC15390	annexin	-1.65	-1.85	1.93	1.48		1.58	-1.55	1.18	
TC18668	Death associated protein 5	-1.74	-1.49	1.95	1.64					
TC9163	protein kinase	-1.43	-1.42	1.39	1.34					1.71
TC13915	adenylate cyclase	1.34	1.38					1.17		
TC16949	thrombospondin	1.46	1.53					1.31		-1.83
TC17619	RNA binding protein	1.35	1.32					1.31		1.48
TC7118	ankyrin repeat-containing	-1.19	-1.22	-1.23				-1.60	1.18	
TC11059	ku P80 DNA helicase			-1.47	-1.44	-1.32		-1.34		
TC13976	programmed cell death protein 2			-1.38	-1.31			-1.24		
TC18058	APEX nuclease			-1.53	-1.31			-1.17		
TC19191	inhibitor of apoptosis 1			-1.78	-1.36			-1.28		1.42
TC7067	glucose-6-phosphate isomerase			-1.47	-1.34			-1.17		
TC12214	Death-associated protein kinase 3		-1.39	2.07	1.47			1.35		
TC14883	Bcl2-associated athanogene 1			1.34	1.31			1.20		
TC17280	family S10 unassigned peptidase			1.21	1.24		1.19	1.18		
TC17560	serine/threonine protein kinase			1.19	1.31			-1.26		-1.09
TC16195	Hepatitis B virus X-interacting protein homolog			2.48	1.95				2.18	2.33
TC17324	programmed cell death protein			2.87	2.47				1.36	1.66
TC9795	Death effector domain-associated factor A		-1.05	1.65	1.52				1.18	1.26
TC12470	ADAMTS14 peptidase (M12 family)			-1.53	-1.62				-1.52	-2.13
TC16813	calmodulin			-1.96	-1.49				-1.50	-1.38
TC17764	synoviolin			-1.84	-1.51				-1.26	-1.34
TC7759	RB1-inducible coiled-coil protein 1	1.33		-1.91	-1.60				-1.25	-1.53

Table 22: ABC drug transporter related significant expression

ABC Transporter Subfamily	Array Feature	Annotation	Protein Encoded	28DPE 1µg/mL PZQ 1h	28DPE 10µg/mL PZQ 1h	28DPE 1µg/mL PZQ 20h	28DPE 10µg/mL PZQ 20h	42DPE Male 1µg/mL PZQ 1h	42DPE Male 10µg/mL PZQ 1h	42DPE Male 1µg/mL PZQ 20h	42DPE Female 1µg/mL PZQ 20h	42DPE Female 10µg/mL PZQ 20h
ABCB1	TC14689	Smp_055780	SMDR2, PGP			-1.17	-1.44				-1.38	-1.73
ABCB1	TC14236	Smp_089200	Other PGP		2.22	-2.86	-2.66				-1.30	-1.42
ABCB7	TC17965	Smp_087930				-1.27	-1.33					
	TC8655	Smp_087930				-1.30	-1.30			-1.14		1.35
ABCB8	None	L26286	SMDR1	Up	Not Significant	Not Significant	Not Significant	Not Tested	Not Tested	Not Tested	Not Tested	Not Tested
ABCC1	None	GU967672	SmMRP1	Up	Up	Down	Not Tested	Not Tested	Not Tested	Not Tested	Not Tested	Not Tested
ABCC1	TC9859	Smp_129820	Other MRP1	1.54		-1.91						-1.24
ABCG1	TC9950	Smp_181150		-1.23		-1.27					1.41	
ABCG2	TC10285	Smp_137890	BCRP	1.47	1.57							2.67
ABCG2	TC8681	Smp_126450	BCRP	-1.29		-1.39	-1.63				-1.22	-1.22

A list of the ABC transporters related to drug transport and the significant expression values from the microarray. Also included are the results of the two drug transporters tested with RT-PCR that were not on the microarray, SMDR1 and SmMRP1. Values for RT-PCR expression are not reported, just the direction of significantly expressed genes from the tested exposure groups.

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