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TOWARDS A BETTER UNDERSTANDING OF THE EFFECTS OF PRAZIQUANTEL ON THE INTERACTION BETWEEN THE HELMINTH PARASITE SCHISTOSOMA MANSONI AND ITS MURINE HOST

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ABSTRACT

Schistosomiasis is a neglected tropical disease (NTD) that infects 206 million individuals worldwide. This disease is caused by dioecious trematodes of the genus *Schistosoma*, and its pathology is associated with the large number of eggs that are released by the female. Entrapped eggs lead to host immune and inflammatory responses resulting in disease progression. Chemotherapy provides the main means of control and praziquantel (PZQ) is the only widely available drug that is used in all mass drug administration (MDA) programs. As the number of individuals receiving PZQ continues to grow, the development of PZQ resistance is a concern and increases as the drug pressure placed on the parasites increases. We employed Next Generation Sequencing (NGS) to provide transcriptomic data related to four areas of attention: 1) What is the effect of PZQ on the murine host- *S. mansoni* interaction, and how does PZQ effect *S. mansoni in vivo*?, 2) What is the effect at the molecular level of PZQ on male *S. mansoni in vivo*?, 3) What is the respective contribution of R- and S- enantiomers to the *S. mansoni* transcriptomic response?, and 4) What is the

molecular basis for reduced PZQ susceptibility in lab strains of *S. mansoni* exposed to increasing sub-lethal doses of the drug?

We found that the murine hepatic response to infection follows a T helper 1 (Th1) response followed by a T helper 2 (Th2) response upon the increase in egg burden that, in turn, leads to significant granuloma formation and fibrosis. While infected mice treated with PZQ have a significantly reduced egg burden and granuloma formation, the immune response remains relatively similar. Additionally quantitative real-time PCR allowed *in vivo* examination of ATP-binding cassette (ABC) transporters in juvenile and adult *S. mansoni*. Results show juvenile schistosomes had induced activity of multiple members of this gene family and provides support that these transporters may provide juveniles natural resistance to PZQ.

The examination of the effects of the enantiomers of PZQ on male *S. mansoni* resulted in more differentially expressed transcripts in the R-PZQ treated parasites then S-PZQ treated compared to control, with a total of 101 up- and down-regulated transcripts in R-PZQ, and 22 in the S-PZQ treated group. The enantiomers shared 4 up-regulated transcripts and 7 down-regulated transcripts, with S-PZQ having one unique up-regulated transcript (Smp_125510, putative cadherin). The up-regulated transcripts identified for R-PZQ contained 26 hypothetical or uncharacterized proteins, 3 major egg antigens, and three heat shock proteins. The R-PZQ enantiomer is the main anti-schistosomal effector, perhaps explaining the differences in transcripts expressed between the parasites treated with each enantiomer.

Lastly the examination of reduced PZQ sensitivity in PR1 *S. mansoni* revealed a shift in sex ratio to a prominently female population combined with reduced length in female

parasites compared to the control parasites. Moreover, we found no association between PZQ insensitivity and specific gene products or pathways perhaps pointing to a single loss of function gene mutation or epigenetic interactions involving multiple genes.

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INTRODUCTION

Schistosomiasis infects 206 million people in approximately 78 countries; of these individuals over 90% live in sub-Saharan Africa (Ross et al., 2017), and it is estimated ~800 million people are at risk of exposure to the disease (Steinmann et al., 2006). Infection occurs during routine exposure (agricultural, domestic, occupational, and recreational) to infected water, and the transmission dynamics make children especially susceptible to infection and account for 111 million cases (Bustinduy et al., 2016). In children the disease has been linked to anemia, stunted growth, and reduced cognitive capacities (Colley et al., 2014). The treatment for schistosomiasis depends almost exclusively on the drug praziquantel (PZQ), and all mass drug administration (MDA) programs rely on praziquantel for the long-term goal of elimination (King, 2017).

Schistosomiasis is considered a Neglected Tropical Disease (NTD) a term used to describe viral, bacterial, and parasitic diseases that together impacts the lives of more than 1 billion people in 149 countries (WHO, 2015). NTDs mainly affect populations in poverty stricken developing regions of the world that lack adequate sanitation, including access to potable water (WHO, 2015). Schistosomiasis is a parasitic disease caused by dioecious trematodes in the Genus *Schistosoma*, with three main species contributing to human cases, *Schistosoma mansoni, Schistosoma haematobium*, and *Schistosoma japonicum*. Schistosomes have a complex lifecycle involving a definitive host, humans in named species, and a gastropod intermediate host. *S. mansoni* and *S. haematobium* infect aquatic freshwater *Biomphalaria* spp and *Bulinus* spp snails respectively, while *S. japonicum* infect amphibious freshwater *Oncomelania* spp snails (Colley et al., 2014). The parasites undergo asexual reproduction in the intermediate host and are released into fresh water as infective cercariae,

which penetrate the skin of definitive hosts and mature into sexually mature adults. The adult parasites pair up and produce eggs in the venules that must be released into the environment. In order to cross the endothelium and lining of organs (intestines or bladder) the eggs must orchestrate an intense cellular response to allow for excretion and continuation of the parasite's lifecycle (File, 1995). In the case of *S. mansoni*, an adult female can lay 100-300 eggs per day but one third to half of the eggs released will not reach the external environment (Cheever et al., 1994; Pearce and MacDonald, 2002); it is these eggs that become trapped in host tissues and elicit a host immune response leading to serve pathology (Burke et al., 2009; Pittella, 1997). Granuloma formation serves to contain proteolytic products resulting from the egg and provide protection for the host (Fallon, 2000). It is known granulomas are a predecessor of fibrotic tissue formation leading to an increase in portal blood pressure and portal systemic shunt formation (Cheever et al., 2002; Wilson et al., 2006).

An overarching observation in *Schistosoma* infections is an initial T-helper1 (Th1) response that is pro-inflammatory, which then switches to a T-helper2 (Th2) response upon egg production (de Oliveira Fraga et al., 2010; La Flamme et al., 2001; Vella and Pearce, 1992). Th2 type responses are needed for minimization of host pathology as seen with studies that showed mice depleted of Th2 type responses mainly interlukin-4 resulted in higher mortality related to pathology from pro-inflammatory Th1 type responses (La Flamme et al., 2001; Patton et al., 2002). Additionally egg antigens are thought to prime activated macrophages resulting in down-regulation of Th1 responses and thus decreasing mortality associated with inflammation (Anthony et al., 2007; Hams et al., 2013). To date, there have been multiple studies involving schistosomiasis immunopathogenesis and all have investigated specific mechanisms or a small number of genes. One of my research aims was

to present a global view of the murine host hepatic immune and fibrotic responses as a result of *S. mansoni* infection and investigate the effects PZQ has on those transcriptomic responses (Chapter 1).

The treatment and control of schistosomiasis has relied on PZQ for over 40 years (Andrews, 1985; Caffrey, 2015; Cioli and Pica-Mattoccia, 2003). The exclusive use of PZQ is based on the efficacy (40mg/kg in humans yields 60-90% cure)(Doenhoff, 1998), safety (Sousa-Figueiredo et al., 2012), convenience, and price (Hotez et al., 2010). Although PZQ has been used for many years the mechanism of action (MOA) remains unknown. The PZQ effects on schistosomes have been previously studied and it has been demonstrated that PZQ causes calcium ion influx, muscle contraction, and surface modifications *in vitro*, and it is thought the disruption of the parasite's tegument may lead to exposure of surface antigens and host recognition and clearance by host immune system (Brindley et al., 1989; Doenhoff et al., 1987; Harnett and Kusel, 1986; Kohn et al., 2001). The drug is synthesized and administered as a racemic compound containing equal parts R- (levorotatory, $_{L}$-PZQ) and S- (dextrorotatory, $_{D}$-PZQ) stereoisomers and studies have shown that the anthelmintic activity of PZQ is associated with R-PZQ, and its side effects, large pill size, and bitter taste being attributed to S-PZQ (Andrews, 1985; Meister et al., 2014; Olliaro et al., 2014).

S. mansoni has been shown to have sex and stage related variability to PZQ sensitivity, and earlier research has shown that *in vivo* fifty percent drug effective dose (ED_{50}) in the infected mouse model to be 80.9 mg/kg in bi-sex adult infections (49 days post infection (dpi)) compared to 2456 mg/kg in bi-sex juvenile infections (28 dpi); in addition female adult unisex infection had an ED_{50} of 1107 mg/kg compared to 197.7 mg/kg in male unisex infections (Pica-Mattoccia and Cioli, 2004; Xiao et al., 1985). Juvenile parasites

natural resistance to PZQ provides an obstacle when it comes to control of schistosomiasis because repeated doses of PZQ are needed to kill all the parasites in an individual, a protocol not currently performed in endemic areas under MDA programs.

MDA programs rely on PZQ to control schistosomiasis and the number of individuals receiving treatment continues to grow raising concern for emergence of PZQ resistance. There have been reports of PZQ insensitivity/resistance in the field (Ismail et al., 1996; Melman et al., 2009; Stelma et al., 1995) but the overwhelming theme is there is no evidence of widespread or enduring PZQ resistance. In addition, PZQ resistance has been experimentally induced in the lab multiple times (Couto et al., 2011; Fallon and Doenhoff, 1994; Ismail et al., 1994). In both field and lab selected PZQ resistance there has been some evidence that PZQ resistance comes at a cost, decreased fitness (Coeli et al., 2013; Melman et al., 2009; William et al., 2001). The lack of sustained evidence for PZQ resistance in the field may be attributed to reduced fitness or may be explained by the large refugia of susceptible parasites resulting in a reduced likelihood that PZQ resistance is sustained. A study examining an endemic area that 10 years earlier reported PZQ resistance (Ismail et al., 1996) found no evidence for PZQ resistance in this area that had received multiple PZQ treatments (Botros et al., 2005) and may provide support for fitness reduction as a tradeoff to resistance.

The mechanisms by which schistosomes acquire PZQ resistance remains unclear however studies have provided some clues regarding PZQ resistant parasites. Two studies have pointed to induction of ATP-binding cassette (ABC) transporters, drug and xenobiotics influx and efflux pumps. Hines-Kay and colleagues found increased levels of two such transporters in juvenile parasites compared to adults *in vitro* (Hines-Kay et al., 2012), and

Kasinathan et al. (Kasinathan et al., 2010) found juvenile parasites express higher levels of a ABC transporter gene (SmMRP1) compared to control parasites. Lastly, a recent study identified characteristic proteins in PZQ resistant *S. mansoni*, including identifying proteins unique to female PZQ resistant parasites (Pinto Almeida et al., 2018).

The goal of this dissertation was to gain a better understanding of the intimate relationship that encompasses the disease triangle of schistosomiasis by looking at the effect of PZQ on the host (murine), and pathogen (*S. mansoni*) by applying Next Generation Sequencing (NGS). The three main areas of focus are to investigate the effect of PZQ on the murine host-*S. mansoni* interaction and on *S. mansoni in vivo* (Chapter 1), examine the effect of R-and S-PZQ on *S. mansoni in vitro* (Chapter 2), and examine the molecular basis for PZQ resistance in *S. mansoni* (Chapter 3). This dissertation contributes to the field of schistosomiasis by providing new insights into the transcription profiles related to the three areas of focus. Ultimately the work conducted has enhanced our understanding of the effect of PZQ on *S. mansoni* and provided transcriptional data as the basis of analyses and meta-analyses.

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Chapter 1

Effect of praziquantel on the differential expression of mouse hepatic genes and parasite ATP binding cassette transporter gene family members during *Schistosoma mansoni* infection

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Abstract

Schistosomiasis is a chronic parasitic disease caused by sexually dimorphic blood flukes of the genus *Schistosoma*. Praziquantel (PZQ) is the only drug widely available to treat the disease but does not kill juvenile parasites. Here we report the use of next generation sequencing to study the transcriptional effect of PZQ on murine hepatic inflammatory, immune and fibrotic responses to *Schistosoma mansoni* worms and eggs. An initial T helper cell 1 (Th1) response is induced against schistosomes in mice treated with drug vehicle (Vh) around the time egg laying begins, followed by a T helper cell 2 (Th2) response and the induction of genes whose action leads to granuloma formation and fibrosis. When PZQ is administered at this time, there is a significant reduction in egg burden yet the hepatic Th1, Th2 and fibrotic responses are still observed in the absence of granuloma formation suggesting some degree of gene regulation may be induced by antigens released from the dying adult worms. Quantitative real-time PCR was used to examine the relative expression of 16 juvenile and adult *S. mansoni* genes during infection and their response to Vh and PZQ treatment *in vivo*. While the response of stress genes in adult parasites suggests the worms were alive immediately following exposure to PZQ, they were unable to induce transcription of any of the 9 genes encoding ATP-binding cassette (ABC) transporters tested. In contrast, juvenile schistosomes were able to significantly induce the activities of ABCB, C and G family members, underscoring the possibility that these efflux systems play a major role in drug resistance.

Author summary

Schistosomiasis is a disease caused by parasitic worms that significantly impacts the lives of approximately 260 million people. During infection, female parasites can release thousands of eggs, a significant number of which become trapped in the liver. Entrapment of eggs triggers immune and inflammatory responses that in turn causes many of the health problems associated with the disease. In this study, we analyzed gene expression profiles of livers from mice infected with the human parasite *Schistosoma mansoni* over multiple time points, beginning when the parasites start to lay eggs. In mice treated with a lethal dose of the anti-schistosomal drug praziquantel, we identified a number of different genes and pathways central to immune and inflammatory responses that are active even in the absence of egg deposition. Praziquantel is the only drug available to treat schistosomiasis, however, it displays bimodal activity in that it is ineffective against juvenile parasites during the early stages of mammalian infection. We also show that development of drug resistance may be due to the improved efficacy of the juvenile parasite to actively excrete the drug. These results provide insights into the effect of praziquantel on the host response to infection as well as the ability of juvenile parasites to overcome the drug's lethal effect.

Introduction

Schistosomiasis is a chronic neglected tropical disease caused by digenetic parasitic flatworms of the genus *Schistosoma.* In 2014, 259 million people were treated for the disease with 91.4 % of those individuals living in Africa where *Schistosoma mansoni* and *S. haematobium* are the major causative agents [1]. Of those infected globally, an estimated 123 million were school-aged children [1]. The human cost of schistosomiasis in 2010 was calculated at approximately 3 million years lived with disability [2], however, accurate numbers are difficult to pin down for a number of reasons, not least of which is the contribution of co-infection with other helminths as well as HIV [3].

The complex life cycle of *Schistosoma spp.* requires that free-living cercariae released from infected fresh water snails burrow through human skin and enter the venous circulation as schistosomulae [4]. Within a week, parasites migrate to the lungs and reach the hepatoportal circulation approximately 14 days after penetration of the skin. In experimental mouse disease models, schistosomes do not develop synchronously though most can be considered as sexually immature juveniles between 14 and 28 days post infection, with sexual maturation occurring from day 28 onward. In the case of *S. mansoni*, sexually mature adult male and female worms reside in the hepatic portal and mesenteric venous systems of the host with the female releasing increasing numbers of fertilized eggs, of which approximately half will migrate through the bowel wall to be excreted in the host stool. The remaining eggs often become trapped in the liver where they trigger host inflammatory responses. Hepatic fibrosis and granuloma formation caused by the deposition of collagen and extracellular matrix components around the eggs leads to the occlusion of the hepatic

portal veins, which in turn causes portal hypertension, enlargement of the spleen, ascites, and gastrointestinal bleeding [4,5].

Numerous studies have tracked the expression of immune and inflammatory hepatic genes during infection, inflammation and granuloma formation. In the early stages of infection, a T-helper cell type 1 response (Th1) against the parasite has been recognized through increased production of pro-inflammatory cytokines (TNFα, IL1α, IL1β and IL6) as well as Signal Transducers and Activators of Transcription 1 (STAT1) and IFNγ [5-7], while elevated Th17 cell numbers have been suggested to mediate bladder pathology during *S. haematobium* infection [8]. As parasite egg deposition commences, a contemporaneous Th2 response characterized by a surplus of chemokine expression as well as IL4, IL5, IL10, IL13 and IL33 production also gets underway [5-7, 9]. In addition, production of IL10 by regulatory T cells (Treg) may help to control pathology along with IL10 independent naturally occurring CD4+ Foxp3+ regulatory T cells [10, 11]. Chuah and colleagues used microarrays to study neutrophil mediated changes in gene expression during granuloma formation in *S. japonicum* infected mice and found a significant up-regulation of Th1, Th2 and Th17 immune genes, as well as inflammatory genes within the granuloma that are spatially and temporally separated [12].

While a number of drugs have been used to treat schistosomiasis only one, praziquantel (PZQ), is widely employed as it is relatively cheap, easy to use, and effective against all schistosome species that infect humans [13]. Its use in the past 10 years has increased significantly as the number of patients treated has grown from approximately 12 million in 2006 to 61.6 million in 2014 when 20.7 % of affected individuals received the drug [1]. This has largely been due to the implementation of mass treatment campaigns with, for example,

an increase of 52.3 % in those receiving treatment in the African region when comparing 2014 with the previous year [1].

Although PZQ brings relief to those treated, it does not provide a cure as juvenile schistosomes are relatively resistant to the anthelmintic effects of the drug [14-17]. When available, administration of PZQ is often limited to a single dose per year and treated individuals, frequently children, will quickly become reinfected through continued and unavoidable exposure to the parasite. Additionally, any juvenile parasites that escape elimination during treatment subsequently mature and begin to release eggs. The frequent exposure and survival of resistant juveniles to PZQ also gives concern that under this ineffective pressure, drug resistance could emerge [18].

In the absence of an anti-schistosomal vaccine, one route to improving treatment of patients is to enhance the efficacy of PZQ by increasing the sensitivity of juvenile parasites to the drug. This approach requires an understanding of the molecular basis of juvenile resistance. While it is possible resistance may be driven by a reduction in expression of the drug target or an increased rate of drug metabolism, we and others have suggested that ATP Binding Cassette (ABC) multi-drug transporters play an important role [19-23]. These trans-membrane proteins work by hydrolyzing ATP and using the energy liberated to move compounds, including drugs, across membranes. Drug resistance results from the amplification, over-expression or modification of some members of this transporter family [24].

In the current study we use next generation sequencing (RNA-Seq) to quantitate the effect of PZQ on the mouse hepatic transcriptomic response to *S. mansoni* infection during the immediate two-week period following the transition of the parasite from sexually

immature juvenile to mature adult. We observe that PZQ has little effect on immune and inflammatory gene regulation in the period immediately following drug treatment, but do note a significant reduction in these responses in the absence of egg deposition two weeks after treatment commenced. In addition, we investigate the response of juvenile and adult schistosomes to treatment with PZQ *in vivo* by quantitative real-time PCR and demonstrate that juvenile schistosomes may protect themselves from the lethal effects of the drug through up-regulation of a number of ABC transporter genes.

Materials and Methods

Ethics statement

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health. Animal use procedures were reviewed and approved by the University of New Mexico Institutional Animal Care and Use Committee.

Mice, parasites and experimental procedures

For all infection experiments, eight to ten-week-old female Swiss Webster outbred mice (Charles River, Kingston, NY) were each infected percutaneously with approximately 150 *S. mansoni* Puerto Rican 1 (PR1) cercariae. Treatment with PZQ (Sigma-Aldrich, St Louis, MO) was administered by gavage at a dose of 250 mg/kg/day in vehicle (Vh) (Cremophor EL, Sigma-Aldrich) for four consecutive days. Control mice were either infected or left uninfected and administered an equivalent volume/kg/day of Vh for four consecutive days.

To determine the effect of PZQ or Vh on the number of parasites present in the livers of mice during and after treatment, PZQ or Vh was administered to two groups of 15 mice infected with *S. mansoni* (PR1) beginning on day 32 post infection (from this point onwards all data points will be defined by the time post cercarial challenge i.e., days 32, 35, 39 or 46). After infection, mice were randomly distributed into the treatment or control group and five mice from each group sacrificed 3 h after the initial treatment on day 32, after the final treatment on day 35 and 14 days after treatment commenced (day 46). After euthanasia with sodium pentobarbital, livers were collected, gently shredded and parasites counted (n = 5 per group).

The number of eggs present in livers of infected mice receiving PZQ or Vh for four consecutive days starting on day 32 was also assessed. Mice were sacrificed (n = 4 per group) on days 32 and 35 (3 h after treatment) and on days 39 and 46. Livers were digested at 37°C overnight in 4 % potassium hydroxide and the number of eggs per gram of liver calculated [25].

To determine changes in expression of hepatic and schistosomal genes during infection and PZQ treatment, two groups of 16 mice were infected with *S. mansoni* and treated with PZQ or Vh for 4 consecutive days beginning on day 32. Mice were sacrificed 3 h after treatment (n = 4 per group) on days 32, 35 and on days 39 and 46. A third group of 16 uninfected mice were treated with a weight related dose of Vh (S1 Fig). Three of the four whole livers were randomly selected and placed in RNA*later* (ThermoFisher Scientific, Waltham, MA) for RNA isolation. The remaining liver was placed in 10 % formalin for histological analysis. Livers from four uninfected Vh treated mice at each time interval were used as controls to provide gene expression and histological baseline data.

Isolation of total RNA

Livers were removed from RNA*later*, weighed then placed in RNeasy lysis buffer with 1 % 2-mercaptoethanol (Sigma-Aldrich) and homogenized. Total RNA was isolated using an RNeasy Maxi Kit (Qiagen) and digestion with RNase-free DNase (Qiagen, Redwood City, CA) according to the manufacturer's instructions. Total RNA was quantified using a ND-1000 spectrophotometer and the quality verified using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). RNA was stored at -80° C. Representative bioanalyzer traces from each treatment group are shown in S2 Fig and a minimal RNA integrity number (RIN) of 6 was the threshold for sample use in this study.

Next generation sequencing: cDNA library construction, sequencing and data processing

RNA sequencing (RNA-Seq) was conducted on three separate biological replicates representing each type of treatment and time-point using the Illumina Next Generation Sequencing (NGS) platform (Illumina, San Diego, CA) and was performed at the National Center for Genome Resources (NCGR, Santa Fe, NM). First strand cDNA libraries were prepared from 500 ng of polyA$^+$ heat-fragmented RNA using Superscript II (Invitrogen, Carlsbad, CA) and random hexamer primers followed by second strand cDNA synthesis with Second Strand Master Mix (Illumina). Universal and bar coded TruSeq Adapters were ligated to cDNA ends and the resulting adapted cDNA libraries were PCR amplified and further purified. Samples were normalized to a concentration of 10 nM and pooled prior to sequencing. Sequencing was performed on a HiSeq 2000 instrument (Illumina) to generate

50 base-pair single-end reads. The raw sequence reads for each sample were checked for quality control using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and mapped to the *S. mansoni* (annotation: Ensembl release 75) and *Mus musculus* (assembly: GRCm38, annotation: Ensembl release 75) genomes using TopHat2 [26]. The resulting alignment files were analyzed with Cufflinks [27] and HTSeq-Count [28] to generate a transcriptome assembly for each data set [29]. Differentially expressed genes (DEG) were identified in PZQ and Vh treated infected transcriptomes after normalization against Vh treated uninfected transcriptomes using three separate analysis tools: Cuffdiff [30], DESeq [31] and edgeR [32] with cutoffs for all programs set at an adjusted $p \leq 0.05$ with $\log_2$ fold change either <-1 (down-regulated genes) or >1 (up-regulated genes). DEG lists for each sample from each program were displayed using a Venn diagram and only genes common in multiple bioinformatics approaches were retained for further analysis. The online Lumenogix platform (api.lumenogix.com) was used to perform all bioinformatics analysis [33]. Two-dimensional principal component analysis (PCA) to characterize inter-variable relationships was performed using R package DESeq2. All high-throughput sequence data sets generated for this study were deposited with Gene Expression Omnibus and can be accessed through series GSE19432.

Pathway analysis

Final DEG lists from RNA-Seq analysis were analyzed with Ingenuity® Pathway Analysis (IPA) tool version 6 (Qiagen). The IPA Knowledge Base incorporates in-house curating and public databases to formulate and update signaling pathways. Overrepresented pathways are measured as the probability of association between experimental gene sets

(DEG) compared to a reference gene set for specific processes or pathways. A right-tailed Fisher's Exact Test resulting in a $p < 0.05$ indicates a statistically significant non-random association.

Gene ontology analysis

Protein Annotation Through Evolutionary Relationship (PANTHER) was used to classify differentially expressed genes with ontology terms to identify biological functions present in the final DEG lists based on the statistical overrepresentation test [34].

Quantitative real-time PCR

Mouse RNA used for quantitative real-time PCR (qRT-PCR) originated from the corresponding liver samples used for Illumina sequencing. qRT-PCR was performed on six mouse genes to validate the expression patterns observed with RNA-Seq analysis. These genes were chosen for validation based on their differentially expressed profile in the Illumina data sets, i.e., having > 20 reads per sample and the qRT-PCR primers passing efficiency tests. One microgram of total RNA was reverse transcribed in a 20 μL reaction using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Predesigned PrimeTime® qRT-PCR gene specific assays (Integrated DNA Technologies; primer sequences are shown in S1 Table) were used for RNA-Seq validation. qRT-PCR was carried out in 20 μL reactions containing 100 ng cDNA, 0.5 μM primer and SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) and performed in biological triplicates with technical duplicates using a C1000 96 Touch Thermo Cycler (Bio-Rad Laboratories). PCR cycling conditions for RNA-Seq

validation were 95°C for 2 min followed by 40 cycles at 95°C for 5 s, 60°C for 30 s and 65°C for 15 s. Relative expression ($2^{-\Delta\Delta Ct}$) was performed using CFX Manager™ Software v3.1 (Bio-Rad Laboratories) to calculate the fold change relative to the reference *Mus musculus* gene glyceraldehyde-3-phosphate dehydrogenase (PrimePCR™ SYBR® Green Assay: GAPDH, Mouse, Bio-Rad Laboratories). Expression of this gene did not vary significantly between treatment groups. Correlation between qRT-PCR and RNA-Seq data was assessed using a Spearman's Rho correlation, as the data were not normally distributed. Data analysis and statistical comparisons were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

The expression of 16 juvenile and adult *S. mansoni* genes were analyzed by qRT-PCR. These represented genes we previously reported to be differentially regulated as a result of PZQ treatment *in vitro* [22] or those identified in the recent literature as being affected by PZQ including ABC multi-drug transporter, calcium regulatory and stress-related family members.

To investigate the effect of drug treatment on juvenile *S. mansoni* gene expression *in vivo*, groups of infected mice were treated with Vh or PZQ on four consecutive days beginning on day 25 as described above. Mice were sacrificed 3 h after treatment (n = 4 per group) on days 25 and 28 and the livers placed in RNA*later* for total RNA isolation. For qRT-PCR analysis, mRNA was reverse transcribed and parasite gene specific cDNA amplified. Similarly, mouse liver RNA samples used for Illumina sequencing that contained infecting adult *S. mansoni* mRNA were reverse transcribed and parasite gene specific cDNA amplified. The general conditions for *S. mansoni* qRT-PCR were as described above. *S.*

mansoni GAPDH was used as a housekeeping gene to calculate relative fold change as its expression did not vary significantly between treatment groups (S2 Table). qRT-PCR cycling conditions for *S. mansoni* gene expression were 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 60°C for 1 min and 60°C for 20 s. PCR primers for *S. mansoni* genes (S3 Table) were designed using the Integrated DNA Technologies OligoAnalyzer tool (www.idtdna.com). Fold change was calculated as described above. One-way analysis of variance (ANOVA) was performed to calculate statistical significance of normalized gene expression between treatment groups and where significance was detected the Fisher's least significant difference post hoc test for multiple comparisons was used with $p < 0.05$ considered statistically significant.

Results and Discussion

Effect of praziquantel on parasitic infection

The initial research aim was to identify a suitable four-day treatment window when the majority of infecting *S. mansoni* would be mature enough to be PZQ sensitive, but young enough that egg accumulation and resultant tissue damage in the liver would be negligible. This would allow a comparison of the host hepatic transcriptome giving insights into development of both the immune and inflammatory response to PZQ treated worms as well as to the ongoing infection in those treated with Vh. In addition, it was intended initially that the worm transcriptomic response to both PZQ and mouse immune and inflammatory reaction to infection might also be acquired.

For these studies mice were infected with approximately 150 *S. mansoni* cercariae. This level of infection will usually result in around 40-60 worms reaching maturity with the host becoming debilitated on or around days 49 to 56 post infection. Preliminary studies were conducted that identified the 32nd day after infection as the optimal time-point to begin PZQ treatment (data not shown). At this stage, infection was still relatively benign as few if any eggs had been deposited in the host liver with minimal signs of animal distress or hepatic damage, yet the majority of worms had matured sufficiently to have acquired sensitivity to the drug. Administering 250 mg/kg PZQ on day 32 resulted in no immediate effect on the number of live schistosomes retrieved by dissection from the liver 3 h after treatment compared with Vh treated infected mice (Fig 1A). After four consecutive daily doses totaling 1000 mg/kg there was a small but significant ($p < 0.05$) drop in the number of live worms retrieved from PZQ treated (22.4 ± 2.8) compared to Vh treated mice (29.1 ± 1.8). This difference was greater still ($p < 0.001$) when mice were sacrificed 14 days (day 46) after the initial treatment when limited numbers of *S. mansoni* were retrieved from the liver of PZQ (8.3 ± 5.2) compared with Vh (54.3 ± 17.4) treated mice. The hepatic egg burden was negligible in controls and drug treated mice at days 32 and 35 but had increased significantly ($p < 0.05$) in Vh compared with PZQ treated mice on days 39 and 46 (Fig 1B). Treatment with PZQ resulted in little accumulation of eggs in the liver during the course of the experiment, however, the number of eggs deposited in the liver of Vh treated mice reached its maximum on day 46 coinciding with a significant ($p < 0.001$) increase in liver weight compared to PZQ treated mice (Fig 1C).

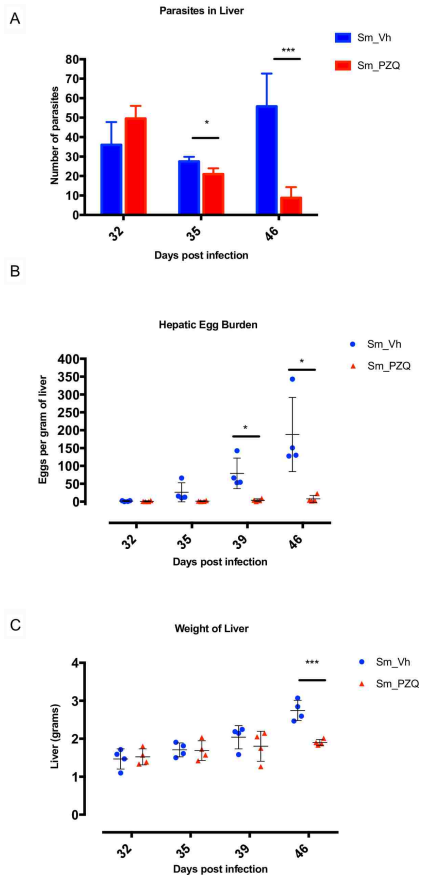

Fig 1. The effects of PZQ on infected host liver. (A) Effect of treatment with Vh and PZQ on the number of parasites present in host livers at days 32, 35 and 46 post infection, n = 5 per treatment group. (B) Number of parasite eggs per gram of liver at days 32, 35, 39 and 46 post infection after treatment with Vh and PZQ (n = 4). (C) Weight of liver at days 32, 35, 39 and 46 post infection after treatment with Vh and PZQ (n = 4). Error bars represent mean with standard deviation. * $p < 0.05$ and *** $p < 0.001$.

Transcriptome sequencing, assembly and analysis

To compare changes in the hepatic transcriptome, cDNA libraries were prepared from the livers of 24 *S. mansoni* infected mice treated with PZQ or Vh, as well as 12 uninfected mice treated with a weight related dose of Vh (S1 Fig). After the removal of adaptor sequences and any ambiguous or low quality reads (Q <20), a total of approximately 920 million 50 base pair single-end reads were obtained (S4 Table). The mean number of reads in each of the 12 treatment groups (n = 3 per group) varied between 23.48 and 27.78 million. Sequence

reads were assigned to the *Mus musculus* genome assembly and 88.2-91.0 % of individual library reads with means of 88.9-90.3 % for each group were successfully mapped. The number of aligned reads per sample was more than sufficient for differential expression analysis with treatment groups each containing three biological replicates [35]. In contrast, only a mean of between 0.01 % (uninfected liver from Vh treated mice on days 39 and 46) and 0.13 % (infected liver from Vh treated mice on days 32 and 35) of reads mapped to the *S. mansoni* genome. While the former number likely represents transcripts of high homology between mouse and parasite as no *S. mansoni* transcripts should be present, the latter was not sufficiently robust to allow for inferences to be made regarding the expression of *S. mansoni* genes during Vh or PZQ treatment.

When the transcriptomes of the 36 individual mouse livers were compared globally, they segregated into three distinct clusters with the 12 uninfected Vh samples grouped together (Fig 2A). Of the infected individuals, the day 32, 35, 39 and 46 transcriptomes from PZQ treated mice grouped together with the day 32, 35 and 39 transcriptomes from Vh treated mice with a high degree of sub-clustering generally coincident with the time and type of treatment. The six day 39 and 46 PZQ treated mice formed a separate sub-cluster indicating their transcriptomes were distinct from the others within this larger subset. The three day 46 Vh samples formed a distinct third cluster and were more similar to each other than any other samples, likely reflecting an expanded gene response to increasing egg deposition in these mice and the ensuing hepatic damage. All three biological replicates associated with each individual treatment and time grouped closely together, suggesting consistency between samples and none were omitted from further analyses. PCA was used to further characterize the relationship between the treatment groups (S3 Fig) and confirmed the internal structure of

the dendrogram (Fig 2A) and that conditions for the day 46 Vh samples accounted for most of the variation.

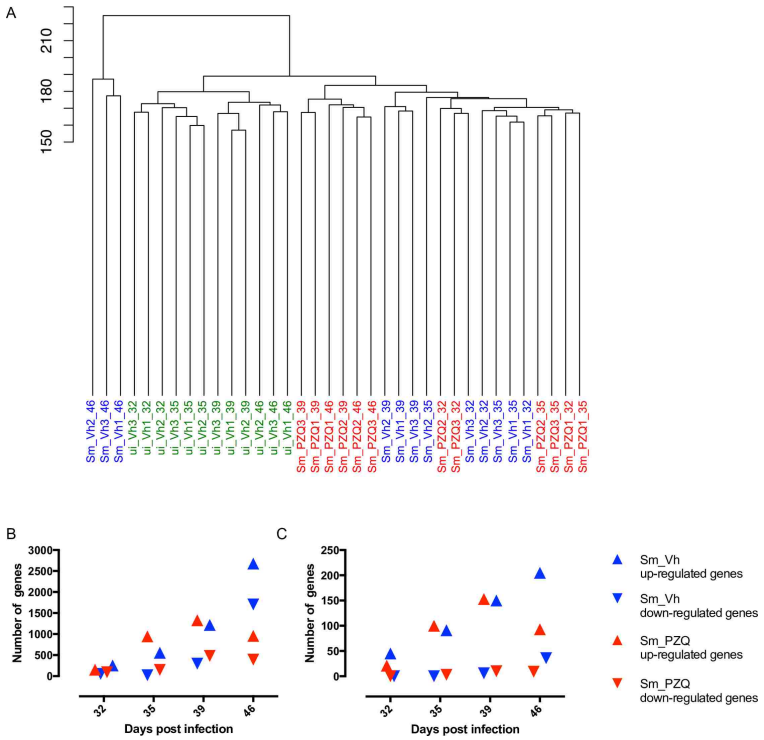

Fig 2. Hierarchical clustering of gene expression between different treatment groups and differentially expressed genes. (A) Dendrogram showing global similarities between hepatic transcriptomic replicates (n = 3) of uninfected Vh treated (ui_Vh), infected Vh treated (Sm_Vh) and infected PZQ treated (Sm_PZQ) mice at days 32, 35, 39 and 46 post infection. (B) The total number of up- and down-regulated hepatic genes in Vh and PZQ treated mice compared to uninfected Vh treated control samples at days 32, 35, 39 and 46 post infection. (C) The total number of up- and down-regulated immune genes identified by GO analysis in Vh and PZQ treated mice compared with uninfected Vh treated control samples at days 32, 35, 39 and 46 post infection.

As it has already been established that PZQ has no significant effect on the uninfected mouse liver transcriptome beyond 3 weeks post drug delivery [36] the transcriptomes of the two infected treatment groups at each time point were normalized against the corresponding uninfected Vh treated samples and differentially regulated genes were then identified. While there is no discernible difference in the numbers of differentially regulated genes between the treatment groups at days 32, 35 and 39, day 46 revealed a substantial increase in the number

of both up- and down-regulated genes in Vh compared with PZQ treated mice (Fig 2B and S5 Table). Gene Ontology (GO) analysis revealed that while the number of differentially regulated genes increased with each time point regardless of treatment, the proportion of each biological process did not alter significantly (S4 Fig). The identity of all differentiated genes at each time point and treatment as well as their fold change is shown in S6 Table. The number of up-regulated genes characterized as 'immune response related' by GO analysis increased over the first three time points irrespective of treatment while the number of down-regulated genes was consistent (Fig 2C and S5 Table). At day 46, however, the number of up-regulated genes in the PZQ samples decreased in comparison to day 39. In contrast, the number of up- and down-regulated immune genes in the Vh samples increased. These results suggest that when viewed globally, PZQ treatment has little impact on the host's immune response to infection until after day 39 when egg accumulation became more pronounced in Vh treated animals.

Expression analysis of immune and fibrotic transcripts

To identify the response of individual genes across treatment and time in greater detail, heat maps were constructed for differentially regulated immune/inflammatory and fibrotic genes (Fig 3). Of the classic inflammatory cytokines, IL1β was up-regulated at days 32 through 46 in Vh samples but was only induced at days 32 and 35 with PZQ treatment. IL6 was only found to be up-regulated in day 46 Vh samples and TNFα was up-regulated at days 32 through 46 with a peak of activity at day 39 in Vh samples, while treatment with PZQ resulted in peak at day 39 but this abruptly disappeared at day 46. (Fig 3A).

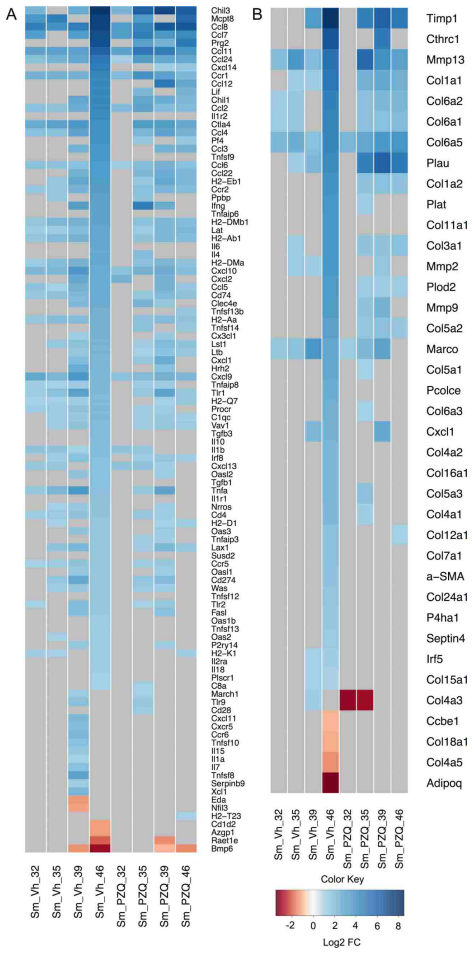

Fig 3. Temporal expression of mouse hepatic genes during infection with *S. mansoni*. Heat maps of (A) immune and (B) fibrotic gene markers depicting differentially expressed genes in infected Vh and PZQ treated mice relative to uninfected mice. Gray map sections represent genes not expressed at that time point or in that treatment group. Regions of blue and red indicate, relative to uninfected Vh treated controls, increased and decreased gene expression respectively. The color scale indicates $\log_2$ fold change (FC) and the profile of each group is the average of three biological replicates. Gene names associated with the figure differ slightly from those used in the text. Relevant differences include: Ccl (rather than CCL as it appears in the main body of text), Cxc (CXC), Cxcl (CXCL), Il1b (IL1β), Tnfa (TNFα), Tgfb (TGFβ), Ifng (IFNγ), Cd (CD) and Timp (TIMP).

Previous studies of the immune response to chronic schistosome infection have indicated that a Th1 response against migrating schistosomulae as well as maturing and mature schistosomes during the first 28-42 days is followed by a Th2 response driven by egg deposition and antigen release from the maturing miracidium contained therein [reviewed by

5]. In this study, Th1 related transcripts, including STAT1, STAT4 and Tbet, generally increased their expression from day 32 onwards compared to uninfected controls after Vh treatment (S5A Fig). Of the classical Th1 markers, only IFNγ was increased significantly and only at days 39 and 46 (Fig 3A and S5A Fig). In contrast, PZQ treatment led to an increase in expression of all four Th1 markers at days 35 and 39 though again only IFNγ changed significantly and at both time points with all falling back to initial expression levels at day 46 (Fig 3A and S5B Fig). Th2 related cytokine and chemokines IL4, CCL12 and CCL22 were elevated most significantly at day 46 in Vh samples but their activities peaked earlier after PZQ treatment (Fig 3A and S5C, D Figs). TGFβ, a negative immune regulator, was only elevated in Vh samples and only at day 46 (Fig 3A).

Burke and colleagues examined the temporal expression of immune (and fibrotic) genes in *S. japonicum* infected mice [7]. These animals were more lightly infected than reported here and gene expression profiles were assessed using microarrays on days 28, 42 and 49 post infection. While granulomas formed as a result of *S. japonicum* infection have been described as more severe and neutrophilic than those associated with *S. mansoni* [37], which are more eosinophilic [38], we observed many of the same transcriptional patterns during the initiation and establishment of immune responses. Burke and colleagues noted the early onset of Th1 associated gene expression (i.e. IFNγ, STAT1 as well as CXCL9) peaked at days 28-42 with increases in expression of Th2 chemokines (i.e., CCL7 and CCL24) peaking at day 42; a similar pattern of expression was observed for these genes in this study (Fig 3A). Indeed, a number of CC and CXC chemokine transcripts were observed in both studies to be elevated at most time points sampled. For example, the early and sustained elevation of T cell attractants CCL8 and CXCL9 and 10 in both treatment groups as well as the concomitant

induction of CD4 agrees with observations in *S. japonicum* [7] and supports the notion that Th recruitment begins independently of egg deposition. Induction of the B cell cytokine CXCL13 was also early and sustained, but no change in the B cell marker CD19 was observed, suggesting no significant recruitment of B cells into the liver as a result of worm infection or egg deposition.

Although this approach gives insight into the differential expression of key individual genes, it does not provide a global overview of the effect of infection, treatment and time on T cell gene networks controlling maturation and differentiation. We used Ingenuity Pathway Analysis to visualize this complexity and two pathways that best encapsulate this data, representing T cell maturation/differentiation and fibrosis, are discussed. Vh treatment results in T cell maturation pathway activity that was likely initiated before day 32 and increased progressively until day 46 (Fig 4A-D). While a number of key genes involved in Th1 differentiation are progressively up-regulated between days 32 and 46, gene regulation leading to Th2 differentiation is only apparent at days 39 and 46. Administration of PZQ did not result in any maturation or differentiation gene activity at day 32, but after completion of treatment, multiple genes in both the maturation and Th1 and Th2 differentiation pathways were substantially up-regulated at day 35 and peaking at day 39 (Fig 5A-D). At day 46, however, the breadth and intensity of these responses declined precipitously.

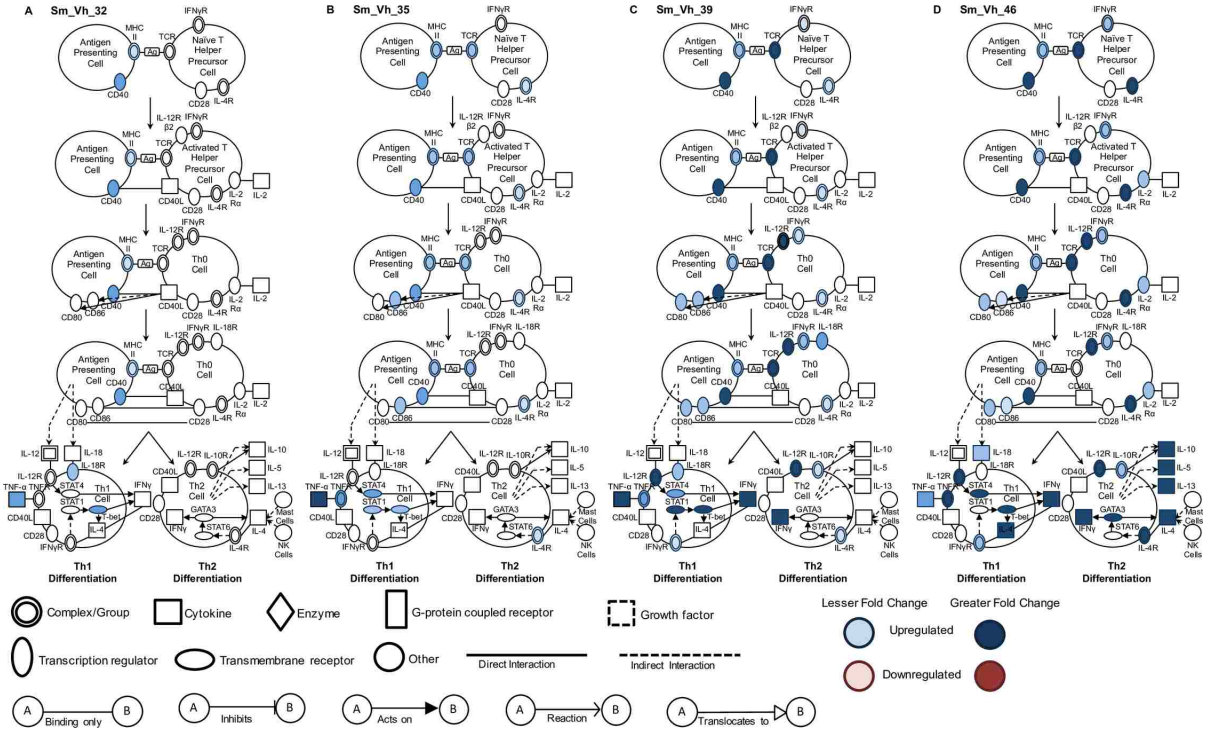

Fig 4. Canonical pathway analysis of T helper cell maturation and differentiation in vehicle treated infected mice. Signaling events in the T cell maturation and differentiation pathway at (A) 32, (B) 35, (C) 39 and (D) 46 days in infected Vh treated (Sm_Vh) mice. Increasing expression in infected mice relative to uninfected mice is indicated by deeper blue shading. None of the genes indicated were down-regulated. Non-expression and non-differential expression is indicated by a lack of shading.

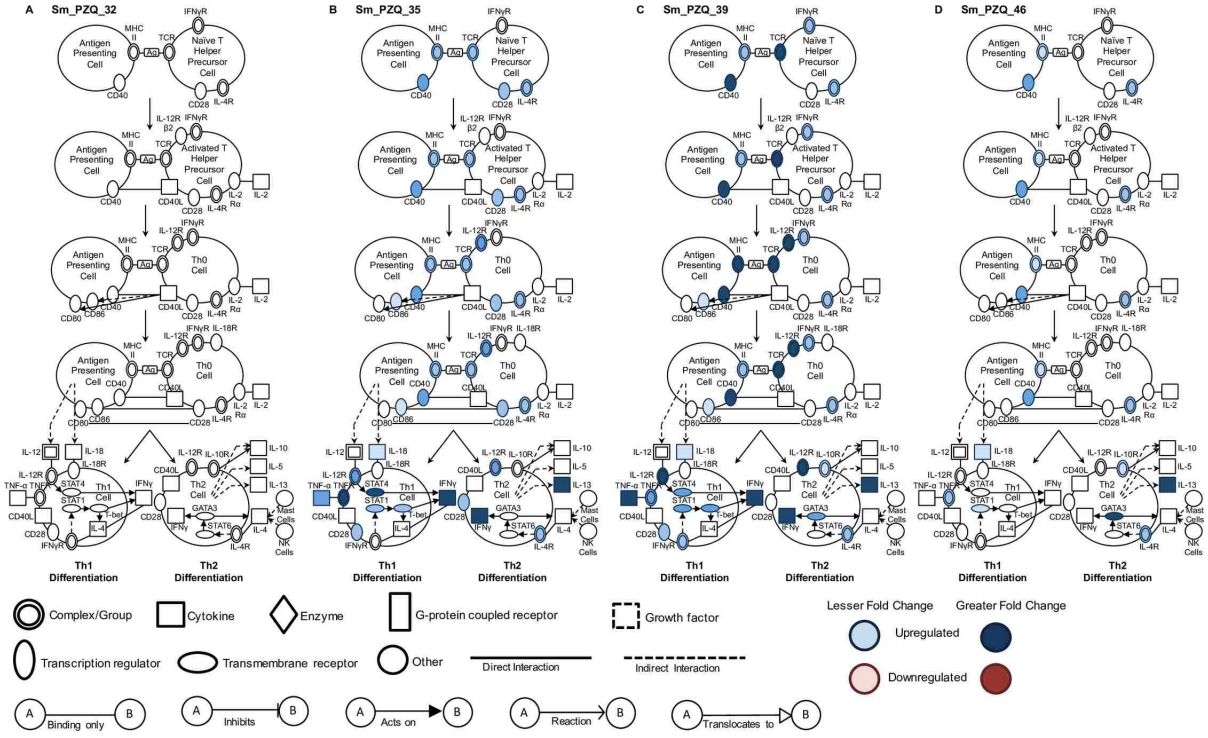

Fig 5. Canonical pathway analysis of T helper cell maturation and differentiation in PZQ treated infected mice. Signaling events in the T cell maturation and differentiation pathway at (A) 32, (B) 35, (C) 39 and (D) 46 days in infected PZQ (Sm_PZQ) treated mice. Increasing expression in infected mice relative to uninfected mice is indicated by deeper blue shading. None of the genes indicated were down-regulated. Non-expression and non-differential expression is indicated by a lack of shading.

Burke and colleagues noted the early and sustained up-regulation of the eosinophil chemo-attractants CCL11 and CCL24 [7] and we also observed this pattern in both treatment groups. It is noteworthy that in the absence of PZQ treatment, CCL11 and CCL24 expression was especially pronounced on day 46, whereas in the presence of the drug, expression of both cytokines peaked earlier (Fig 3A). As noted above, eosinophils have a significant presence in *S. mansoni* induced granulomas [38] and our data confirms they play a role in both the initial anthelmintic response as well as establishment of fibrosis. As discussed above, treatment with Vh resulted in a significant accumulation of parasite eggs in the livers of infected animals at day 46. Egg accumulation in turn led to development of granulomas

with significant fibrotic pathology (comparing S6A and C Fig). For hepatic fibrotic markers there was again substantial differential regulation of individual genes in day 46 Vh samples (Fig 3B). Many of this subset were members of the collagen gene family including *Col1a1* and *Col3a1* that encode type I and III collagens respectively. Histological examination of Vh day 46 liver sections using Picrosirius Red highlighted the deposition of type I and type III collagen in the surrounding matrix of the granulomas (comparing S6B and D Fig). Other genes with peak expression coinciding with the fibrotic response in Vh treated animals included those encoding matrix metalloproteinases (Mmp) 2, 9 and 13 as well as tissue inhibitors of matrix metalloproteinases (TIMP) 1 but not TIMP 2. These transcripts have previously been shown to be abundant in *S. mansoni* and *S. japonicum* induced fibrosis [6, 39, 40]. A number of genes including *Col1a1*, *Col3a1*, *Mmp2, 9* and *13* as well as *TIMP1* were induced after treatment with PZQ and in the absence of egg deposition or gross fibrosis suggesting that their expression is also driven by the breakdown of *S. mansoni* after drug treatment.

Pathway analysis was employed to provide a global overview of the differential expression of genes previously identified as being involved in early signaling in hepatic stellate cells, the major cell type involved in liver fibrosis [41]. Thirty-two days after infection and three hours after treatment with PZQ or Vh, there was no substantial differential expression of genes involved in fibrotic pathways (Figs 6A and 7A). Thereafter, treatment with Vh was associated with a gradual increase in differential expression of numerous genes across fibrotic networks (Fig 6B-D). In contrast, treatment with PZQ led to an earlier more pronounced increase in fibrotic markers at day 35 on cessation of treatment and this level of activity was maintained until day 46 (Fig 7B-D). Examination of these

pathways at 46 days post infection revealed a 10-fold expansion in the number of genes across multiple pathways that are differentially regulated irrespective of treatment. A comparative analysis of different treatments at this time point revealed that Vh treatment led to a greater diversity and a more pronounced expression of genes across multiple fibrotic signaling pathways. These observations suggest that treatment with PZQ coincident with female parasite sexual maturation and egg release dampens but does not eliminate the occurrence of hepatic fibrotic events.

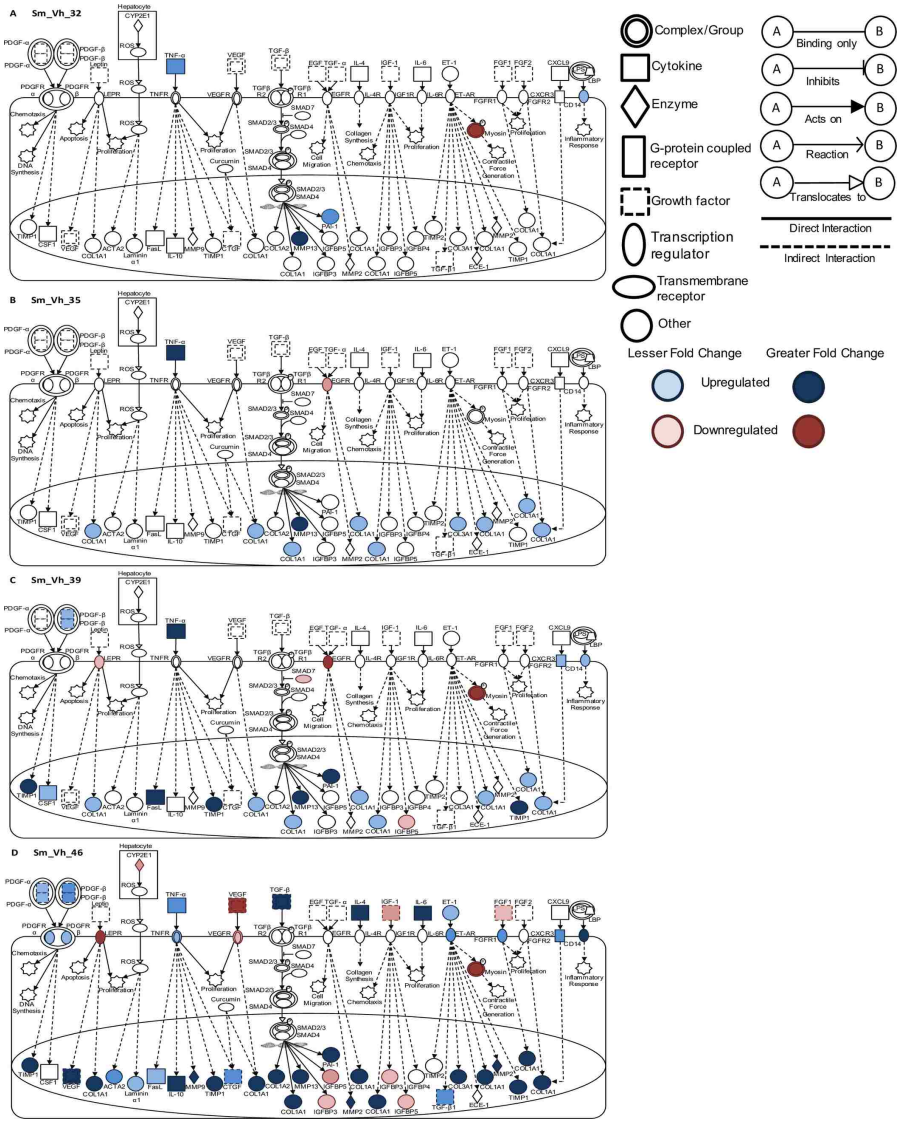

Fig 6. Canonical pathway analysis of hepatic fibrosis and stellate cell activation in vehicle treated infected mice. Signaling events in the fibrotic and stellate cell activation pathway at (A) 32, (B) 35, (C) 39 and (D) 46 days post infection in infected Vh treated (Sm_Vh) mice. Increasing expression in infected mice relative to uninfected mice is indicated by deeper blue shading. Decreased expression is shown in dark red. Non-expression and non-differential expression is indicated by a lack of shading.

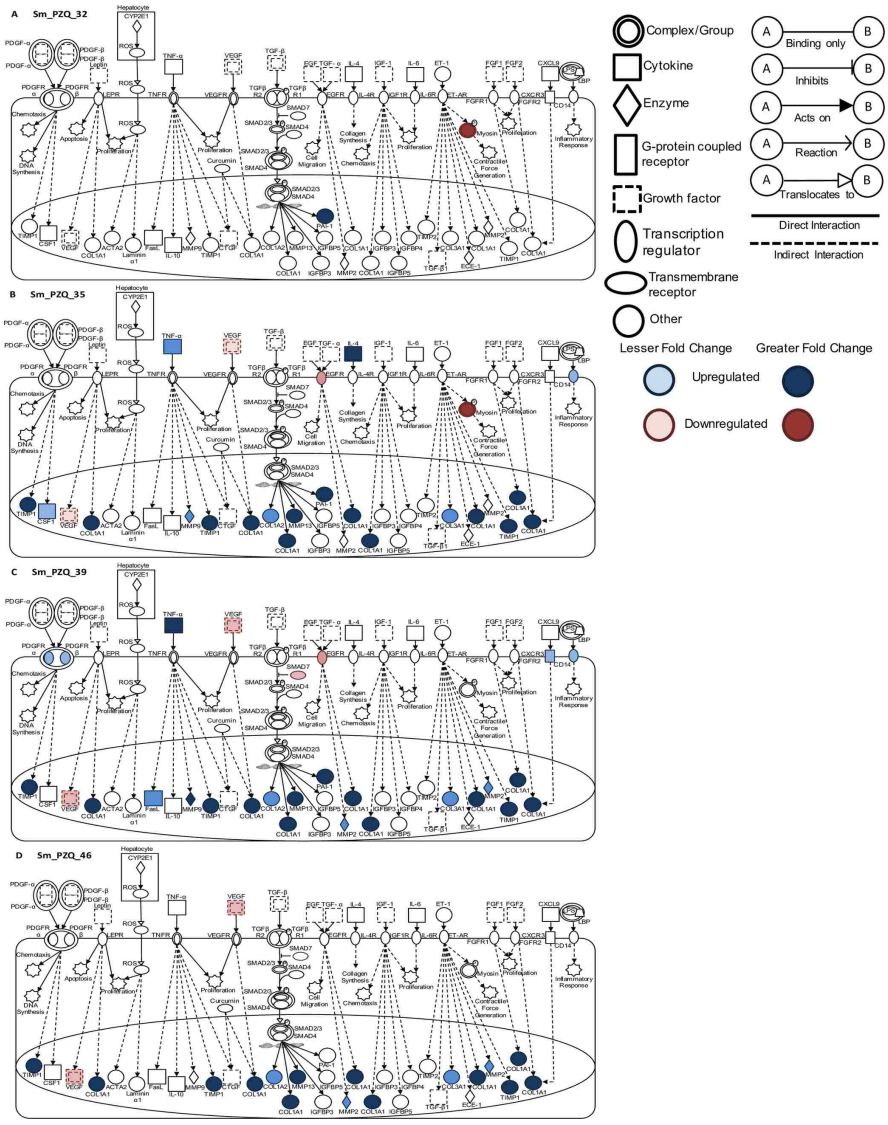

Fig 7. Canonical pathway analysis of hepatic fibrosis and stellate cell activation in PZQ treated infected mice. Signaling events in the fibrotic and stellate cell activation pathway at (A) 32, (B) 35, (C) 39 and (D) 46 days post infection in infected PZQ (Sm_PZQ) treated mice. Increasing expression in infected mice relative to uninfected mice is indicated by deeper blue shading. Decreased expression is shown in dark red. Non-expression and non-differential expression is indicated by a lack of shading.

Figures 8A and B summarizes Th1, Th2 and fibrotic gene responses in relation to worm presence and/or elimination and egg burden during and after Vh or PZQ treatment. As anticipated, markers of Th1 and Th2 immune function and hepatic fibrosis rise with time in

response to increasing hepatic egg burden and perhaps continued parasite presence (Fig 8A). With PZQ treatment, expression of T cell networks peaks earlier as drug treatment eliminates the majority of parasite burden (Fig 8B). Surprisingly, some fibrotic markers also rise in the absence of egg burden, perhaps in response to mechanical breakdown of parasites in the liver although no visible fibrosis was present.

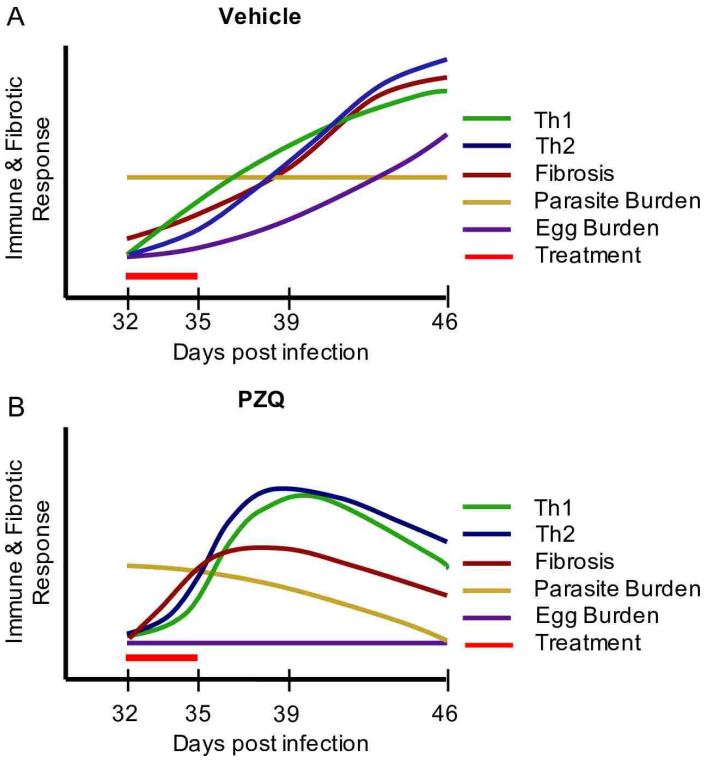

Fig 8. Model of Th1, Th2 and fibrotic responses during *S. mansoni* infection and treatment. A global representation of Th1, Th2 and fibrotic gene responses during *S. mansoni* infection at 32, 35, 39 and 46 days post infection in (A) Vehicle and (B) PZQ treated mice.

Results obtained from NGS analysis were validated by qRT-PCR of six transcripts (CCL7, Col1a1, Col6a5, IFNγ, IL1β and Krt4) over each time point for samples from both Vh and PZQ treated animals (S7 Fig). Patterns observed in the qRT-PCR data mirrored those obtained by NGS and showed a significant correlation (S8 Fig) between the two across both treatments (Vh: $r = 0.70$, $p < 0.0001$; PZQ; $r = 0.61$, $p < 0.0001$).

S. mansoni gene expression *in vivo*

It had been our intention originally to investigate changes in the *S. mansoni* transcriptome using the NGS data generated from infected mouse livers. As outlined above and in S4 Table, too few reads were generated to provide a robust data set and we decided to use the *S. mansoni* RNA contained within the hepatic samples as template for qRT-PCR analysis of individual genes of interest, especially those encoding ABC transporters. In a review of the ABC transporter family in *S. mansoni* and *S. japonicum,* Greenberg reported the presence of 21 and 19 family members in each species respectively [42]. He also matched existing transcripts in the schistosome and NCBI gene and transcript databases with their human homologs and we have relied on an expansion of that nomenclature to identify the transcripts quantified to avoid confusion (S3 Table).

While we and others have examined the effect of PZQ on a limited number of ABC transporters in *S. mansoni* treated *ex vivo*, this, as far as we are aware, is the first study to examine a suite of transporters during drug treatment *in vivo*. As there is also the potential that differences in ABC transporter expression may underpin juvenile resistance to PZQ, we extended our experiments to parasite RNA extracted from livers derived from mice treated with PZQ and Vh on days 25-28 of infection (Fig 9A). Fig 9B-J shows the differential expression of nine transporters in juvenile and adult *S. mansoni* 3 h after the first and fourth doses of PZQ or Vh. After four consecutive days of PZQ treatment there was a significant increase in expression of ABCB1-1, B8, C1-1, C1-2, G1 and G2 transcripts in juvenile schistosomes. No ABC transporter gene showed a reduction in the level of transcription. In contrast, no ABC transporter gene showed an increase in transcription in adult worms

exposed to PZQ for 4 consecutive days while ABCB1-3 showed a significant fall. ABCB1-3 and C1-1 showed a significantly higher level of activity in adult compared to juvenile worms on the first day of treatment with no juvenile genes showing greater activity. In contrast, when comparing gene expression between juvenile and adult worms on the fourth day of treatment, genes encoding ABCB1-1, B8, C1-1, C1-2 and G2 all show significantly greater levels of activity in juveniles with no gene activity significantly greater in adult than juvenile worms. Essentially, our data indicates that treatment with a 'lethal' dose of PZQ over a 4 day period leads to a significant increase in transcription associated with several ABC transporter genes belonging to the ABCB, C and G families in juvenile but not adult parasites. These families are especially noteworthy as some members (ABCB1, C1 and G2) are strongly associated with the transport of xenobiotics and multi-drug resistance in cancer treatment [43]. Our data is not in complete agreement with several published studies that have investigated the response of individual *S. mansoni* transporters to PZQ. Schistosome ABCB1-1 transporter (also named SMDR2) and ABCC1-1 (also named SmMRP1) transcript and protein levels have been shown to be transiently increased in adult schistosomes following exposure to a sub-lethal dose of PZQ *in vitro* [19, 20] while the drug has also been found to be a substrate of ABCB1-1 when this transporter is expressed in CHO cells [21]. Kasinathan and colleagues also demonstrated that *S. mansoni* ABCB1-1 and C1-1 were expressed at higher levels in juveniles compared with adult worms and that adult males, but not females show a significant increase in the level of ABCC1-1 on exposure to a sub-lethal dose of PZQ [20]. They suggested this data was '…consistent with the hypothesis that increases in levels of schistosome multidrug transporters may be involved in development or maintenance or reduced susceptibility to PZQ'. This hypothesis was further supported by

additional data reported by Kasinathan and colleagues in which juvenile and adult *S. mansoni* ABC transporter activities were inhibited pharmacologically in the presence of sub-lethal PZQ concentrations *ex vivo* [23]. With adult worms, these conditions resulted in an exacerbation of the effects of PZQ including a loss of motility and tegument disruption, while juveniles became paralyzed when PZQ doses that they would normally prove refractory to were applied. In contrast, adult worms in which expression of five ABC transporters including ABCB1-1 and C1-1 were knocked down showed increased responsiveness to PZQ. This *ex vivo* data taken together with the *in vivo* results reported here, lends further support to the hypothesis that ABC transporter family members play a significant role in modulating the response of juvenile and adult schistosomes to PZQ and underpin juvenile resistance.

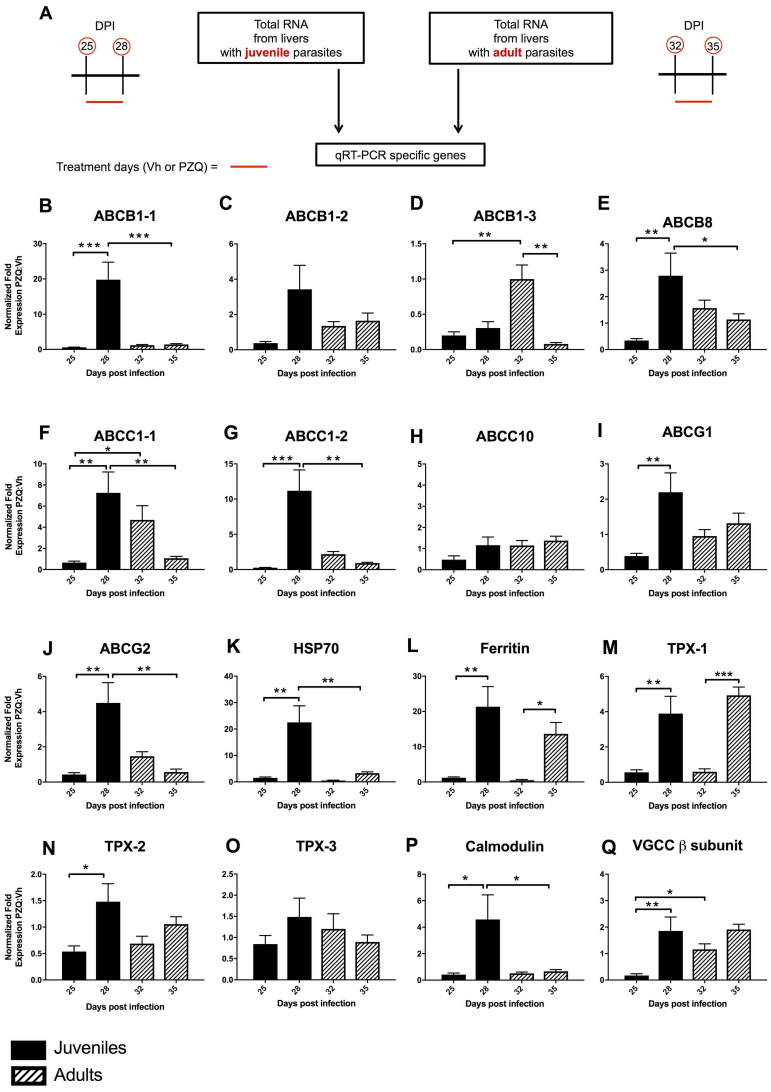

Fig 9. Quantitative real-time PCR analysis of *S. mansoni* gene expression. Results are normalized to *S. mansoni* GAPDH and fold change in gene expression in PZQ treated schistosomes is shown relative to Vh treated groups. (A) Schematic diagram explaining the source of *S. mansoni* RNA for qRT-PCR analysis. (DPI = days post infection). B-Q. Gene expression in juvenile schistosomes at days 25 and 28 is shown with shaded bars and adults at days 32 and 35 are shown hatched. (B-D) and (F-H) show the response of full ABC drug transporters while (E), and (I, J) are half transporters each with homology to a member of the human B, C and G families. (K-O) show the response of stress genes HSP70, ferritin and thioredoxin peroxidase (TPX-1, 2 & 3), (P) the response of calmodulin and (Q) response of voltage-gated Ca^{2+} channel β subunit respectively. Statistical differences in fold change between adult and juvenile *S. mansoni* were assessed using one-way ANOVA followed by Fisher's least significant difference post hoc test for multiple comparisons. Error bars represent +1 standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In addition to nine ABC transporters, the expression of seven other *S. mansoni* genes was examined. Three of these (HSP70, ferritin and thioredoxin peroxidase 1) were selected (Fig 9K-M) as we observed previously that exposure of *S. mansoni* to PZQ results in induction of these stress response genes [17, 22]. This observation was broadly repeated in this study, with all 3 genes being induced significantly in juveniles with ferritin and thioredoxin peroxidase 1 being induced significantly in adult after four days of treatment suggesting that both juveniles and adults were still alive at this time. The expression of two other potential *S. mansoni* thioredoxin peroxidase homologs (TPX-2 and TPX-3) were also quantified but only juvenile TPX-2 was found to be differentially expressed after treatment (Fig 9N, O). Finally, as schistosomes exposed to PZQ have been demonstrated to undergo a Ca^{2+} - dependent contraction that appears to be mediated by a voltage gated Ca^{2+} channel [44], we also measured expression of a schistosomal calmodulin and voltage gated Ca^{2+} channel β subunit transcripts. Binding of Ca^{2+} is essential for the activation of calmodulin which functions as a regulator of many different proteins and pathways. Calmodulin and Ca^{2+} channel β subunit transcript expression was induced significantly in juveniles after 4 days of treatment (Fig 9P, Q), however, neither were differentially regulated in adults.

In conclusion, the analysis of livers from mice infected with *S. mansoni* and treated with PZQ or its Vh enabled a comprehensive study of the effect of the drug on anthelmintic transcriptomic responses in the presence and absence of developing granulomas. While the immune and fibrotic responses in the absence of the drug are significant leading to a severe pathology that will, in time, prove fatal for the animals, many of the same genes and gene networks are active in PZQ treated mice. Furthermore, we have demonstrated that several ABC transporters, especially members of the ABCB, C and G families, are transcriptionally

enriched in juvenile compared to adult worms after exposure to PZQ. This lends further support to the hypothesis that differential expression of these transporters underpins the resistance of juveniles to the drug.

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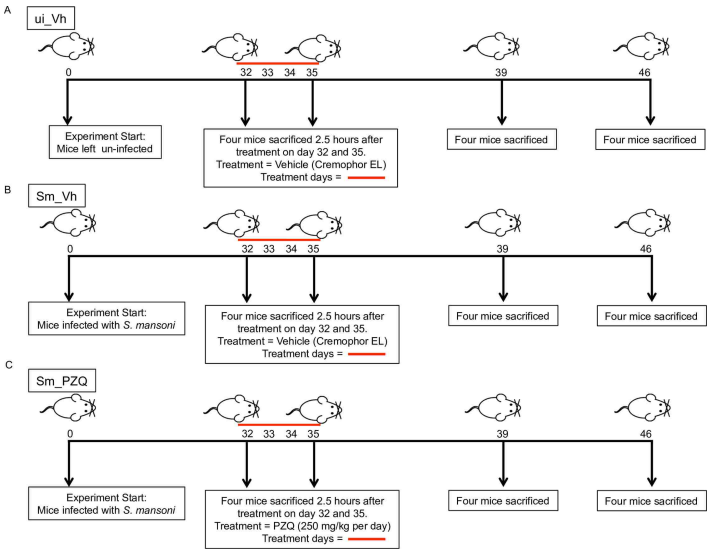
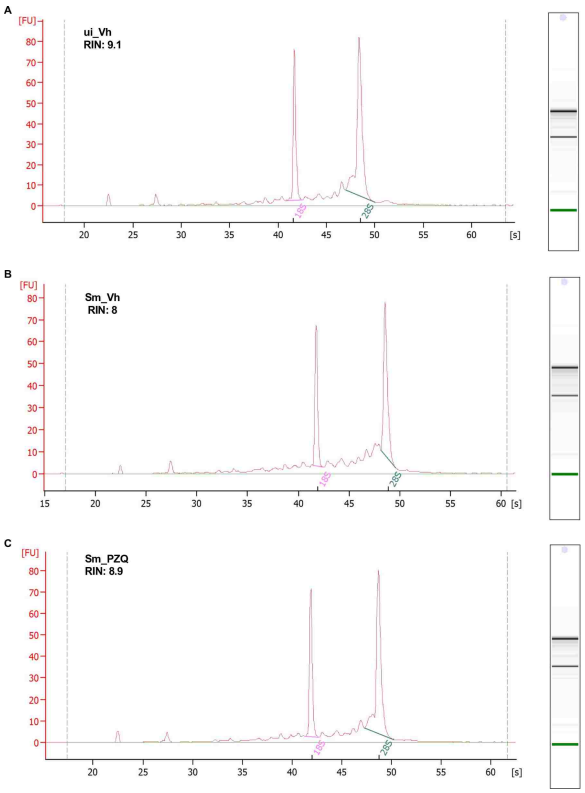
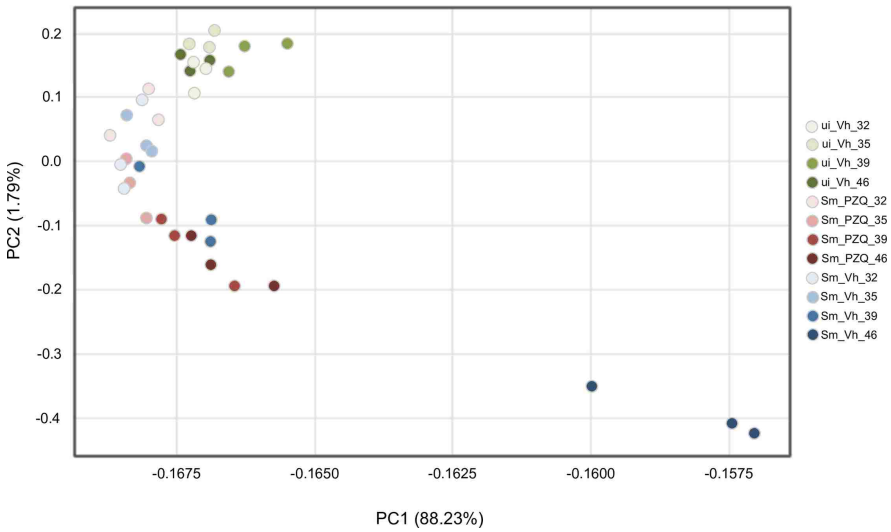
Supporting information



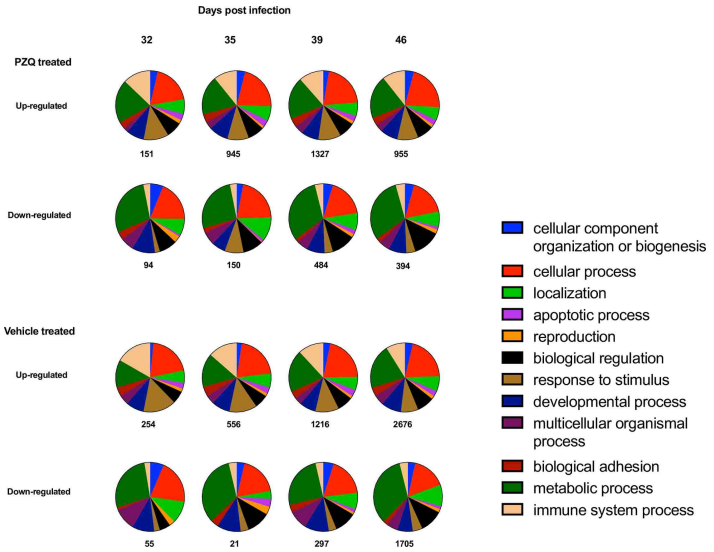
S1 Fig. Schematic diagram of mouse treatments to generate RNA-Seq data. Three distinct groups of 16 mice contributed to the RNa-Seq experiment. These included (A) uninfected mice treated with PZQ vehicle (Cremaphor EL) on days 32-35 post infection with 4 mice each being sacrificed on days 32, 35, 39 and 46 post infection; (B) *S. mansoni* infected mice treated with PZQ vehicle (Cremaphor EL) on days 32-35 post infection with 4 mice each being sacrificed on days 32, 35, 39 and 46 post infection and (C) *S. mansoni* infected mice treated with PZQ on days 32-35 post infection with 4 mice each being sacrificed on days 32, 35, 39 and 46 post infection.



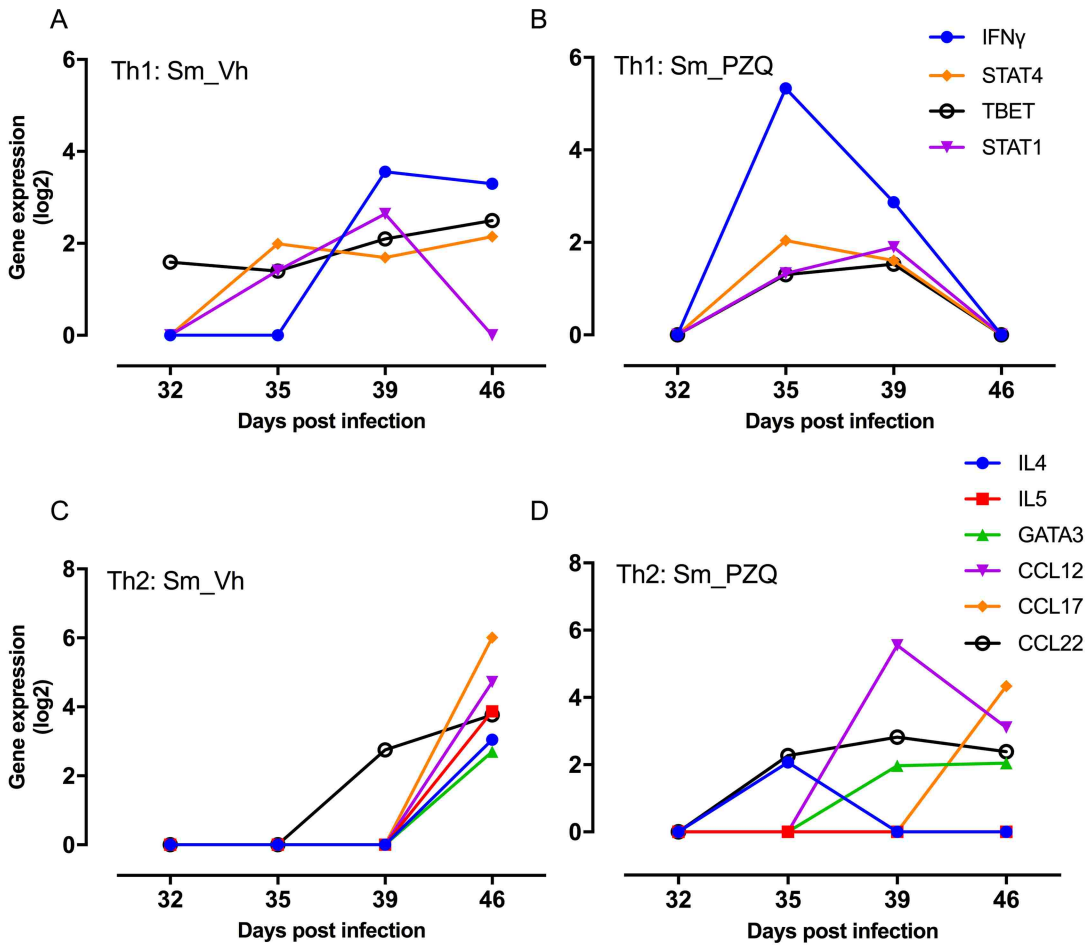
S2 Fig. Representative Bioanalyzer traces of total RNA from murine livers with the corresponding RNA integrity number (RIN) values from (A) an uninfected vehicle treated, mouse; (B) a *S. mansoni* infected vehicle treated mouse and (C) a *S. mansoni* infected PZQ treated mouse.



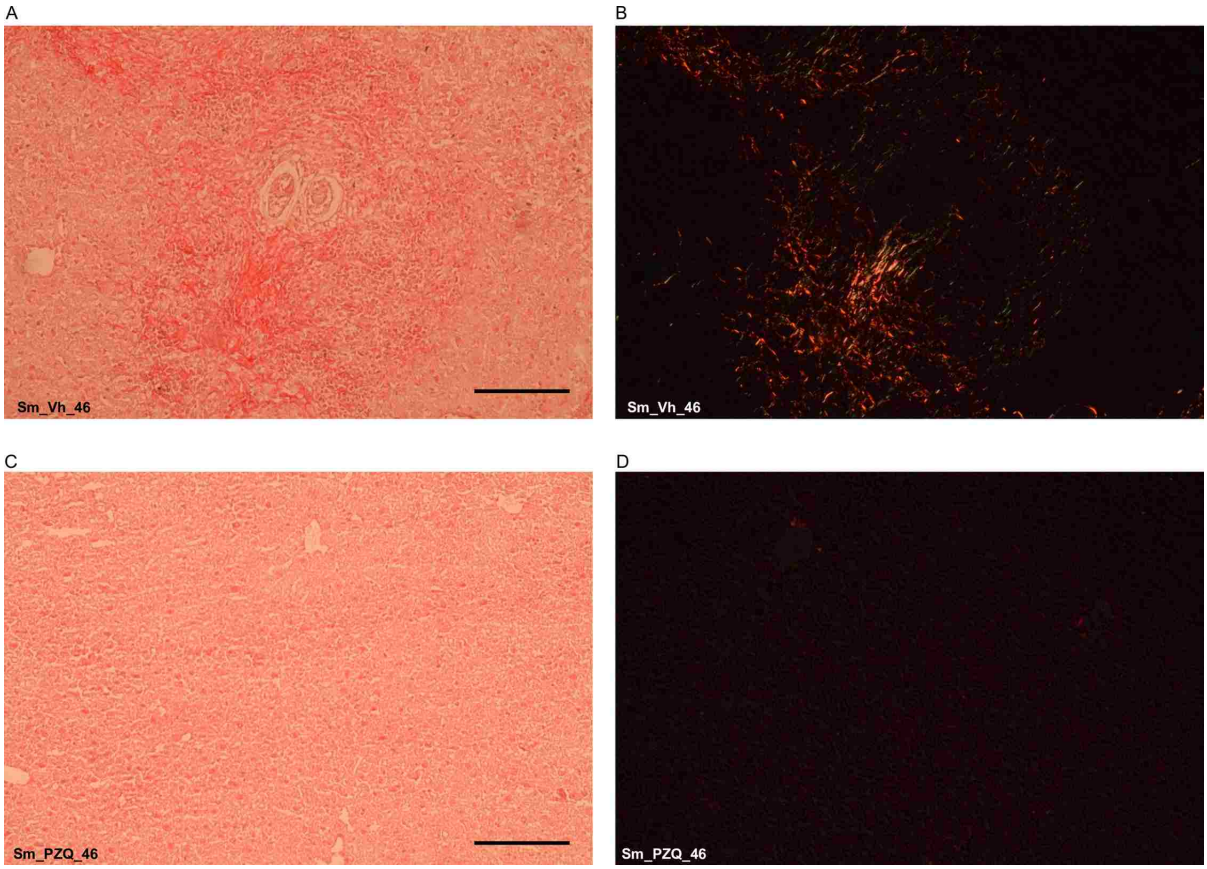
S3 Fig. Two dimensional principal component analysis (PCA). Visualization of the clustering or scattering of hepatic transcriptome replicates.



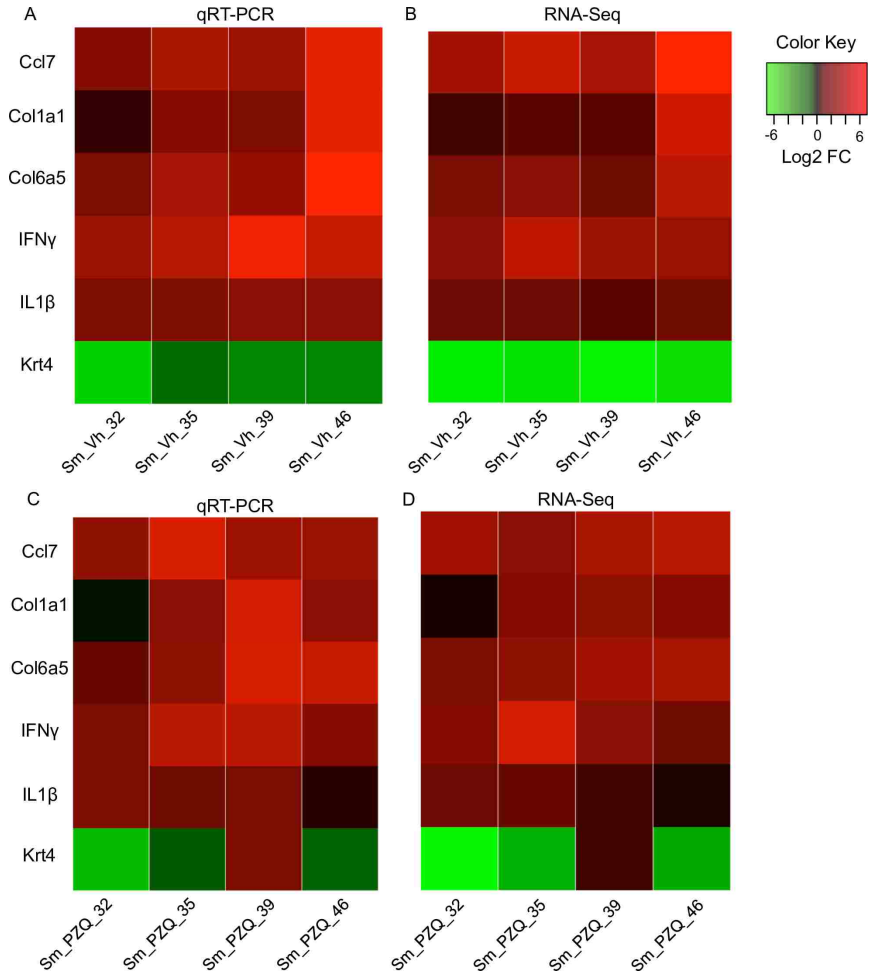
S4 Fig. Biological process GO terms associated with differentially regulated genes during *S. mansoni* infection. Pie charts of the enriched biological processes for genes significantly up- and down-regulated at days 32, 35, 39 and 46 in infected PZQ and Vh treated mice. Data were generated using gene ontology (GO) analysis with a Bonferroni-adjusted p value ≤0.05.



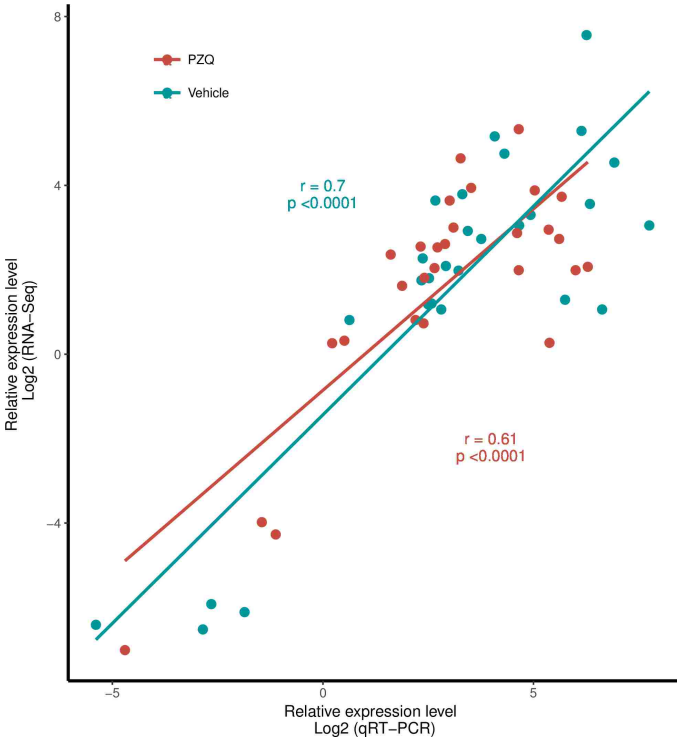
S5 Fig. Change in expression of Th1 and Th2 markers as a result of Vh or PZQ treatment. (A) and (B) show the change in IFNγ, STAT1, STAT4 and TBET expression during and after treatment with Vh (Sm_Vh) and PZQ (Sm_PZQ) respectively. (C) and (D) show the change in expression of IL4, IL5, GATA3, CCL12, CCL17 and CCL22 expression during and after treatment with Vh (Sm_Vh) and PZQ (Sm_PZQ) respectively.



S6 Fig. Granuloma formation in the murine liver during *S. mansoni* infection. Picrosirius staining (PolySciences Inc., Washington, PA) was performed according to the manufacturer's protocol to determine hepatic fibrosis progression. (A) Bright red stain around the two schistosome eggs in the center field indicates picrosirius dye binding to collagen fibrils within the granuloma. The section was taken from the liver of an infected mouse treated with Vh 45 days after infection. (B) The same field of view shown in (A) but under polarizing light. Yellow-orange birefringence indicates type I collagen fibers while green birefringence indicates type III. (C) and (D). Section from PZQ treated mouse liver 45 days after *S. mansoni* infection. No granuloma or collagen fibrils were evident. Slides were visualized on a Zeiss Axio Scope.A1 using a 20x objective and images were acquired with a Nikon D5200 Camera fitted with a MM-SLR Adapter. Scale bar = 130 μm.



S7 Fig. Validation of RNA-Seq gene expression data by quantitative real-time PCR (qRT-PCR). Log_2 changes in expression of genes encoding chemokine Ccl7, collagen type I pro-α chain (col1a1), collagen type VI α5 chain (Col6a5), interferon γ (IFNγ) interleukin 1β (IL1β) and keratin 4 (Krt4) in Vh and PZQ treated infected mice at days 32, 35, 39 and 46 post infection. The gene expression profile at each point is the average of three biological replicates. For both RNA-Seq and qRT-PCR data, regions of red and green indicate gene expression has increased and decreased respectively.



S8 Fig. Correlation between NGS and quantitative real-time PCR (qRT-PCR). Relationship between NGS and qRT-PCR data for six genes over each time point and for both treatments was established using Spearman's Rho correlation.

S1 Table. Primers used in *Mus musculus* quantitative real-time PCR reactions. GAPDH (ENSMUSG00000057666) primer set is part of the PrimePCR™ Probe Assay (BIORAD qMmuCED0027497).

S1 Table. Primers used in *Mus musculus* quantitative real-time PCR reactions.

Ensemble Gene ID	Gene Symbol	Forward Primer	Reverse Primer
ENSMUSG00000035373	Ccl7	TTTGTTTCTTGACATAGCAGCAT	TCTCACTCTCTTTCTCCAACCA
ENSMUSG00000001506	Col1α1	GAAACCTCTCTCGCCTCTTG	TGGTGAAGCAGGCAAGC
ENSMUSG00000091345	Col6α5	TCCACGATTTCCACTTGATCC	GCCGTCCTAGCTCCAAAG
ENSMUSG00000055170	IFNγ	CTCTTCTCATGGCTGTTTCT	TTCTTCCACATCTATGCCACTT
ENSMUSG00000027398	IL1β	CCACCTCAATGGACAGAATATCA	CCCAAGGCCACAGGTATTT
ENSMUSG00000059668	Krt4	CTCTGCATCAGCCACAGAT3	GATAGCCTGAAGACCACCAAG

S2 Table. Real time PCR cycle threshold (Cq) raw data for *S. mansoni* reference gene GAPDH.

S2 Table. Real-time PCR cycle threshold (Cq) raw data for *S. mansoni* reference gene GAPDH.

Well	Fluor	Target	Content	Sample	Biological Set Name	Cq	Cq Technical Mean	Cq Technical Std. Dev	Cq Biological Mean	Cq Biological Std. Dev
A01	SYBR	GAPDH5	Unkn-01	25V1	Sm_Vh_25	24.45	24.43	0.02	23.95	0.45
A03	SYBR	GAPDH5	Unkn-01	25V1	Sm_Vh_25	24.42	24.43	0.02	23.95	0.45
A05	SYBR	GAPDH5	Unkn-02	25V2	Sm_Vh_25	24.01	24.00	0.01	23.95	0.45
A06	SYBR	GAPDH5	Unkn-02	25V2	Sm_Vh_25	23.99	24.00	0.01	23.95	0.45
A07	SYBR	GAPDH5	Unkn-03	25V3	Sm_Vh_25	23.42	23.42	0.00	23.95	0.45
A08	SYBR	GAPDH5	Unkn-03	25V3	Sm_Vh_25	23.43	23.42	0.00	23.95	0.45
A10	SYBR	GAPDH5	Unkn-04	28V1	Sm_Vh_28	23.53	23.55	0.03	23.36	0.19
A11	SYBR	GAPDH5	Unkn-04	28V1	Sm_Vh_28	23.57	23.55	0.03	23.36	0.19
B02	SYBR	GAPDH5	Unkn-05	28V2	Sm_Vh_28	23.11	23.13	0.02	23.36	0.19
B03	SYBR	GAPDH5	Unkn-05	28V2	Sm_Vh_28	23.14	23.13	0.02	23.36	0.19
B05	SYBR	GAPDH5	Unkn-06	28V3	Sm_Vh_28	23.40	23.40	0.00	23.36	0.19
B06	SYBR	GAPDH5	Unkn-06	28V3	Sm_Vh_28	23.39	23.40	0.00	23.36	0.19
B08	SYBR	GAPDH5	Unkn-07	32V1	Sm_Vh_32	24.86	24.84	0.02	23.70	0.90
B09	SYBR	GAPDH5	Unkn-07	32V1	Sm_Vh_32	24.83	24.84	0.02	23.70	0.90
B11	SYBR	GAPDH5	Unkn-08	32V2	Sm_Vh_32	22.96	22.98	0.04	23.70	0.90
B12	SYBR	GAPDH5	Unkn-08	32V2	Sm_Vh_32	23.01	22.98	0.04	23.70	0.90
C01	SYBR	GAPDH5	Unkn-09	32V3	Sm_Vh_32	23.29	23.26	0.04	23.70	0.90
C03	SYBR	GAPDH5	Unkn-09	32V3	Sm_Vh_32	23.24	23.26	0.04	23.70	0.90
C05	SYBR	GAPDH5	Unkn-10	35V1	Sm_Vh_35	22.50	22.48	0.03	23.36	0.74
C06	SYBR	GAPDH5	Unkn-10	35V1	Sm_Vh_35	22.46	22.48	0.03	23.36	0.74
C07	SYBR	GAPDH5	Unkn-11	35V2	Sm_Vh_35	24.13	24.12	0.01	23.36	0.74
C08	SYBR	GAPDH5	Unkn-11	35V2	Sm_Vh_35	24.11	24.12	0.01	23.36	0.74
C11	SYBR	GAPDH5	Unkn-12	35V3	Sm_Vh_35	23.47	23.47	0.01	23.36	0.74
C12	SYBR	GAPDH5	Unkn-12	35V3	Sm_Vh_35	23.46	23.47	0.01	23.36	0.74
D02	SYBR	GAPDH5	Unkn-13	25P1	Sm_PZQ_25	22.91	22.94	0.04	23.73	0.64
D03	SYBR	GAPDH5	Unkn-13	25P1	Sm_PZQ_25	22.97	22.94	0.04	23.73	0.64
D05	SYBR	GAPDH5	Unkn-14	25P2	Sm_PZQ_25	24.32	24.34	0.03	23.73	0.64
D06	SYBR	GAPDH5	Unkn-14	25P2	Sm_PZQ_25	24.36	24.34	0.03	23.73	0.64
D07	SYBR	GAPDH5	Unkn-15	25P3	Sm_PZQ_25	23.88	23.91	0.05	23.73	0.64
D09	SYBR	GAPDH5	Unkn-15	25P3	Sm_PZQ_25	23.94	23.91	0.05	23.73	0.64
D11	SYBR	GAPDH5	Unkn-16	28P1	Sm_PZQ_28	24.38	24.39	0.01	24.20	0.36
D12	SYBR	GAPDH5	Unkn-16	28P1	Sm_PZQ_28	24.39	24.39	0.01	24.20	0.36
E01	SYBR	GAPDH5	Unkn-17	28P2	Sm_PZQ_28	24.48	24.48	0.00	24.20	0.36
E03	SYBR	GAPDH5	Unkn-17	28P2	Sm_PZQ_28	24.49	24.48	0.00	24.20	0.36
E05	SYBR	GAPDH5	Unkn-18	28P3	Sm_PZQ_28	23.73	23.74	0.02	24.20	0.36
E06	SYBR	GAPDH5	Unkn-18	28P3	Sm_PZQ_28	23.75	23.74	0.02	24.20	0.36
E07	SYBR	GAPDH5	Unkn-19	32P1	Sm_PZQ_32	22.71	22.70	0.01	23.94	1.03
E09	SYBR	GAPDH5	Unkn-19	32P1	Sm_PZQ_32	22.70	22.70	0.01	23.94	1.03
E11	SYBR	GAPDH5	Unkn-20	32P2	Sm_PZQ_32	25.01	24.99	0.03	23.94	1.03
E12	SYBR	GAPDH5	Unkn-20	32P2	Sm_PZQ_32	24.97	24.99	0.03	23.94	1.03
F01	SYBR	GAPDH5	Unkn-21	32P3	Sm_PZQ_32	24.15	24.12	0.03	23.94	1.03
F03	SYBR	GAPDH5	Unkn-21	32P3	Sm_PZQ_32	24.10	24.12	0.03	23.94	1.03
F04	SYBR	GAPDH5	Unkn-22	35P1	Sm_PZQ_35	23.79	23.79	0.01	23.57	0.44
F06	SYBR	GAPDH5	Unkn-22	35P1	Sm_PZQ_35	23.78	23.79	0.01	23.57	0.44
F07	SYBR	GAPDH5	Unkn-23	35P2	Sm_PZQ_35	23.04	23.01	0.03	23.57	0.44
F08	SYBR	GAPDH5	Unkn-23	35P2	Sm_PZQ_35	22.99	23.01	0.03	23.57	0.44
F10	SYBR	GAPDH5	Unkn-24	35P3	Sm_PZQ_35	23.89	23.91	0.04	23.57	0.44
F12	SYBR	GAPDH5	Unkn-24	35P3	Sm_PZQ_35	23.94	23.91	0.04	23.57	0.44

S3 Table. Primers used in *S. mansoni* quantitative real-time PCR reactions.

S3 Table. Primers used in *S. mansoni* quantitative real-time PCR reactions.

Gene ID	Gene	Forward Primer	Reverse Primer
Smp_055780	ABCB1-1 (SMDR2)	CGGTGCTACTCAGGGTTATTT	TCACCAAGGAAGCAATGACTAA
Smp_170820	ABCB1-2	TTTGGGAGCTGCAATGTTTATC	CCTCCGAGTGTTGTTCCAATA
Smp_089200.1	ABCB1-3	GGTTCAGCTGCGAAATGTATTC	CACAACCAGAAGGTCCAACTA
Smp_063000	ABCB8 (SMDR1)	CTTATTCGGTGCCATTCTGG	TTCGTGAGTCACAACGCATC
Smp_171740	ABCC1-1 (SmMRP1)	GGTCGTACTGGTTCGGGTAA	TGAACGTAACGTGCCAGAG
Smp_129820.1	ABCC1-2 (SmMRP2)	TTATGGCGTGCAATAGAATCTG	AGATTTGCTCCACCTTCACC
Smp_147250	ABCC10	CCTCCCTGGACCTTATACAAAC	CGAAGTAAGGTGAGAGGAACAG
Smp_181150	ABCG1	GTTTAGTCCCTTACCTCGCATC	ATTCACCACAAGCCACTTCC
Smp_126450	ABCG2 (BCRP)	AGACCATTCCCACAACACTATC	CGTCCAGCCAACACATCTAA
Smp_186020.1	Heat Shock Protein 70	ACTTAATCGGACCATACACGG	GCAAACATTCCACGTCTCAAAC
Smp_047660.1	Ferritin 2 (soma)	TGTCAATGGAGAAGGCAGTG	TGTCAATGGAGAAGGCAGTG
Smp_059480.1	Thioredoxin Peroxidase-1	GCTGACCGTAAACAGGAGATT	TTCACCGGACACACTTCAC
Smp_158110.1	Thioredoxin Peroxidase-2	CTTGACGAACAGGAAGGTCAT	GAGAGAGAAGCAACAGGATCAG
Smp_004470.1	Thioredoxin Peroxidase-3	GGTGGTGGATGGCCAATTTA	GTTTCTGATAGTCCGCCAAGAG
Smp_026560	Calmodulin-1	CAGAATCCTACAGAGGCTGAAC	TCGCCCAAGTTAGTCATTACAT
Smp_141660.1	VGCC β Subunit	GGTTGTTTTCGTCGTGCGAA	TCGCTGGTGACGGAATAAAG
Smp_056970.1	SmGAPDH	GTCATTCCAGCACTAAACGG	CCTTCCCTAACCTACATGTCAG

S4 Table. Summary of Illumina read counts for each sequenced sample.

Please see publication Sanchez, M.C., Krasnec, K.V., Parra, A.S., von Cabanlong, C., Gobert, G.N., Umylny, B., Cupit, P.M., Cunningham, C., 2017. Effect of praziquantel on the differential expression of mouse hepatic genes and parasite ATP binding cassette transporter gene family members during Schistosoma mansoni infection. PLOS Neglected Tropical Diseases 11, e0005691. https://doi.org/10.1371/journal.pntd.0005691.s014

S5 Table. Number of differentially regulated genes in Vh and PZQ treated samples.

S5 Table. Number of differentially regulated genes in Vh and PZQ treated samples.

Timepoint Treatment	Total genes upregulated	Total genes downregulated	Immune genes upregulated	Immune genes downregulated
Sm_Vh_32	248	47	45	0
Sm_Vh_35	546	21	91	0
Sm_Vh_39	1191	293	150	6
Sm_Vh_46	2633	1687	205	36
Sm_PZQ_32	147	80	21	0
Sm_PZQ_35	927	149	100	3
Sm_PZQ_39	1304	474	153	10
Sm_PZQ_46	937	387	93	9

S6 Table. Identities of all differentially expressed hepatic genes at each time point and treatment.

Please see publication Sanchez, M.C., Krasnec, K.V., Parra, A.S., von Cabanlong, C., Gobert, G.N., Umylny, B., Cupit, P.M., Cunningham, C., 2017. Effect of praziquantel on the differential expression of mouse hepatic genes and parasite ATP binding cassette transporter gene family members during Schistosoma mansoni infection. PLOS Neglected Tropical Diseases 11, e0005691. https://doi.org/10.1371/journal.pntd.0005691.s014

Chapter 2

Initial Analysis: The transcriptomic response of *Schistosoma mansoni* exposed to R- and S- praziquantel *in vitro*.

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Abstract

Schistosomiasis is a highly prevalent neglected tropical disease caused by blood-dwelling helminths of the genus *Schistosoma.* Praziquantel (PZQ) is the only drug that is widely available for the treatment of the disease and is administered as large, bitter tasting tablets which many patients, especially children, find hard to swallow. The drug is synthesized and administered in its racemic form combining R- and S- stereoisomers in equal amounts, however, while the anthelmintic activity of PZQ is associated with the R-enantiomer, its side effects and bitter taste have been attributed to the S-enantiomer. Previously, we have published a protocol for the synthesis of highly pure gram amounts of the individual isomers. In this preliminary analysis we have used these synthetic compounds to investigate the effect of each isomer on the transcriptome of sexually mature *Schistosoma mansoni* PR1. Treatment with R-PZQ resulted in the differential regulation of almost 5-fold more transcripts than S-PZQ and while there was little overlap in the gene sets we note that many of the most highly differentially regulated transcripts were affected by both the R- and S-isomers. One concern is that, unlike previous studies with racemic PZQ we saw no evidence of differential regulation of specific stress response genes such as ferritin or thioredoxin peroxidase. Thus, we will shortly reexamine our data to make sure that in using relatively conservative cut-off parameters to judge whether genes are differentially regulated, we have not missed a more informative gene set.

Keywords: Praziquantel, enantiomers, transcriptome, proteome, Schistosoma, helminth

1. Introduction

Schistosomiasis is a water borne parasitic disease that affects more than 200 million people, mainly in sub-Saharan Africa, with approximately 111 million being school aged children [1]. While the level of mortality is not as significant as that associated with HIV/AIDS or malaria [2], it is a disease associated with poverty and poor sanitation, infection with multiple overlapping parasites and a significant economic and health load for affected populations with, for example, a calculated global disease burden of up to 56 million disability adjusted life years lost in 2010 [3].

The causative agents of schistosomiasis are digenetic trematode worms belonging to the genus *Schistosoma* with *Schistosoma mansoni*, *S. japonicum* and *S. haematobium* being responsible for the majority of cases. Unfortunately, there is no vaccine and little prospect of one becoming available in the near future. Treatment of the disease relies on a single drug, praziquantel (PZQ), that is cheap, widely available, easy to use and effective against all schistosome species that infect humans [4]. In 2016, approximately 64 million people were treated with PZQ [1] leading to a significant drop (from 249 million including 114 million schoolchildren in 2014) in the number of people with the disease. While this is a success to be celebrated, there remain concerns that increasing intensity of use may eventually cause PZQ to be lost to resistance in sexually mature parasites. In fact, one drawback of the PZQ is its failure to kill sexually immature, juvenile parasites [5,6]. Thus a single treatment, which is usual in poor, remote communities, often does not effect a complete cure but does reduce the worm burden of an individual. While the molecular basis of juvenile resistance is unclear we and others have suggested that sexually immature but not mature schistosomes induce

transcription of ATP Binding Cassette (ABC) transporter efflux pumps to keep the intracellular concentration of the drug at sub-lethal levels [7-10].

Currently PZQ is synthesized and employed as a racemic mixture of R- and S-stereoisomers. The anthelmintic activity of the drug is associated with the R- isomer [11,12], while the inactive S-isomer has been implicated in the drug's side effects [13] and extremely bitter taste [14] which, together with its large size [15], often makes it difficult to swallow, especially for children. We have previously published a protocol for the small-scale synthesis and separation of both the R- and S-PZQ enantiomers [16]. Each isomer can be produced in gram amounts with an optical purity of greater than 99.999%. Thus while the S-isomer contains trace R-isomer contamination, it is present in such low amounts that it is likely, therapeutically insignificant. We calculated the EC50 of racemic PZQ to be 2.5 μM in *in vitro* assays while the R-isomer was 0.5 μM and approximately 160–fold more effective than the S-isomer [16]. We also noted that like racemic PZQ, R- but not S-PZQ causes worms to become paralyzed at sub-lethal concentrations, presumably due to the influx of Ca^{2+} ions that has been observed on drug exposure [17].

We have previously examined the effect of racemic PZQ on the transcriptome of *S. mansoni in vivo* [10] and *in vitro* [9]. In these studies we were able to observe the differential regulation of stress response, ABC transporter and Ca^{2+} regulated genes among many others. What was unclear was which of these genes were being differentially regulated by the R- and/or S- enantiomer. Having highly pure, gram amounts of each has allowed us to begin to tease out the relative contribution of each enantiomer to this response and we present our initial findings in this manuscript.

2. Materials and Methods

2.1. Ethics statement

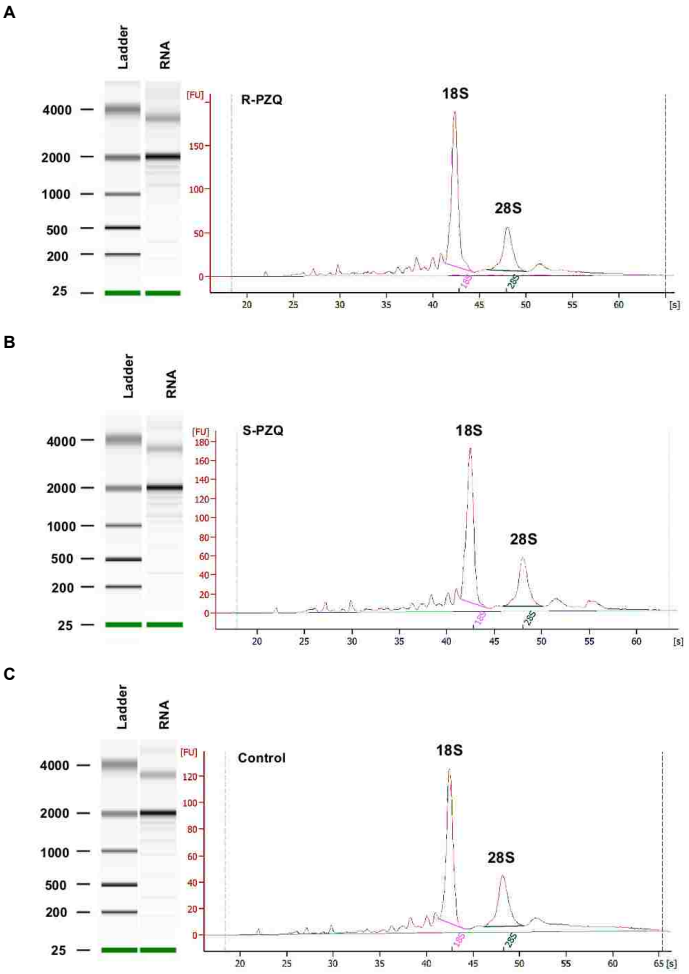
This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health. The University of New Mexico and University of California, San Diego Institutional Animal Care and Use Committees reviewed and approved all animal procedures used in this study.

2.2. Isolation and treatment of schistosomes in vitro with R- and S-PZQ in vitro

Praziquantel enantiomers (R) – [-] PZQ and (S) – [+] PZQ were synthesized and purified as described by Sharma et al. [16]. For use *in vitro*, each enantiomer was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) to produce stock concentrations of 50 μM.

Swiss Webster (SW) outbred mice (Charles River, Kingston, NY) were each infected with approximately 150 *S. mansoni* Puerto Rican 1 (PR1) cercariae per mouse. Mice were euthanized and perfused with RPMI 1640 media (RPMI; Sigma-Aldrich) at 49 days post infection (dpi). Male parasites were randomly divided into three groups of four biological replicates (~40 parasites per replicate) and placed in enriched RPMI (RPMI containing 5% Fetal Bovine Serum (FBS; Sigma-Aldrich) and 1% Penicillin/Streptomycin (Sigma-Aldrich)) overnight at 37 °C and 5% CO_2. Next day each group was incubated in 5 mL fresh, enriched RPMI containing final concentrations of either 1% DMSO (control treatment), 0.5 μM R-PZQ in 1% DMSO or 0.5 μM S-PZQ in 1% DMSO. Parasites were incubated for 18 h at 37 °C and 5% CO_2 then rinsed three times with RPMI 1640 and placed in RNeasy lysis buffer

(Qiagen, Redwood City, CA) with 1% 2-mercaptoethanol (Sigma-Aldrich) and stored at -80 °C prior to total RNA extraction.



S1 Fig. Total RNA qualitative and quantitative analysis. Bioanalyzer data showing total RNA gel image (left) with ladder for reference and electrogram (right) from representative samples (A) R-PZQ, (B) S-PZQ and (C) Control. [FU] = fluorescent units and [s] = retention time in seconds.

Total RNA was isolated using an RNeasy Mini Kit (Qiagen) including a digestion with RNase-free DNase (Qiagen) according to the manufacturer's instructions. RNA was further purified and concentrated (Zymo Research, Irvine, CA), and total RNA quantified using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA) and the quality verified with an

Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Bioanalyzer traces from each treatment showed no significant RNA degradation (Supplementary Figure 1; S1 Fig.). RNA was stored at -80 °C until required.

2.3. Next generation sequencing: cDNA library construction, sequencing and data processing

RNA sequencing (RNA-Seq) was conducted on four separate biological replicates representing each type of treatment using the Illumina Next Generation Sequencing (NGS) platform (Illumina, San Diego, CA). RNA-Seq libraries were generated using a KAPA Stranded mRNA-Seq Kit (KAPA Biosystems, Wilmington, MA). Poly-A mRNA was isolated using oligo dT magnetic beads, fragmented, and first strand cDNA synthesis primed using random hexamers. Second strand synthesis was performed and 2nd strand marked with dUTP and dAMP was added to the 3' end of the double stranded cDNA. Unique bar-coded adapters (Integrated DNA Technologies, Inc., Skokie, IL) were ligated to the cDNA and the resulting product amplified by PCR. The resulting libraries were quality controlled using a Qubit Fluorometric analysis and Bioanalyzer to determine quantity and fragment size. Libraries were further quantified using KAPA Library Quantification Kit (KAPA Biosystems) for Illumina platform. All samples passing the quality control were pooled. Samples were sequenced using an Illumina NextSeq 500 (Illumina) to generate 75 bases paired-end reads. The raw sequence reads for each sample were checked for quality control using Trimmomatic v0.36 [18] and mapped to the *S. mansoni* transcriptome annotated from assembly GCA_000237925.2 ASM23792v2 (NCBI) using Bowtie2 [19]. Gene expression levels were estimated with RSEM v1.18.1 [20]. Differentially expressed genes (DEG) were

identified in R-PZQ and S-PZQ transcriptomes after normalization against control transcriptomes using three separate analysis tools: DESeq2 v1.18.1 [21], EBSeq v1.18.0 [22] and EdgeR v3.20.8 [23]. Results were organized using SARTools [24], with cutoffs for all programs set at an adjusted p ≤0.05 with log$_2$fold change either <-1 (down-regulated genes) or >1 (up-regulated genes). DEG lists for each sample from each program were displayed using a Venn diagram and only genes common in at least two bioinformatics approaches were retained for further analysis. All high-throughput sequence data sets generated for this study were deposited with NCBI Gene Expression Omnibus and can be accessed through accession number GSE114506.

2.4. Gene Ontology analysis

Blast2GO [25] version 5.0.13 was used to classify DEG with ontology terms to identify biological functions present. A statistical assessment was conducted with the FatiGO package in Blast2GO to identify statistical over or under represented ontology terms in the final DEG lists using Fisher's Exact Test correcting for multiple testing with a FDR ≤ 0.05) [26].

2.5. RNA-Seq Validation

S. mansoni RNA samples for quantitative real-time PCR (qRT-PCR) originated from the corresponding samples used for RNA-Seq in order to validate Illumina data set expression patterns. Genes were chosen based on qRT-PCR primers passing efficiency tests and their differential expression patterns profile in the RNA-Seq data sets, i.e., being expressed in both R-PZQ and S-PZQ samples. One microgram total RNA was reverse transcribed in a 20 µL reaction using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA)

according to the manufacturer's instructions. qRT-PCR was performed in biological triplicates with technical duplicates. *S. mansoni* gene specific qRT-PCR primers (Table S1) were designed using the Integrated DNA Technologies OligoAnalyzer tool (www.idtdna.com).

S1 Table. Primers used in *S. mansoni* quantitative real-time PCR reactions.

Gene ID	Gene	Forward Primer	Reverse Primer
Smp_056970	SmGAPDH	GTCATTCCAGCACTAAACGG	CCTTCCCTAACCTACATGTCAG
Smp_123260	AN1 type zinc finger and ubiquitin	ATCCGCGGTTTATCTGGTGC	TGGTCAGTTAATTCAACACCGG
Smp_170340	Collagen alpha 1(IV) chain	GAAGAAGGCTGTTGTGGTGC	TTCTCCCACACCATCGATGC
Smp_144700	5' AMP activated protein kinase subunit gamma	ACAGCACCGTCCATATCACC	GTACACTGGGTGACTGTCGG
Smp_160360	Sodium:chloride dependent neurotransmitter	CGAACTGCATTACTTGATGGTGC	TTGAGTTCCTGCATCCGACC
Smp_008070	Thioredoxin	CGACAAACTTGAAGAGACCGC	AGAAGCCCCAACAACATCCC

Each 20 μL reaction contained SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad Laboratories), 100 ng cDNA, and 0.5 μM primer and was performed using a C1000 96 Touch Thermo Cycler (Bio-Rad Laboratories). qRT-PCR cycling conditions were as follows; 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 60°C for 1 min and 60°C for 20 s. Relative expression ($2^{-\Delta\Delta Ct}$) was performed using CFX Manager™ Software v3.1 (Bio-Rad Laboratories) to calculate the fold change relative to the reference *S. mansoni* gene glyceraldehyde-3-phosphate dehydrogenase (SmGAPDH). Expression of this gene did not

vary significantly between treatment groups (Table S2).

S2 Table. Real-time PCR cycle threshold (Cq) raw data for *S. mansoni* reference gene GAPDH

Well	Fluor	Target	Content	Sample	Biological Set Name	Cq	Cq Mean	Cq Std. Dev
A01	SYBR	GAPDH	Unkn-01	C_S1	control	21.65	21.68	0.064
A02	SYBR	GAPDH	Unkn-01	C_S1	control	21.75	21.68	0.064
A03	SYBR	GAPDH	Unkn-02	C_S1	control	21.63	21.68	0.064
A04	SYBR	GAPDH	Unkn-02	C_S2	control	20.45	20.47	0.025
A05	SYBR	GAPDH	Unkn-03	C_S2	control	20.48	20.47	0.025
A06	SYBR	GAPDH	Unkn-03	C_S2	control	20.50	20.47	0.025
A07	SYBR	GAPDH	Unkn-04	R-PZQ_S1	R-PZQ	21.43	21.38	0.044
A08	SYBR	GAPDH	Unkn-04	R-PZQ_S1	R-PZQ	21.36	21.38	0.044
A09	SYBR	GAPDH	Unkn-05	R-PZQ_S1	R-PZQ	21.35	21.38	0.044
A10	SYBR	GAPDH	Unkn-05	R-PZQ_S2	R-PZQ	21.83	21.82	0.060
A11	SYBR	GAPDH	Unkn-06	R-PZQ_S2	R-PZQ	21.76	21.82	0.060
A12	SYBR	GAPDH	Unkn-06	R-PZQ_S2	R-PZQ	21.88	21.82	0.060
B01	SYBR	GAPDH	Unkn-07	R-PZQ_S3	R-PZQ	20.70	20.74	0.053
B02	SYBR	GAPDH	Unkn-07	R-PZQ_S3	R-PZQ	20.72	20.74	0.053
B03	SYBR	GAPDH	Unkn-08	R-PZQ_S3	R-PZQ	20.80	20.74	0.053
B04	SYBR	GAPDH	Unkn-08	R-PZQ_S4	R-PZQ	21.48	21.51	0.066
B05	SYBR	GAPDH	Unkn-09	R-PZQ_S4	R-PZQ	21.47	21.51	0.066
B06	SYBR	GAPDH	Unkn-09	R-PZQ_S4	R-PZQ	21.59	21.51	0.066
B07	SYBR	GAPDH	Unkn-10	S-PZQ_S1	S-PZQ	20.45	20.42	0.046
B08	SYBR	GAPDH	Unkn-10	S-PZQ_S1	S-PZQ	20.45	20.42	0.046
B09	SYBR	GAPDH	Unkn-11	S-PZQ_S1	S-PZQ	20.37	20.42	0.046
B10	SYBR	GAPDH	Unkn-11	S-PZQ_S2	S-PZQ	20.70	20.72	0.017
B11	SYBR	GAPDH	Unkn-12	S-PZQ_S2	S-PZQ	20.73	20.72	0.017
B12	SYBR	GAPDH	Unkn-12	S-PZQ_S2	S-PZQ	20.73	20.72	0.017
C01	SYBR	GAPDH	Unkn-13	S-PZQ_S3	S-PZQ	20.93	20.86	0.064
C02	SYBR	GAPDH	Unkn-13	S-PZQ_S3	S-PZQ	20.81	20.86	0.064
C03	SYBR	GAPDH	Unkn-14	S-PZQ_S3	S-PZQ	20.83	20.86	0.064
C04	SYBR	GAPDH	Unkn-14	S-PZQ_S4	S-PZQ	20.05	20.05	0.045
C05	SYBR	GAPDH	Unkn-15	S-PZQ_S4	S-PZQ	20.09	20.05	0.045
C06	SYBR	GAPDH	Unkn-15	S-PZQ_S4	S-PZQ	20.00	20.05	0.045

The $\log_2$-fold change of qRT-PCR was derived on the basis of expression values from treatment averaged from three samples and expressed as the ratio compared to the control

group. Correlation between qRT-PCR and RNA-Seq data was assessed using a Spearman's Rho correlation, as the data were not normally distributed. Data analysis and statistical comparisons were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

3. Results and Discussion

We have previously demonstrated that, when tested *in vitro* against sexually mature *S. mansoni* PR1, the EC50 of the synthetic R–and S-PZQ available for these experiments was 0.5 μM and 78.1 μM respectively [16]. This difference in efficacy is unsurprising as R-PZQ was identified at an early stage as having the significantly greater anthelmintic activity [11,12]. As PZQ is administered to patients and animals as a racemic mixture containing approximately equal amounts of R- and S-PZQ we decided to identify the transcriptomes induced by each enantiomer at 0.5 μM to compare their effects relative to each other at a single dose, (due to cost and enantiomer availability), that is sub-lethal. We acknowledge this may result in a loss of data associated with S-PZQ exposure at higher concentrations, however, we felt it would more realistically capture the relative contribution of each enantiomer to the schistosomal response to the drug when it is administered *in vivo*.

3.1. Transcriptome sequencing and analysis

cDNA libraries were prepared from pooled, mixed sex mature schistosomes after overnight exposure *in vitro* to 0.5 μM of each enantiomer. On removing adapter sequences and any ambiguous, low quality reads (Q<20), a total of 512.6 million, 75 base paired-end reads were obtained (S3 Table).

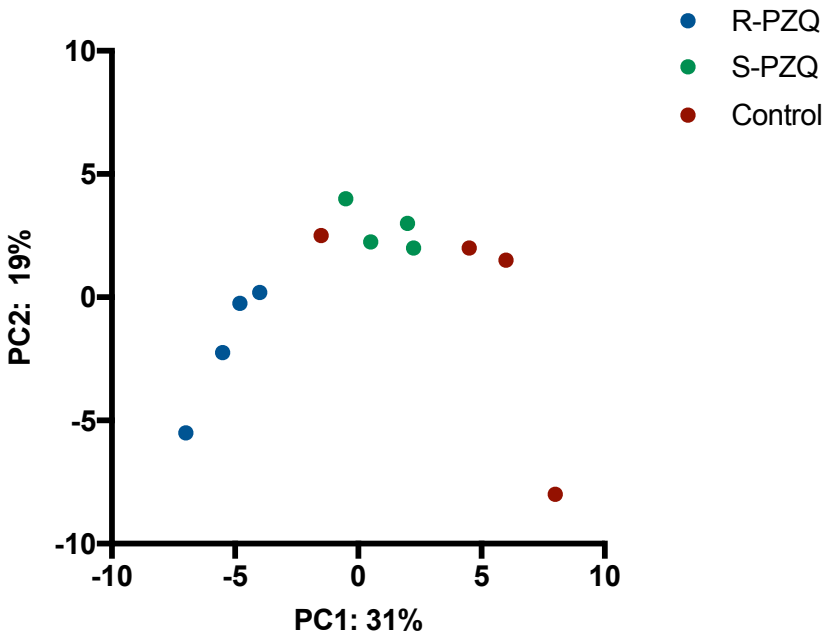
S3 Table. Summary of Illumina read counts and mapped reads for each sequenced sample

Sample	Total quality-trimmed paired reads (Q>20)	Percentage of *S. mansoni* mapped reads
C_S1	50,037,818	60.02
C_S2	30,637,990	64.25
C_S3	47,228,652	62.25
C_S4	39,101,959	62.16
R-PZQ_S1	44,144,467	64.03
R-PZQ_S2	40,990,919	61.50
R-PZQ_S3	42,779,709	61.08
R-PZQ_S4	40,770,368	63.98
S-PZQ_S1	44,441,980	66.00
S-PZQ_S2	48,958,544	63.04
S-PZQ_S3	45,114,001	66.41
S-PZQ_S4	38,466,340	65.09

The mean number of reads in the control, R- and S-PZQ groups was 41.8, 42.2 and 44.3 million respectively with a mean of 62.2, 62.7 and 65.1 % paired end reads mapped to the *S. mansoni* genome. Each group contained 4 biological replicates and the number of reads provided enough coverage for differential expression analysis [27].

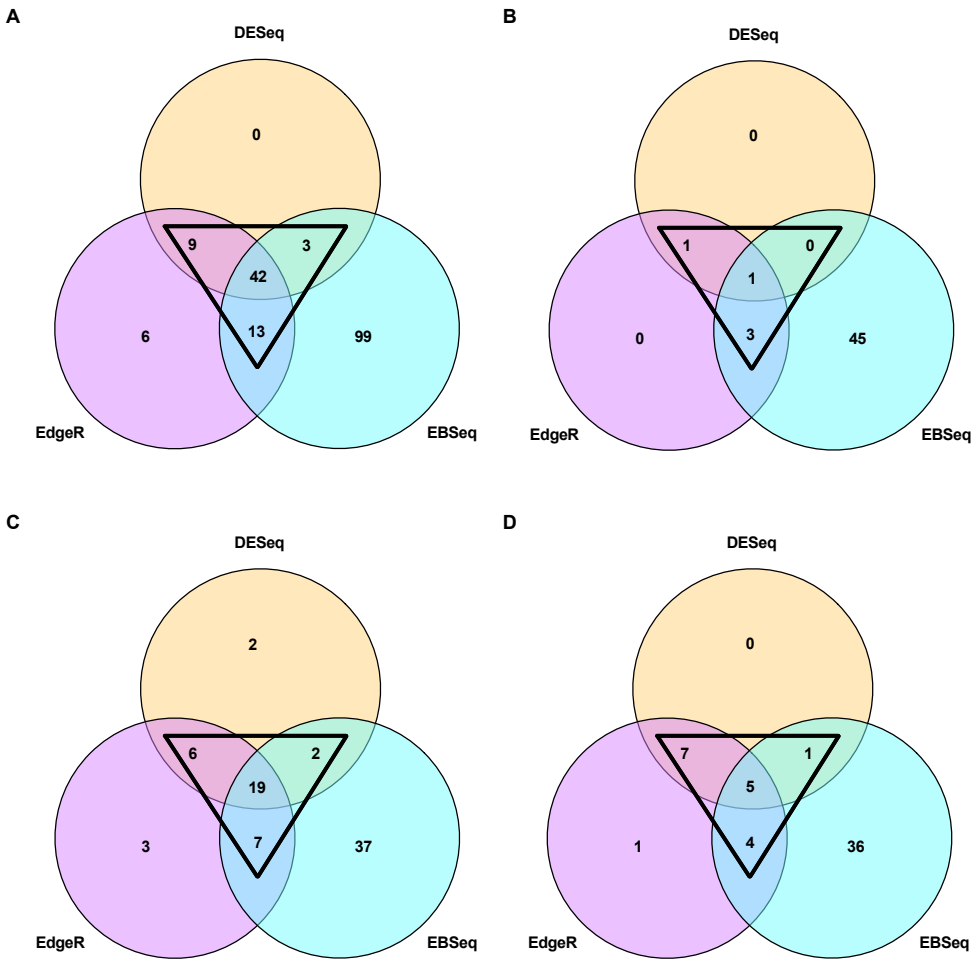
Principal component analysis of the transcriptomes indicated that one of the control samples was a significant outlier to the others which clustered tightly with the 4 S-PZQ samples (S2 Fig). The R-PZQ samples clustered together but separately from the S-PSQ and control samples. In this initial analysis, all samples were retained for further analyses to

maintain an *n* of 4 across all groups, however, prior to publication the data will be reanalyzed using an *n* of 3 across all groups after discarding the outlier control sample and one of both the R- and S- samples to establish if the outlier is causing an under- or over-reporting of DEGs.



S2 Fig. Principal component analysis. Two-dimensional principal component (PC) was used to characterize the scattering or clustering of replicates in each treatment groups.

Three programs (DESeq, EBSeq, and EdgeR) were used to identify differential transcript expression and PZQ treated samples were normalized to controls for each isomer. The number of DEGs observed using each program is shown in S3 Fig and transcripts that were identified by at least 2 of the programs were retained for further analyses. The number of differentially regulated genes associated with R- and S-PZQ treatment was 101 and 22 respectively (S4 Table).



S3 Fig. Venn diagrams displaying selection process of differentially expressed genes in this study. Three programs (DESeq, EBSeq, and EdgeR) were employed to find differentially expressed genes in R- and S-PZQ treated schistosomes compared to controls. Only differentially expressed genes that were seen in at least two of these programs were maintained for the study. Separated differentially expressed genes in this study are shown in the triangle, (A) R-PZQ up-regulated, (B) S-PZQ up-regulated, (C) R-PZQ down-regulated, and (D) S-PZQ down-regulated.

S4 Table. Identities of all differentially expressed genes for R-PZQ and S-PZQ

Passage	Total genes up-regulated	Total genes down-regulated
R-PZQ	67	34
S-PZQ	5	17

This lower number of DEGs associated with the S-PZQ was perhaps unsurprising given the relatively weak anthelmintic effect of the compound at the concentration employed. An additional observation is that though treatment with S-PZQ resulted in the differential regulation of only 22 genes, 11 of these were shared with R-PZQ suggesting that while S-PZQ may not have the anthelmintic specificity, both compounds may share 'off target' effects on the transcriptome (Fig. 1).

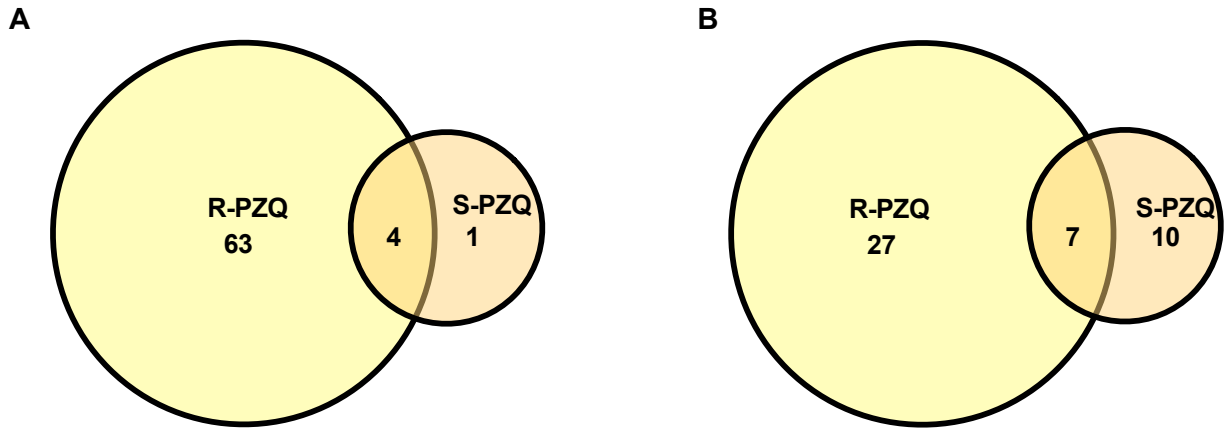


Fig 1. Venn diagrams of differentially expressed genes. Indicated in the Venn diagrams are the numbers of (A) up-regulated and (B) down-regulated differentially expressed genes shared or exclusive to each treatment. The diameter of each circle is proportional to the number of transcripts it represents.

No Gene Ontology analysis of overrepresented GO terms is provided as only between 1-3 genes were selected for any particular Biology Process term rendering a limited analysis.

Heat maps were constructed for all differentially regulated genes. Among the 68 up-regulated genes, 24 were hypothetical or unnamed (Fig. 2, Table S5). The two genes showing the greatest level of up-regulation after treatment with R-PZQ, Smp_174240 ($log_2 = 5.5$) and Smp_041730 ($log_2 = 4.4$) were also the two highest differentially regulated after S-PZQ treatment ($log_2 = 4.4$ and 4.1 respectively). While Smp_174240 encodes a hypothetical protein, Smp_041730 encodes a homolog of putative pre-mRNA-splicing factor cwc-21. The third most induced transcript in the R-PZQ cohort was splicing factor 3b (Smp_141630, $log_2 = 4.1$) and the induction of two putative arginine/serine-rich splicing factors (Smp_036270, $log_2 = 1.20$ and Smp_032320, $log_2 = 1.02$), two DNA-binding Early Growth Response proteins (Smp_094930, $log_2 = 1.54$ and Smp_134870, $log_2 = 1.40$), two basic helix-loop-helix dimerization regions that characterize dimerizing transcription factors (Smp_132810, $log_2 = 3.05$ and Smp_067430, $log_2 = 2.69$) as well as cut-like 1 ($log_2 = 1.27$) and Far Upstream element (FUSE) (Smp_044550, $log_2 = 1.06$) DNA binding proteins confirms that one general effect of PZQ exposure is increased levels of transcription and transcript processing.

A second group of genes differentially regulated by R-PZQ were those encoding heat shock proteins including Smp_049240 ($log_2 = 2.94$), Smp_186020 ($log_2 = 2.1$) and Smp_049250 ($log_2 = 1.49$). One of these, Smp_186020 which encodes Heat Shock Protein 70 was upregulated in juvenile *S. mansoni* in the 3 h following treatment with racemic PZQ in vivo [10]. No other previously identified stress response genes such as ferritin (Smp_047660) or thioredoxin peroxidase -1 (Smp-059480) that have been shown to be induced after PZQ treatment were elevated in this study.

Other induced genes of note included Receptor for activated Protein Kinase C (Smp_102040, $\log_2 = 3.88$), and three major egg antigen transcripts (Smp_049300.1, $\log_2 = 2.98$; Smp_049300.2, $\log_2 = 2.51$ and Smp_049300.3, $\log_2 = 1.85$). Receptor for activated Protein Kinase C has been associated with PZQ resistance [28], however, its up-regulation in this non-PZQ selected strain suggests its differential regulation is unrelated to resistance. Major egg antigen (Smp40) has been mooted as a potential vaccine candidate [29] and its increased transcription after drug treatment gives support to the idea that any vaccine based on egg antigen may be enhanced by treatment with PZQ to stimulate production of the target antigen by the pathogen.

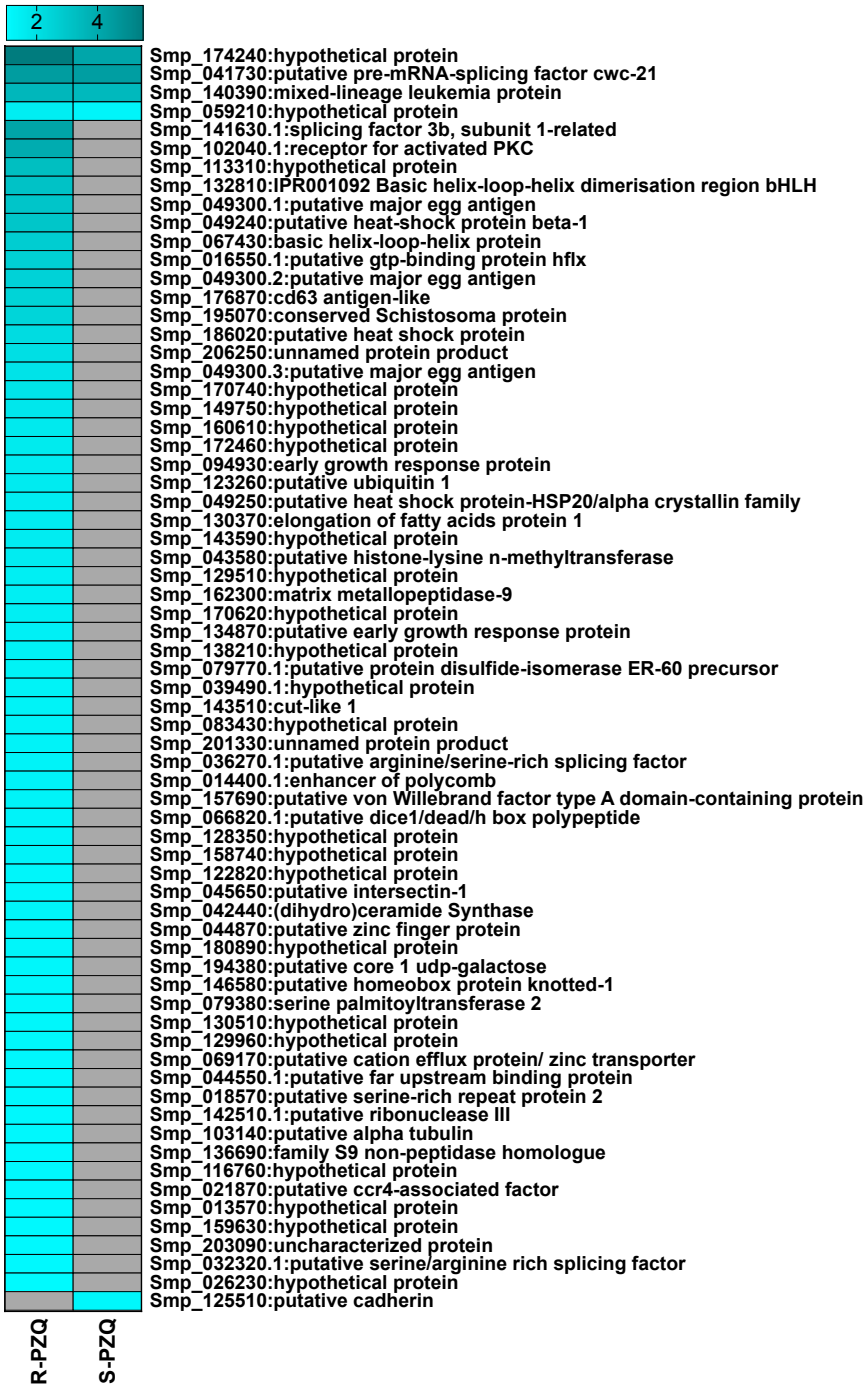


Fig 2. Expression of *S. mansoni* genes after treatment with R- and S-PZQ. Heat map of all up-regulated genes in R and S-PZQ treated schistosomes relative to controls. Genes not differentially expressed are colored grey. The color scale indicates $\log_2$ fold change in the average of four biological replicates.

S5 Table. List of genes up-regulated after treatment of *S. mansoni* with R- or S-PZQ

XM_Id	GeneDB_Id	log2FC	p-adj	NCBI_annotation
R-PZQ				
XM_018792500.1	Smp_174240.1	5.484	1.751E-06	hypothetical protein
XM_018797184.1	Smp_041730.1	4.379	7.082E-03	putative pre-mRNA-splicing factor cwc-21
XM_018794188.1	Smp_141630.1	4.117	1.245E-02	splicing factor 3b, subunit 1-related
XM_018792424.1	Smp_102040.1	3.882	2.708E-03	receptor for activated PKC
XM_018791620.1	Smp_140390.1	3.474	1.441E-02	mixed-lineage leukemia protein
XM_018794745.1	Smp_113310	3.219	7.285E-03	hypothetical protein
XM_018791956.1	Smp_132810	3.053	3.744E-04	Basic helix-loop-helix dimerisation region bHLH
XM_018794696.1	Smp_049300.1	2.981	3.645E-21	putative major egg antigen
XM_018794684.1	Smp_049240	2.942	1.659E-17	putative heat-shock protein beta-1
XM_018799842.1	Smp_067430	2.692	3.795E-04	basic helix-loop-helix protein
XM_018797404.1	Smp_016550.1	2.551	2.902E-02	putative gtp-binding protein hflx
XM_018794700.1	Smp_049300.2	2.505	1.770E-02	putative major egg antigen
XM_018791281.1	Smp_176870	2.45	1.189E-03	cd63 antigen-like
XM_018789885.1	Smp_195070	2.383	1.770E-02	conserved Schistosoma protein, unknown function
XM_018794694.1	Smp_186020	2.123	9.412E-16	putative heat shock protein
XM_018795708.1	Smp_206250	1.991	4.884E-02	unnamed protein product
XM_018794699.1	Smp_049300.3	1.854	6.074E-09	putative major egg antigen
XM_018797437.1	Smp_170740	1.798	2.606E-03	hypothetical protein
XM_018796585.1	Smp_149750	1.694	2.151E-07	hypothetical protein
XM_018799180.1	Smp_160610	1.597	7.363E-03	hypothetical protein
XM_018798203.1	Smp_172460	1.561	3.193E-18	hypothetical protein
XM_018797509.1	Smp_094930	1.544	4.041E-10	early growth response protein
XM_018797982.1	Smp_123260	1.543	2.694E-08	putative ubiquitin 1
XM_018794689.1	Smp_049250	1.494	2.912E-13	putative heat shock protein-HSP20/alpha crystallin family
XM_018799836.1	Smp_130370	1.484	6.736E-03	elongation of fatty acids protein 1
XM_018796297.1	Smp_143590	1.471	9.808E-03	hypothetical protein
XM_018797690.1	Smp_043580	1.468	9.501E-03	putative histone-lysine n-methyltransferase
XM_018796971.1	Smp_059210.1	1.45	6.352E-04	hypothetical protein
XM_018799097.1	Smp_129510	1.425	1.484E-08	hypothetical protein
XM_018797880.1	Smp_162300	1.425	5.019E-04	matrix metallopeptidase-9
XM_018795017.1	Smp_170620	1.382	4.603E-03	hypothetical protein
XM_018799619.1	Smp_134870	1.349	1.914E-09	putative early growth response protein
XM_018799499.1	Smp_138210	1.333	1.342E-06	hypothetical protein
XM_018799452.1	Smp_079770.1	1.279	6.492E-03	putative protein disulfide-isomerase ER-60 precursor
XM_018797750.1	Smp_039490.1	1.273	9.980E-06	hypothetical protein
XM_018796310.1	Smp_143510	1.27	7.509E-03	cut-like 1
XM_018790738.1	Smp_083430	1.249	2.150E-02	hypothetical protein
XM_018791691.1	Smp_201330	1.247	1.858E-03	unnamed protein product
XM_018794202.1	Smp_036270.1	1.243	1.371E-02	putative arginine/serine-rich splicing factor
XM_018790795.1	Smp_014400.1	1.238	3.458E-03	enhancer of polycomb, putative
XM_018789482.1	Smp_157690	1.217	1.146E-02	putative von Willebrand factor type A domain-containing protein

XM_Id	GeneDB_Id	log2FC	p-adj	NCBI_annotation
XM_018793974.1	Smp_066820.1	1.202	1.397E-02	putative dice1/dead/h box polypeptide
XM_018788885.1	Smp_128350	1.199	4.272E-05	hypothetical protein
XM_018788508.1	Smp_158740	1.189	8.512E-15	hypothetical protein
XM_018791522.1	Smp_122820	1.152	3.173E-03	hypothetical protein
XM_018797672.1	Smp_045650	1.148	6.359E-03	putative intersectin-1
XM_018797081.1	Smp_042440	1.144	2.186E-02	(dihydro)ceramide Synthase
XM_018795610.1	Smp_044870	1.138	9.686E-06	putative zinc finger protein
XM_018795382.1	Smp_180890	1.127	1.858E-03	hypothetical protein
XM_018795041.1	Smp_194380	1.116	2.480E-03	putative core 1 udp-galactose:n-acetylgalactosamine-alpha-r beta 1,3-galactosyltransferase
XM_018795673.1	Smp_146580	1.112	1.705E-02	putative homeobox protein knotted-1
XM_018793229.1	Smp_079380	1.1	7.032E-03	serine palmitoyltransferase 2
XM_018790009.1	Smp_130510	1.096	1.947E-05	hypothetical protein
XM_018788454.1	Smp_129960	1.095	8.077E-03	hypothetical protein
XM_018796790.1	Smp_069170	1.068	1.143E-03	putative cation efflux protein/ zinc transporter
XM_018788610.1	Smp_044550.1	1.059	6.359E-03	putative far upstream (fuse) binding protein
XM_018798920.1	Smp_018570	1.055	3.293E-06	putative serine-rich repeat protein 2
XM_018797573.1	Smp_142510.1	1.049	1.739E-03	putative ribonuclease III
XM_018797511.1	Smp_103140	1.035	1.601E-03	putative alpha tubulin
XM_018791807.1	Smp_136690	1.035	3.669E-02	family S9 non-peptidase homologue
XM_018798673.1	Smp_116760	1.033	2.400E-04	hypothetical protein
XM_018795348.1	Smp_021870	1.032	4.788E-07	putative ccr4-associated factor
XM_018799096.1	Smp_013570	1.03	3.064E-04	hypothetical protein
XM_018798142.1	Smp_159630	1.029	1.247E-02	hypothetical protein
XM_018798499.1	Smp_203090	1.029	1.524E-02	uncharacterized protein
XM_018790092.1	Smp_032320.1	1.02	1.501E-07	putative serine/arginine rich splicing factor
XM_018799314.1	Smp_026230	1.002	1.623E-04	hypothetical protein
S-PZQ				
XM_018797184.1	Smp_041730.1	4.338	7.465E-03	putative pre-mRNA-splicing factor cwc-21
XM_018792500.1	Smp_174240.1	4.038	1.034E-02	hypothetical protein
XM_018791620.1	Smp_140390.1	3.42	3.103E-02	mixed-lineage leukemia protein
XM_018796971.1	Smp_059210.1	1.178	1.148E-02	hypothetical protein
XM_018796291.1	Smp_125510	1.075	6.607E-03	putative cadherin

A total of 44 genes were down-regulated across both treatments and 22 were classed as either hypothetical, uncharacterized or unnamed. (Fig. 3 and Table S6). Seven transcripts were present in both the R- and S- transcriptomes and six of these were the most down-regulated proteins in both data sets. Three of these six, Smp_152150, Smp_131110 and Smp_014610, were hypothetical with $\log_2$ = -4.3, -4.2 and –3.3 respectively for R-PZQ and -

9.9, -4.7 and -3.6 for S-PZQ treatment. The remaining 3 genes encoded a putative WD-containing protein (Smp_032800), an eggshell precursor (Smp_000430) and eggshell protein (Smp_191910). Lamberton et al. [30] noted that treatment with racemic PZQ had an effect on worm fecundity as measured by the number of hatching eggs recovered from the spleen and liver of infected mice. Down-regulation of eggshell proteins by both R- and S-PZQ treatment (R-PZQ also down-regulated Smp_077890, Trematode eggshell synthesis domain containing protein) suggests that any effect of the drugs on egg production may be independent of the killing mechanism of the R-PZQ and an inherent property of both isomers.

Three transporter proteins were also down-regulated after treatment with R-PZQ including a sodium/chloride dependent neurotransmitter transporter (Smp_160360, $\log_2 = -1.2$) and a putative glucose transporter (Smp_105410, $\log_2 = -1.0$). Of most note was the down regulation of the ABC superfamily member ABCB1 (Smp_170820, $\log_2 = -1.2$). Members of the ABCB, ABCC and ABCG subfamilies have previously been shown to excrete structurally unrelated drugs and have the potential to confer drug resistance on cells and organisms when aberrantly over expressed [31]. *S. mansoni* ABCB, ABC and ABCG homologs, including Smp_170820, have been shown previously by us to be up-regulated in juvenile schistosomes which are refractory to the drug [10]. In contrast there was little evidence of transporter activity in mature schistosomes with the exception of a single ABCB1 homolog (Smp_089200). Pinto-Almeida et al., [2015] found ABCB1 homolog SmMDR2 (Smp_055780) to be up-regulated on exposure to PZQ, but in a PZQ resistant strain. The absence of up-regulated ABC genes in the current study is consistent with our previous findings when using standard *S. mansoni* PR1 that have not been selected for PZQ resistance are exposed to the drug [10].

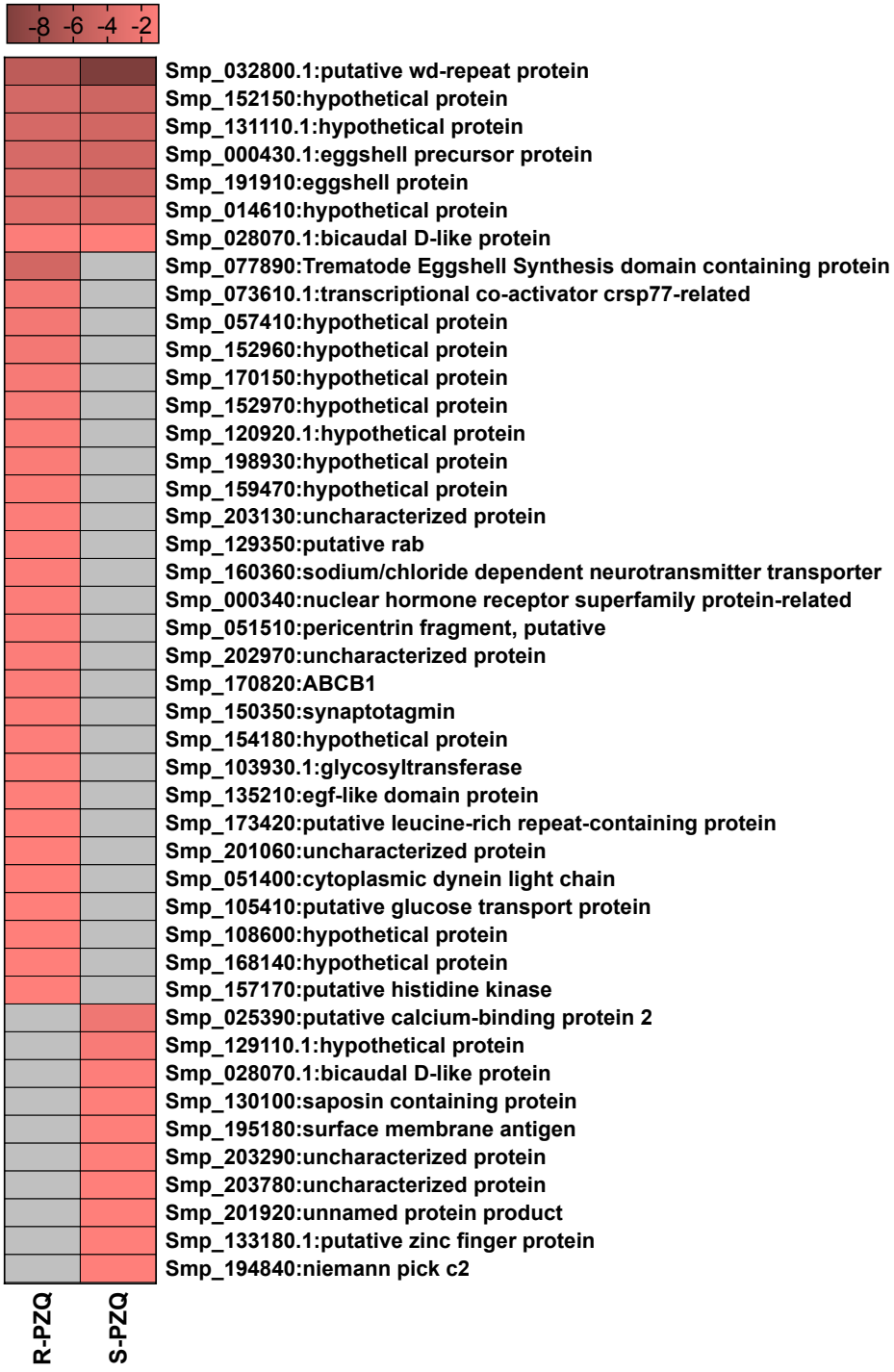


Fig 3. Expression of *S. mansoni* genes after treatment with R- and S-PZQ. Heat map of all down-regulated genes in R and S-PZQ treated schistosomes relative to controls. Genes not differentially expressed are colored grey. The color scale indicates $\log_2$ fold change in the average of four biological replicates.

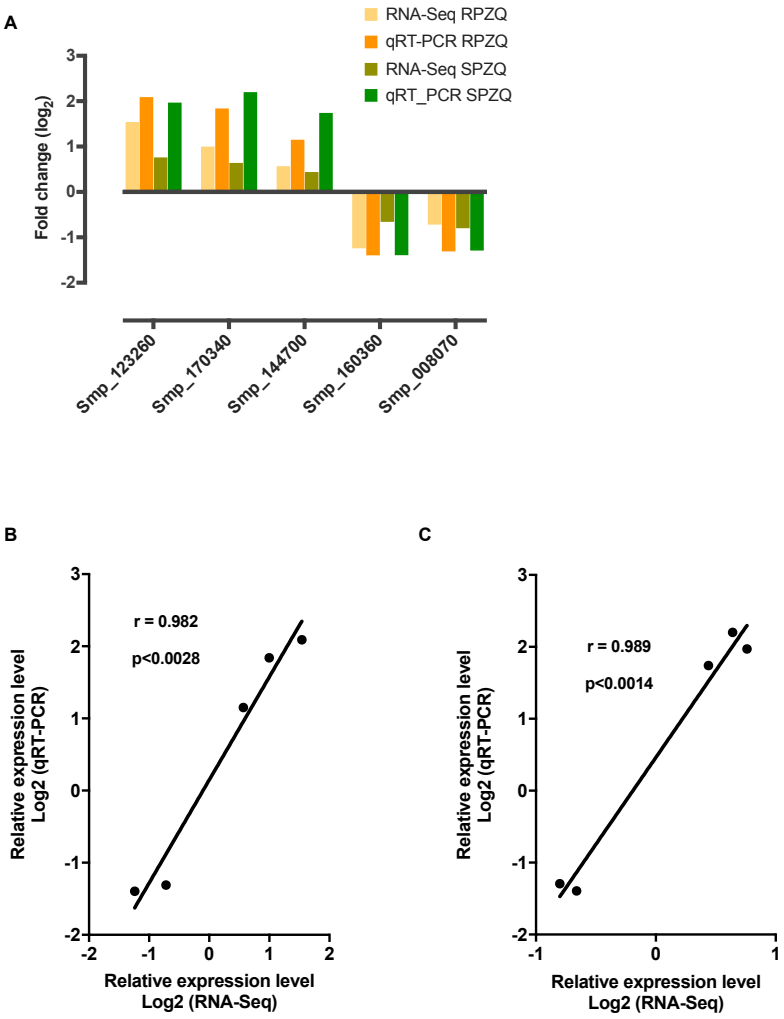
S6 Table. List of genes down-regulated after treatment of *S. mansoni* with R- or S-PZQ

XM_Id	GeneDB_Id	log2FC	p-adj	NCBI_annotation
R-PZQ				
XM_018795263.1	Smp_032800.1	-5.871	2.003E-11	putative wd-repeat protein
XM_018791417.1	Smp_077890	-4.335	3.454E-02	Trematode Eggshell Synthesis domain containing protein
XM_018793003.1	Smp_152150	-4.282	6.505E-03	hypothetical protein
XM_018797389.1	Smp_131110.1	-4.181	2.085E-02	hypothetical protein
XM_018790417.1	Smp_000430.1	-4.104	1.442E-02	eggshell precursor protein
XM_018790420.1	Smp_191910	-3.546	2.684E-02	eggshell protein
XM_018795711.1	Smp_014610	-3.274	1.609E-02	hypothetical protein
XM_018793199.1	Smp_073610.1	-1.972	4.529E-02	transcriptional co-activator crsp77-related
XM_018798680.1	Smp_057410	-1.881	1.035E-09	hypothetical protein
XM_018798679.1	Smp_152960	-1.866	1.648E-09	hypothetical protein
XM_018790590.1	Smp_170150	-1.657	1.253E-09	hypothetical protein
XM_018798678.1	Smp_152970	-1.62	3.336E-04	hypothetical protein
XM_018793894.1	Smp_120920.1	-1.428	2.765E-02	hypothetical protein
XM_018798598.1	Smp_198930	-1.377	1.793E-05	hypothetical protein
XM_018788481.1	Smp_159470	-1.367	1.480E-02	hypothetical protein
XM_018798004.1	Smp_203130	-1.29	4.047E-09	uncharacterized protein
XM_018799126.1	Smp_129350	-1.267	9.024E-09	putative rab
XM_018789587.1	Smp_160360	-1.243	1.617E-04	sodium/chloride dependent neurotransmitter transporter
XM_018794141.1	Smp_028070.1	-1.236	3.830E-05	bicaudal D-like protein
XM_018791409.1	Smp_000340	-1.234	2.245E-03	nuclear hormone receptor superfamily protein-related
XM_018792625.1	Smp_051510	-1.223	3.661E-08	pericentrin fragment
XM_018798000.1	Smp_202970	-1.22	7.146E-10	uncharacterized protein
XM_018788897.1	Smp_170820	-1.203	2.057E-04	ABCB1
XM_018789676.1	Smp_150350	-1.165	1.253E-09	synaptotagmine
XM_018791285.1	Smp_154180	-1.15	8.878E-04	hypothetical protein
XM_018792740.1	Smp_103930.1	-1.101	1.610E-02	glycosyltransferase
XM_018789656.1	Smp_135210	-1.08	2.554E-03	egf-like domain protein
XM_018793971.1	Smp_173420	-1.058	1.739E-03	putative leucine-rich repeat-containing protein
XM_018794538.1	Smp_201060	-1.057	4.204E-03	uncharacterized protein
XM_018790629.1	Smp_051400	-1.038	1.188E-05	cytoplasmic dynein light chain
XM_018795817.1	Smp_105410	-1.033	2.104E-05	putative glucose transport protein
XM_018790768.1	Smp_108600	-1.029	2.160E-09	hypothetical protein
XM_018789841.1	Smp_168140	-1.012	2.499E-02	hypothetical protein
XM_018789610.1	Smp_157170	-1.002	6.558E-04	putative histidine kinase
S-PZQ				
XM_018795263.1	Smp_032800.1	-9.905	3.268E-02	putative wd-repeat protein
XM_018793003.1	Smp_152150	-4.726	1.909E-03	hypothetical protein
XM_018797389.1	Smp_131110.1	-4.5	1.411E-02	hypothetical protein
XM_018790420.1	Smp_191910	-4.467	2.845E-02	eggshell protein

XM_Id	GeneDB_Id	log2FC	p-adj	NCBI_annotation
XM_018790417.1	Smp_000430.1	-4.423	1.406E-02	eggshell precursor protein
XM_018795711.1	Smp_014610	-3.575	5.692E-03	hypothetical protein
XM_018789515.1	Smp_025390	-2.226	8.802E-06	putative calcium-binding protein 2
XM_018799286.1	Smp_129110.1	-1.594	6.607E-03	hypothetical protein
XM_018794143.1	Smp_028070.1	-1.143	1.627E-03	bicaudal D-like protein
XM_018795705.1	Smp_130100	-1.093	3.119E-09	saposin containing protein
XM_018797927.1	Smp_195180	-1.083	1.304E-05	surface membrane antigen
XM_018798695.1	Smp_203290	-1.065	1.324E-02	uncharacterized protein
XM_018794141.1	Smp_028070.1	-1.061	3.557E-02	bicaudal D-like protein
XM_018789716.1	Smp_203780	-1.05	3.632E-04	uncharacterized protein
XM_018791483.1	Smp_201920	-1.044	2.845E-02	unnamed protein product
XM_018793438.1	Smp_133180.1	-1.017	3.825E-02	putative zinc finger protein
XM_018790325.1	Smp_194840	-1.008	2.801E-03	niemann pick c2

3.2. *RNA sequence validation*

Quantitative real-time PCR (qRT-PCR) was employed to validate results obtained by RNA-Seq (S4 Fig). Five transcripts encoding homologs of ubiquitin 1 (Smp_123260), Collagen alpha 1 (V) chain (Smp_170340), AMP-activated protein kinase gamma regulatory subunit (Smp_144700), sodium/chloride dependent neurotransmitter transporter (Smp_160360), and Thioredoxin 1 (Smp_008070) were validated based on their expression in *S. mansoni* after treatment with R- and S-PZQ (S4A Fig). Patterns observed in the qRT-PCR data matched those obtained in the RNA-Seq data set and showed significant correlation for R-PZQ (r = 0.982, $p<0.0028$; S4B Fig) and S-PZQ (r = 0.989, $p<0.0014$;S4 C Fig.]).



S4 Fig. Validation between RNA-Seq and qRT-PCR transcription of six expressed genes.
(A) Log2 fold changes of expression of 5 selected *S. manoni* transcripts from R- and S-PZQ treated parasites relative to control parasites assessed by RNA-Seq and qRT-PCR. qRT-PCR fold changes were normalized to GAPDH (housekeeping gene), and gene expression profiles at each point are the average of three biological replicates. Correlations between RNA-Seq and qRT-PCR transcription each of the genes in (B) R-PZQ, and (C) S-PZQ were determined using Pearson's correlation.

4. Conclusion

We observed that the anthelmintic enantiomer R-PZQ, induces a significantly greater change in the transcriptome of *S. mansoni* than the inactive S-PZQ. With the parameters employed, however, the number of DEGs in both data sets was relatively limited. For

example, at the single, sub-lethal concentration tested we saw a significant induction of genes encoding transcriptional regulatory proteins but surprising little evidence of a stress response to either enantiomer. Enhanced transcriptional regulation comes as no surprise given our previous findings that treatment of *S. mansoni* PR1 *in vitro* with 3 μM racemic PZQ (effectively 1.5 μM of each enantiomer) for up to 20 h resulted in the differential expression of 658 genes, including those involved in stress responses [9]. Similarly, we saw a stress response in male *S. mansoni* exposed to a lethal dose of racemic PZQ *in vivo* [10]. While we cannot be sure of the mouse plasma concentration of the enantiomers in this latter work, we note that Andrews [32] calculated a maximum of 3 μM unmetabolized drug in plasma was achieved in uninfected humans following administration of 46 mg/Kg with 1 μM (0.5 μM of each enantiomer) the probable therapeutic threshold. Of course, it is possible the absence of the stress response (or any significant change in expression of genes regulated by Ca^{2+}influx) in the experiment reported here is an indication that it is a synergistic effect of both enantiomers and that each enantiomer individually may not have achieved a critical intracellular threshold to trigger the response. As noted earlier, we will reanalyze our data omitting the single outlying data point from the control and each of the treated groups. This may improve the sensitivity of our analysis and provide a larger cohort of DEGs to analyze. Alternatively, we have used relatively conservative cut off values for assigning a gene as differentially expressed or not and using a more liberal approach may yield a more informative data set (a baby and bathwater conundrum). Also, both enantiomers may actually have had only relatively minor effects on transcription dynamics or we may also have used a dose of each enantiomer that was too low to be effective. Our previous *in vitro* experiments with racemic PZQ suggests these latter scenarios to be unlikely [9], however, a final

conclusion on the respective effects of each enantiomer on the *S. mansoni* transcriptome will only be made after further analysis.

Conflict of interest

The authors declare that there is no conflict of interest.

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Chapter 3

Transcriptomic analysis of reduced sensitivity to praziquantel in *Schistosoma mansoni*

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Abstract

Schistosomiasis is an intravascular parasitic infection estimated to affect over 206 million people, the majority of whom live in Africa where the trematode worms *Schistosoma mansoni* and *Schistosoma haematobium* are the major causative agents. While a number of drugs have been used to treat schistosomiasis, praziquantel (PZQ) is the only one that is widely available, relatively cheap, and easy to use. The reliance on a single drug for the treatment of such a prevalent disease is a cause for concern due to the potential for resistance to render PZQ ineffective. In this study, we examine the transcriptome of three generations of a laboratory strain of *S. mansoni* (PR1) whose susceptibility to PZQ has been diminished across 9 passages through exposure to increasing sub-lethal doses of the drug. Miracidial susceptibility was significantly reduced after exposure to 2 x 50 mg/Kg PZQ during the first passage. Susceptibility of worms *in vivo* was first assessed during passage 5 when mice infected with PZQ-selected schistosomes were dosed with a lethal dose of 3 x 300 mg/Kg PZQ resulting in only a 10% reduction in worm number compared to control treatment. The emergence of reduced sensitivity was marked by a shift in sex ratio from a predominantly male to a female population, a reduction in the length of females and ultimately the loss of the PZQ-selected line after passage 9. Analysis of differentially regulated transcripts did not suggest that any particular gene product or pathway was associated with drug resistance suggesting either a loss of function mutation to a single gene or an epistatic interaction of multiple gene products as the underlying cause of reduced susceptibility.

Keywords: Praziquantel, Schistosoma, drug resistance, schistosomiasis, helminth, transcriptome

1. Introduction

Schistosomiasis is a chronic neglected parasitic disease caused by digenetic blood flukes of the genus *Schistosoma.* As of 2016, the disease was estimated to affect approximately 206 million people worldwide though it is most prevalent in sub-Saharan Africa where *Schistosoma mansoni* and *Schistosoma haematobium* are the major causative agents [1]. This is a significant improvement on the previous estimate of approximately 259 million infected in 2014 [2]. One reason for this decline is that the number of individuals receiving treatment for the disease has risen from approximately 62 million in 2014 to 89 million in 2016 [1,2].

Praziquantel (PZQ) is the most widely available drug for the treatment of schistosomiasis and remains effective against all schistosomes that infect humans [3]. While PZQ has been used for over 30 years the exact mechanism of action of the drug remains unknown. One drawback is that sexually immature, juvenile schistosomes are resistant to its action [4,5] and, as the drug is usually administrated in endemic areas as a single dose, complete effectiveness is unlikely with a reservoir of PZQ resistant juvenile parasites likely remaining in the host.

With no other readily available treatment options, and as the number of PZQ tablets dispensed increases each year, there are significant concerns that the drug's usefulness may be diminished or lost to resistance. A number of reports of schistosomes with reduced PZQ sensitivity have appeared in the literature though not all of these document increasing resistance. For example, Stelma et al. [6] reported a cure rate of only 18% with PZQ in Senegal in 1991 and suggested PZQ resistance as one explanation, however, Fallon et al. [7] reported that isolates from this population had a slower maturation rate rendering the schistosomes less sensitive to the drug at the times tested. Of more concern were the eight

isolates derived from Egyptian patients who had received multiple, curative doses of PZQ [8]. These had less sensitivity to the drug compared to controls and retained reduced sensitivity over multiple passages through mice. In a subsequent study, William et al., [9] found 3 of the 6 strains retained reduced PZQ sensitivity in the absence of selective PZQ pressure but this came at the cost of diminished reproductive fitness which may account for the failure to find PZQ resistance in the same area of Egypt 10 years after the initial finding [10]. Melman et al. [11] reported a correlation between the number of PZQ treatments received by Kenyan patients and the sensitivity of miracidia hatched from eggs isolated form patient fecal samples. Similar to the experience reported by William et al. [9], a line of *S. mansoni* derived from one patient that showed reduced PZQ sensitivity could not be maintained in the lab beyond a few generations.

While it has proven difficult to find and maintain PZQ resistant Schistosoma *spp.* in field isolates, resistance has been successfully generated in the laboratory by maintaining schistosomes in mice with exposure to increasing sub lethal doses of PZQ over several generations [12-15]. The *S. mansoni* isolates generated by Coeli et al. [13] were also unstable and showed reduced genetic diversity. Remarkably, Couto et al. [16] generated *S. mansoni* with reduced sensitivity after one exposure to PZQ in infected snails.

The molecular mechanism underpinning resistance in field or laboratory-induced isolates is unknown and it may be that there is more than one route to reduced sensitivity. ATP-binding cassette (ABC) proteins, which are known to be involved in the transport of a wide variety of compounds, including drugs, across membranes have been implicated in the natural resistance of juvenile *S. mansoni* [17-20]. We have suggested that the aberrant expression of ABC transporters may underlie acquired resistance in adults [21] and, using a

laboratory selected strain of PZQ resistant *S. mansoni* Pinto-Almeida et al. [15] demonstrated the involvement of ABC transporter SmMDR2, belonging to the ABCB1 (Pgp-like) subfamily, in male worms displaying the resistance phenotype.

As indicated above, PZQ resistance has been shown to come with a fitness tradeoff [9,11,13,22], and may provide one explanation for the absence of sustained PZQ resistance in the field where there is a large refugium of drug sensitive schistosomes. As the number of individuals treated with PZQ continues to rise, the question of resistance in field isolates remains important for both researchers and government agencies trying to implement policy for the control of schistosomiasis. In the absence of alternative drugs or a vaccine, and as the number of PZQ tablets dispensed continues to rise [1], a better understanding of PZQ resistance is needed to understand the risk that it poses. Here we present data that outlines the production of a PZQ-selected line of *S. mansoni* with reduced susceptibility to the drug and chart the change in the transcriptome of the line over 3 passages in comparison to drug sensitive controls.

2. Materials and Methods

2.1. Ethics statement

All animal procedures in this study were reviewed and approved by the University of New Mexico Institutional Animal Care and Use Committee. This study followed the U.S. National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

2.2. Experimental procedures for selection of reduced PZQ sensitivity in S. mansoni

Passage 1: Thirty Swiss Webster (SW) 10–12 week old outbred male mice (Charles River, Kingston, NY) were each infected percutaneously with 100-150 *S. mansoni* Puerto Rican 1 (PR1) cercariae. Mice were randomly divided into 2 groups of 15 and treated by oral gavage at 35 and 37 days post infection (dpi) with either 2% Cremaphore EL (Sigma-Aldrich, St. Louis, MO) in aqueous suspension (control group) or 50 mg/kg PZQ (PZQ-selected group) in aqueous suspension with 2% Cremaphore EL. Mice were euthanized and perfused with RPMI 1640 media (RPMI; Sigma-Aldrich) at 49 dpi. Adult parasites were collected by perfusion, counted and sexed, then placed in RNeasy lysis buffer with 1% 2-mercaptoethanol (Sigma-Aldrich) and stored at -80 °C for RNA isolation. In addition, 10 parasites of each sex and treatment were chosen randomly and placed in RPMI for measurement of their length and breadth (see below).

After perfusion, mouse livers were harvested for egg collection. The eggs were pulse blended in artificial spring water (ASW) and placed under direct light for 1 h to hatch. Miracidia were collected, pooled and used to infect 24 laboratory reared *Biomphalaria glabrata* for each treatment group by exposing individual snails to 5 miracidia overnight. Snails were housed in 10-gallon tanks in ASW and after 8 weeks placed in individual wells under direct light to induce cercarial shedding. Cercariae from each treatment were pooled and used to infect mice as described above.

Passage 2–9: For each successive passage (P), groups of 10 SW mice were infected percutaneously with 100-150 *S. mansoni* PR1 cercariae per mouse derived from either the PZQ-selected or control line of the previous generation. Animals in the control group received 2% Cremaphore EL in aqueous suspension and those in the selected group received PZQ with the dose and time of treatment varying depending on the passage number.

Mice were perfused and euthanized at 49 dpi and the parasites and livers processed as described for P1. Miracidia derived from eggs obtained from livers of PZQ-selected or control mice were used to found the next generation of PZQ-selected and control schistosomes respectively. A 2-way ANOVA followed by a Sidak's multiple comparisons test was used to compare the number of recovered parasites from the control and PZQ-selected groups across each passage. All statistical analyses were conducted using GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

2.3. Dimensions of S. mansoni

Parasites (n = 10 parasites/sex/treatment/generation) were photographed 1 h after perfusion using a Zeiss SteREO Discovery V12 microscope and AxioCam HRc camera (Carl Zeiss Microscopy, Thornwood, NY). Parasite length and breadth were measured using AxioVision 4.8.2 software (Carl Zeiss Microscopy) with at least 3 and up to 5 measurements made per worm for length and breadth. The significance of observed differences between control and PZQ-selected parasites across sex and generation was assessed using 2-way ANOVA followed by Sidak's multiple comparisons test.

2.4. In vivo assay of S. mansoni PZQ resistance

From P5 onwards, to estimate the effectiveness of the PZQ dosing regime in producing drug resistant parasites, mice were infected with approximately 100-150 control or PZQ-selected cercariae (n = 5 per group) derived from the previous generation and subsequently received 3 doses of 300 mg/kg PZQ by oral gavage at 28, 35 and 37 dpi. The mice were

perfused and euthanized at 49 dpi and the number of parasites that survived counted and parasite burden reduction calculated using the following modified formula [11]

% parasite burden reduction =

$$\frac{\text{(Mean \# adult parasites in treatment group) – (Mean \# parasites in adult assay)}}{\text{Mean \# adult parasites in treatment group}} \times 100$$

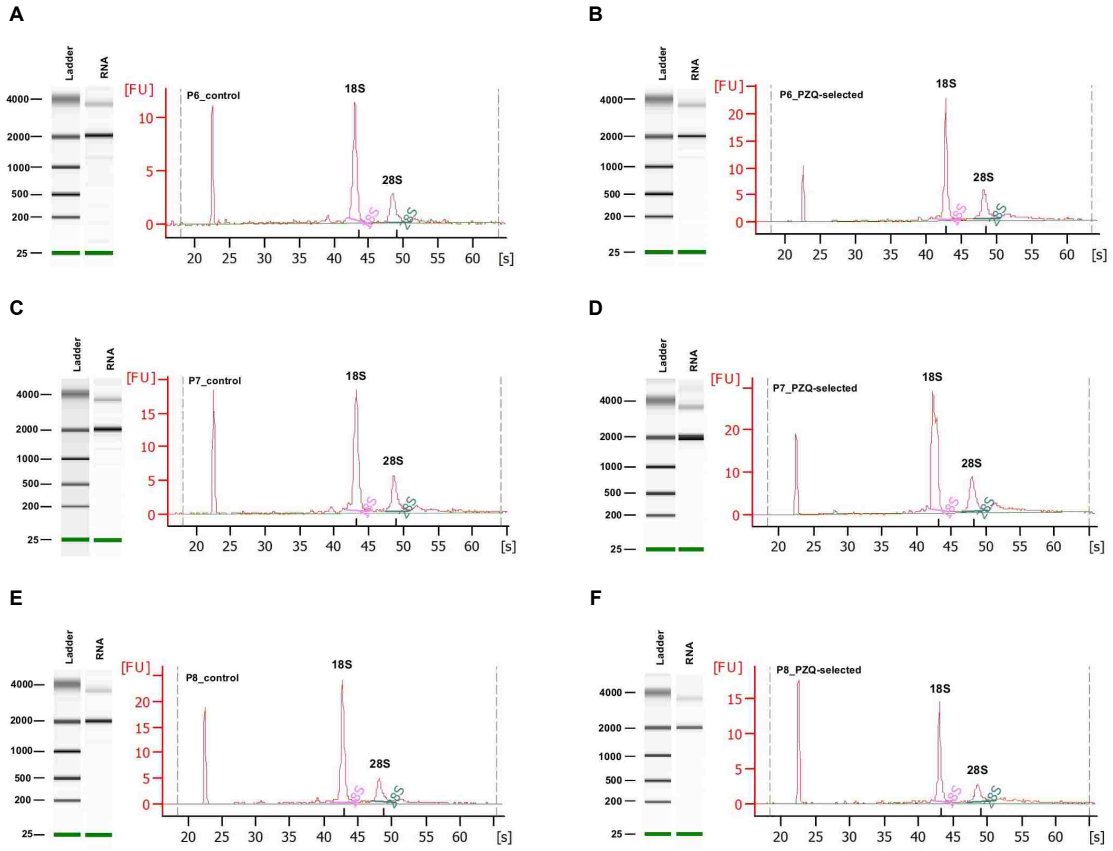
A 2-way ANOVA followed by Sidak's multiple comparisons test was used to compare the number of recovered parasites from the control and selected groups across generations.

2.5. PZQ susceptibility assay of S. mansoni miracidia

Miracidia were tested for PZQ sensitivity using a modified version of the protocol developed by Melman et al. [11]. Three to five PZQ-selected or control miracidia were placed in 40 μL ASW in up to 12 wells of a 96-well plate with a final concentration of 0 or 10^{-5} M PZQ. Dimethyl sulfoxide (DMSO) at 1% was used to prepare 10^{-4} M PZQ stock and all wells received a final concentration of 0.1% DMSO including those with no drug. A dissecting microscope was used to observe the miracidia prior to administration (t=0) and after 20 min incubation. Miracidia were counted blind as alive or dead i.e. remained immobile. The percentages of surviving miracidia were calculated as described by Mwangi et al. [14]. The surviving miracidia percentages were compared using a multiple t-test with the Holm-Sidak method.

2.6. RNA isolation and sequencing

Isolation of parasite total RNA was performed using an RNeasy Mini Kit (Qiagen, Redwood City, CA) according to the manufacturer's instructions and included incubation with RNase-free DNase (Qiagen) followed by RNA cleanup and concentration (Zymo Research, Irvine, CA).



S1 Fig. Total RNA qualitative and quantitative analysis. Bioanalyzer results showing total RNA gel-image (left) with ladder for reference and electrogram (right) from representative samples (A) P6 control, (B) P6 PZQ-selected, (C) P7 control, (D) P7 PZQ-selected, (E) P8 control, and (F) P8 PZQ-selected. FU = fluorescent units, and s = retention time in seconds.

Total RNA was quantified and quality assessed using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA) and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) respectively. Bioanalyzer traces from each generation and treatment showed no significant

RNA degradation (Supplementary Figure 1; S1 Fig.). RNA was stored at -80 °C until required.

RNA sequencing (RNA-Seq) was performed on 4 biological replicates of control and PZQ-selected schistosomes from P6, 7, and 8 using an Illumina NextSeq 500 instrument (Illumina, San Diego, CA). Libraries and indexing were prepared using the KAPA mRNA HyperPrep kit (KAPA Biosystems, Wilmington, MA) following the manufactures instructions. A Qubit Fluorometer and KAPA Library Quantification Kit for Illumina Platform were employed to quantify the libraries. The quality and fragment size for each library was examined with the Agilent 2100 Bioanalyzer. Samples passing quality control were pooled and sequenced to generate 150 base paired-end reads at the Molecular Biology Core Facility, University of New Mexico, NM.

2.7. RNA-Seq data processing

Raw sequence reads were trimmed and filtered using Trimmomatic v0.36 [23] with a slide window of 4 nucleotides, average score above 20 and minimum length of 36 nucleotides. Reads passing filters were mapped to the *S. mansoni* transcriptome annotated from assembly GCA_000237925.2 ASM23792v2 (NCBI) using Bowtie2 v2.2.9 [24]. Gene expression levels were estimated using RSEM v1.2.31 [25] and differential gene expression analysis performed using DESeq2 v 1.18.1 [26] EdgeR v 3.20.8 [27], and EBSeq v1.18.0 [28]. Results were organized using SARTools [29] with cutoffs for all programs set at an adjusted $p \leq 0.05$ with $\log_2$ fold change of either <-1 (down-regulated genes) or >1 (up-regulated genes). Differentially expressed gene (DEG) lists for the average (4 replicates per treatment/generation) of each treatment resulting from each program were displayed by Venn

diagram and only genes common to at least two bioinformatic approaches were retained for further analysis. All sequencing data sets generated for this study were deposited with Gene Expression Omnibus and can be accessed through accession number GSE120682.

2.8. Functional annotation

DEG lists and annotation file *S. mansoni* ASM23792v2 were imported into Blast2GO v5.0.13 [30] to classify genes with ontology terms in order to identify functionality. We performed Fisher's Exact Test (FET) with multiple corrections to identify Gene Ontology (GO) terms significantly ($p < 0.05$) over-represented in the DEG list compared to the whole gene set [31].

2.9. Quantitative real-time PCR

RNA samples for quantitative real-time PCR (qRT-PCR) originated from the corresponding samples used for RNA-Seq in order to validate Illumina data set expression patterns. Genes were chosen based on qRT-PCR primers passing efficiency tests and their differential expression patterns at P6-8. qRT-PCR was performed on 1 μg total RNA reverse transcribed to cDNA (20 μL reactions) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. All qRT-PCR reactions were performed in biological triplicates with technical duplicates using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories). Each 20 μL reaction contained of 100 ng cDNA and 0.5 μM primer. Reactions were performed using a C1000 96 Touch Thermo Cycler (Bio-Rad Laboratories) using *S. mansoni* gene qRT-PCR

primers (S1 Table) designed with the Integrated DNA Technologies OligoAnalyzer tool (www.idtdna.com).

S1 Table. Primers used in *S. mansoni* quantitative real-time PCR reactions

Smp_056970	SmGAPDH	GTCATTCCAGCACTAAACGG	CCTTCCCTAACCTACATGTCAG
Smp_144000	Cytochrome oxidase C subunit	GCCATCCTCAACCATTTGCC	ACCCGTTTCCAATGACTCGC
Smp_119130	Cercarial elastase	TTCTCACACTGAGGAACGGC	ATCCACTGAGTGTCTGTGCG
Smp_062250	Myosin 2 light chain	TTTATTTCCCCCGGACAGGC	AGCCGCTTATCGTCTTACCG
Smp_180180	SIL 1	ATCGCCATCTGGTTTCGAGC	AGGATTCGAACCCGTAAGCC
Smp_197070	Kinesin protein K1F6	GCTGAGGGATAGTTTAGGCGG	ATTAAGGCAACACGCTGTGC
Smp_191310	Histone deacetylase 4	CATGCAGAACCAGGTCAAGC	TGATCGAACAGGGTGGTTGC

Cycling conditions for *S. mansoni* gene expression were as follows; 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min and 60 °C for 20 s. CFX Manager™ Software v3.1 (Bio-Rad Laboratories) was used to calculate fold change ($2^{-\Delta\Delta Ct}$ method) relative to the reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Expression of this gene did not vary significantly between treatment groups (S2 Table).

S2 Table. Real-time PCR cycle threshold (Cq) raw data for *S. mansoni* reference gene GAPDH

Well	Fluor	Target	Content	Sample	Biological Set Name	Cq	Cq Mean	Cq Std. Dev
A01	SYBR	GAPDH	Unkn-01	P6_S1	PZQ-selected	20.79	20.79	0.007
A02	SYBR	GAPDH	Unkn-01	P6_S1	PZQ-selected	20.78	20.79	0.007

A03	SYBR	GAPDH	Unkn-02	P6_S2	PZQ-selected	19.98	19.99	0.008
A04	SYBR	GAPDH	Unkn-02	P6_S2	PZQ-selected	19.99	19.99	0.008
A05	SYBR	GAPDH	Unkn-03	P6_S3	PZQ-selected	20.30	20.32	0.033
A06	SYBR	GAPDH	Unkn-03	P6_S3	PZQ-selected	20.35	20.32	0.033
A07	SYBR	GAPDH	Unkn-04	P6_C1	control	20.14	20.10	0.057
A08	SYBR	GAPDH	Unkn-04	P6_C1	control	20.06	20.10	0.057
A09	SYBR	GAPDH	Unkn-05	P6_C2	control	19.49	19.50	0.014
A10	SYBR	GAPDH	Unkn-05	P6_C2	control	19.51	19.50	0.014
A11	SYBR	GAPDH	Unkn-06	P6_C3	control	18.93	18.92	0.013
A12	SYBR	GAPDH	Unkn-06	P6_C3	control	18.91	18.92	0.013
B01	SYBR	GAPDH	Unkn-07	P7_S1	PZQ-selected	19.84	19.83	0.015
B02	SYBR	GAPDH	Unkn-07	P7_S1	PZQ-selected	19.81	19.83	0.015
B03	SYBR	GAPDH	Unkn-08	P7_S2	PZQ-selected	18.52	18.54	0.036
B04	SYBR	GAPDH	Unkn-08	P7_S2	PZQ-selected	18.57	18.54	0.036
B05	SYBR	GAPDH	Unkn-09	P7_S3	PZQ-selected	19.18	19.14	0.055
B06	SYBR	GAPDH	Unkn-09	P7_S3	PZQ-selected	19.11	19.14	0.055
B07	SYBR	GAPDH	Unkn-10	P7_C1	control	20.40	20.39	0.014
B08	SYBR	GAPDH	Unkn-10	P7_C1	control	20.38	20.39	0.014
B09	SYBR	GAPDH	Unkn-11	P7_C2	control	20.15	20.16	0.009
B10	SYBR	GAPDH	Unkn-11	P7_C2	control	20.16	20.16	0.009
B11	SYBR	GAPDH	Unkn-12	P7_C3	control	19.44	19.43	0.002
B12	SYBR	GAPDH	Unkn-12	P7_C3	control	19.43	19.43	0.002
C01	SYBR	GAPDH	Unkn-13	P8_S1	PZQ-selected	20.70	20.65	0.074
C02	SYBR	GAPDH	Unkn-13	P8_S1	PZQ-selected	20.60	20.65	0.074
C03	SYBR	GAPDH	Unkn-14	P8_S2	PZQ-selected	20.27	20.28	0.016
C04	SYBR	GAPDH	Unkn-14	P8_S2	PZQ-selected	20.30	20.28	0.016
C05	SYBR	GAPDH	Unkn-15	P8_S3	PZQ-selected	20.65	20.62	0.041
C06	SYBR	GAPDH	Unkn-15	P8_S3	PZQ=selected	20.59	20.62	0.041
C07	SYBR	GAPDH	Unkn-16	P8_C1	control	19.14	19.14	0.001
C08	SYBR	GAPDH	Unkn-16	P8_C1	control	19.14	19.14	0.001
C09	SYBR	GAPDH	Unkn-17	P8_C2	control	19.11	19.10	0.020
C10	SYBR	GAPDH	Unkn-17	P8_C2	control	19.09	19.10	0.020
C11	SYBR	GAPDH	Unkn-18	P8_C3	control	19.20	19.18	0.026
C12	SYBR	GAPDH	Unkn-18	P8_C3	control	19.16	19.18	0.026

Statistical analysis was performed using a Student's t-test for normalized gene expression between treatment groups, and Pearson's correlation was used to examine the correlation between qRT-PCR and RNA-Seq data.

3. Results and Discussion

3.1. Selection of praziquantel resistance in S. mansoni

The initial goal of this study was to select for a PZQ resistant line of *S. mansoni* using a modification of the protocol employed by Fallon and Doenhoff [12]. In the original protocol, isolates of *S. mansoni* from Puerto Rico, Brazil, Kenya and Egypt were used to provide sufficient genetic variation to facilitate selection of resistance after 6 passages with increasing drug pressure. Using a similar protocol, we were able to induce resistance in *S. mansoni* derived from 7 Kenyan field isolates within 5 generations [14]. In contrast, at least 2 other studies have generated PZQ resistant schistosomes by selecting on a single (LE) strain [13,16]. Due to the success of these latter experiments in selecting for resistance on (probable) limited genetic variation, we chose *S. mansoni* PR1 as the sole source of genetic material for our experiments. Survival of at least 90% of schistosomes after exposure to 3 x 300 mg/kg PZQ on 28, 35 and 37 dpi was chosen as the indicator of acquisition of PZQ resistance as we found that this regime would reduce PR1 burden from 69.3 $\pm$ 2.5 schistosomes in control mice to 1.3 $\pm$ 2.3 in PZQ treated mice 10 days after the final treatment ($P < 0.0001$). This was also the drug regimen chosen in 2 previous studies to assess drug resistance [12,13]. The number and gender of parasites recovered across 9 passages from control and PZQ-selected treatment lines are shown in Table 1.

Table 1. Worms recovered from *S. mansoni* infected mice after treatment with PZQ over 9 passages

Passage	Treatment (mg/kg PZQ)	Mean ± SD Total No. of worms	Male:Female
1	-	36 ± 36	1.11
	2 x 50[a]	42 ± 22	1.16
2	-	43 ± 17	1.52
	2 x 100[a]	31 ± 19	1.21
3	-	23 ± 4	1.55
	2 x 200[a]	40 ± 17	2.00
4	-	47 ± 8	2.23
	2 x 250[a]	40 ± 17	0.74
5	-	51 ± 11	1.68
	3 x 300[b]	42 ± 21	0.62
6	-	22 ± 9	1.20
	3 x 300[b]	36 ± 15	0.90
7	-	35 ± 17	1.92
	3 x 300[b]	26 ± 5	0.59
8	-	44 ± 13	1.32
	3 x 300[b]	27 ± 10	0.80
9	-	20 ± 4	1.86
	3 x 300[b]	17 ± 6	0.31

[a]PZQ in 2% Cremaphore EL or 2% Cremaphore EL alone were administered at 35 and 37 dpi by oral gavage.
[b]PZQ in 2% Cremaphore EL or 2% Cremaphore EL alone were administered at 28, 35 and 37 dpi by oral gavage.

During P1-4, administration of 50-250 mg/kg PZQ administered on 35 and 37 dpi did not result in a significant reduction of parasite burden compared with the control group. Through the first 3 passages there was a male bias in both treatment groups which is in accord with the sex distribution associated with bisexual infection [32]. By P4, however, the PZQ-selected group showed a female bias for the first time. Beginning P5, the dose of PZQ was increased to 3 x 300 mg/kg on days 28, 35 and 37 dpi. Egg laying begins at around 28 dpi and this

earlier start to treatment ensured that few eggs were deposited prior to the application of drug pressure. Again, there was no significant difference in the number of worms recovered from PZQ treated and control mice at 49 dpi and, as with P4, drug pressure during P5-9 resulted in a female bias that was not observed with the control treatment. For example, after P9 there were 13 $\pm$ 3 male and 7 $\pm$ 2 female worms isolated form the control line but 4 $\pm$ 2 male and 13 $\pm$ 4 females from the PZQ-selected line. Two earlier studies also observed a decrease in male/female ratio following PZQ treatment [22,33]. One explanation could be that female parasites are less susceptible to PZQ *in vivo* due simply to reduced exposure to the drug as they sit in the male gynecophoric canal. Alternatively, reduced PZQ sensitivity may be an inherent trait and Pica-Mattoccia and Cioli [4] observed that the *in vitro* ED_{50} of immature females derived from bisexual infections is three times that of male parasites while Shaw and Erasmus [34] reported that the tegument of male worms is more susceptible to PZQ than that of females. The observation of reduced female sensitivity is not universal. Coeli et al. [13] found more males in the 11^{th} passage of LE strain worms selected for PZQ resistance suggesting sex selection through drug pressure may be a more complex phenomenon than can be explained simply by the sensitivity or degree of exposure of each sex to the drug.

To confirm that the PZQ-selected line had reduced sensitivity to the drug, mice infected with either the PZQ-selected or control lines were treated with 3 x 300 mg/kg PZQ on days 28, 35 and 37 dpi, euthanized at 49 dpi and the number of surviving worms calculated (Table 2).

Table 2. Percentage reduction in parasite burden of control and selected parasites in passages 5-9 after treatment with PZQ.

Passage (P)_Line	Treatment PZQ (mg/kg)	Mean ± SD Parasite number	% Reduction	Statistical Significance (p-value)
P5_control	-	51.0 ± 11	46	0.0006[b]
	3 x 300[a]	27.8 ± 15		
P5_PZQ-selected	-	41.7 ± 20.5	10	NS[c]
	3 x 300	37.4 ± 11.6		
P6_control	-	21.9 ± 8.9	83	0.0063
	3 x 300	3.8 ± 3.0		
P6_PZQ-selected	-	35.6 ± 15.3	0	NS
	3 x 300	36.5 ± 10.5		
P7_control	-	34.9 ± 17	44	0.0201
	3 x 300	19.6 ± 5.2		
P7_PZQ-selected	-	26.2 ± 4.8	0	NS
	3 x 300	31.8 ± 3.7		
P8_control	-	43.6 ± 13.0	67	<0.0001
	3 x 300	14.2 ± 4.1		
P8_PZQ-selected	-	27.4 ± 9.9	20	NS
	3 x 300	21.8 ± 2.1		
P9_control	-	19.9 ± 4.3	37	NS
	3 x 300	12.6 ± 4.7		
P9_PZQ-selected	-	16.9 ± 5.8	4	NS
	3 x 300	16.2 ± 5.6		

[a]PZQ was administered on 28, 35 and 37 dpi. Animals were euthanized and worm burden counted at 49 dpi.
[b]Statistical significance of parasite burden in control and PZQ-selected lines was determined using the Holm-Sidak method with alpha = 0.05.
[c]NS = not significant.

While there was a 46 ($P < 0.001$) and 83% ($P < 0.01$) reduction in worm burden of control line mice, there was only a non-significant 10 and 0% reduction in the PZQ-selected line at P5 and 6 respectively. Similar significant reductions were seen in the control line at P7 and 8 but not in the PZQ line. Although there was a 37% reduction in worm burden in the control line at P9, this failed to reach statistical significance and there was only a 4% reduction in worm burden in the PZQ line at this point. These results confirm that after the

5th passage the PZQ-selected line had become significantly less susceptible to the drug and this status was maintained until P9.

Unfortunately, we were unable to maintain the PZQ-selected line after P9 as the few eggs that were extracted from the liver of infected mice did not hatch. This is similar to the experience of Coeli et al. [13] who lost their line after 11 passages. Loss may be due to a trade-off between PZQ 'resistance' and fitness cost [9] and/or a skewed sex ratio leading to a loss of fecundity [13,22] as observed in this study.

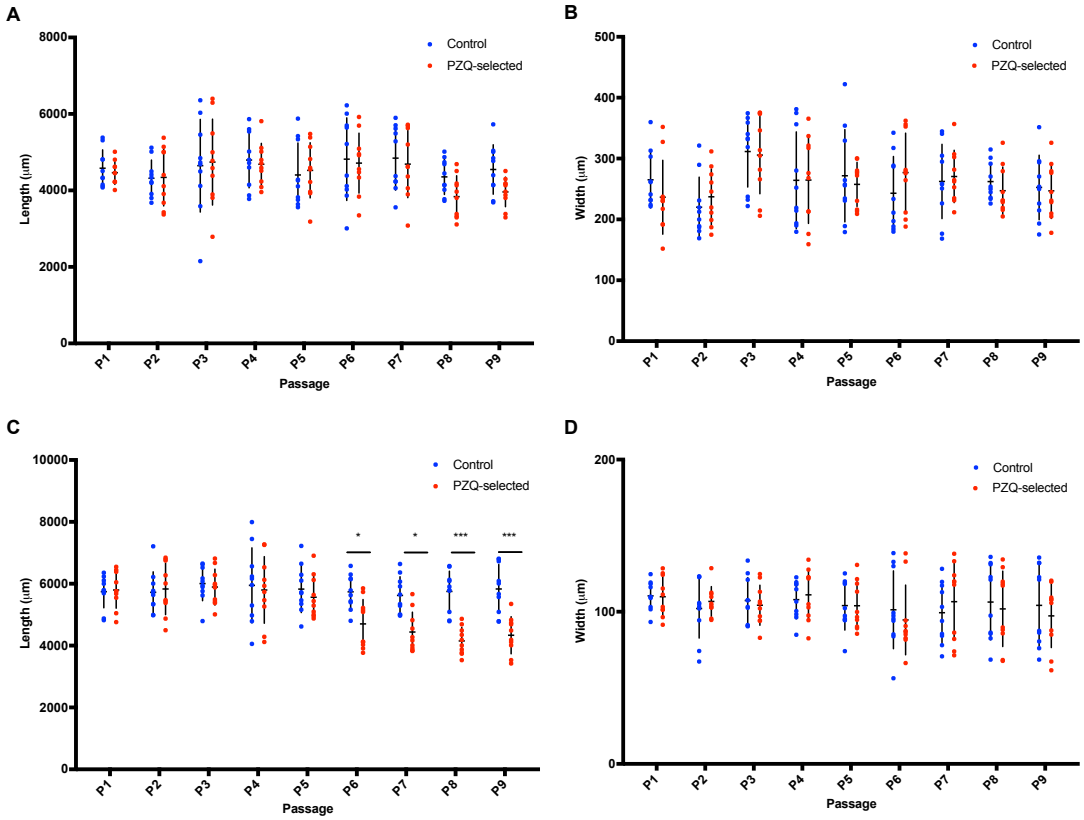


Fig. 1. Changes in *S. mansoni* length and width during PZQ-selection. The length and width of male and female schistosomes isolated during passages (P) 1-9 are shown. (A) Length of male parasites. (B) Width of male parasites. (C) Length of female parasites. (D) Width of female parasites. Significance of observed differences between control and PZQ-selected schistosomes were assessed with 2-way ANOVA with Sidak's multiple comparisons test. Error bars represent mean with standard deviation, n = 10 sex/treatment/passage. * = $p < 0.05$, and *** = $p < 0.001$.

In addition to the shift towards females from P4 onwards, we also observed that while the length (Fig. 1A) and width (Fig. 1B) of PZQ-selected and control male worms did not differ over the 9 passages, females became significantly shorter in the PZQ-selected cohort compared to controls from P6 onwards (Fig. 1C). The greatest difference was seen at P8 where the female schistosomes in the control line had a mean length of 5751.8 $\pm$ 654.2 μm compared to 4150.4 $\pm$ 440.3 μm ($p < 0.001$) in the PZQ-selected line. At P9, the mean length of females was 5832.9 $\pm$ 790.7 μm, while those from the PZQ-selected line were 4336.2 $\pm$ 599.9 μm ($p < 0.001$). No differences in the width of female worms were observed between the two groups (Fig. 1D). While these data are suggestive of a fitness cost we cannot rule out that the stunted growth was due to the female schistosomes apparent need for the presence of males to reach maturity [35].

In vitro treatment of miracidia hatched from control and PZQ-selected eggs were assessed for their ability to survive 20 min exposure to 10^{-5} M PZQ as an indication of PZQ sensitivity. Remarkably, our results suggest the PZQ-selected miracidia were less susceptible to the drug after the first exposure of the adults to 2 x 50 mg/kg on 35 and 37 dpi and across most subsequent generations tested (P3, 4, 6, 7 and 8) with the exception of P5 (Fig. 2 A-G). The rapid acquisition of reduced miracidial PZQ sensitivity was surprising to us and contrary to the observation of Mwangi et al. [14] who only observed a reduction in sensitivity after exposure of mouse infections to 2 x 250 mg/kg during P4 and 3 x 300 mg/kg during P5.

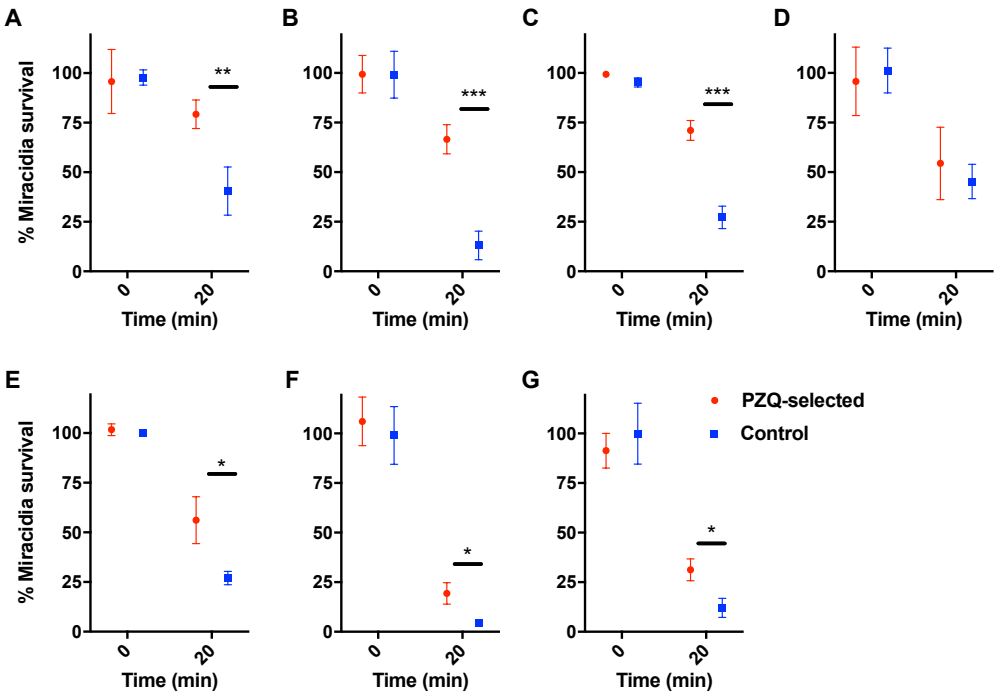


Fig. 2. *In vitro* assay of *S. mansoni* miracidia survival after exposure to PZQ. Miracidia were hatched from control and PZQ-selected groups and exposed to 10^{-5} M PZQ for 20 min and the number surviving counted. (A) P1, (B) P3, (C) P4, (D) P5, (E) P6, (F) P7, and (G) P8. Passage 2 and P9 data are not included due to low miracidia yield. Statistical significance determined between control and PZQ-selected parasites using the Holm-Sidak multiple comparisons method with alpha = 0.05. Error bars represent mean with standard deviation. * = $p< 0.05$, ** = $p< 0.01$, and *** = $p< 0.001$.

Overall, our results suggest that sexually mature *S. mansoni* PR1 had acquired significantly reduced sensitivity to PZQ by the completion of P5 and this was maintained through P9. Thus, transcriptomic analysis of adult schistosomes from control and PZQ-selected lines was undertaken to identify genes associated with reduced sensitivity.

3.2. Transcriptome sequencing and analysis

To compare changes in the *S. mansoni* transcriptome, cDNA libraries were prepared from pooled, mixed sex schistosomes derived from 10 mice infected with PZQ-selected or control lines at P6, 7 and 8. On removing adapter sequences and any ambiguous, low quality reads

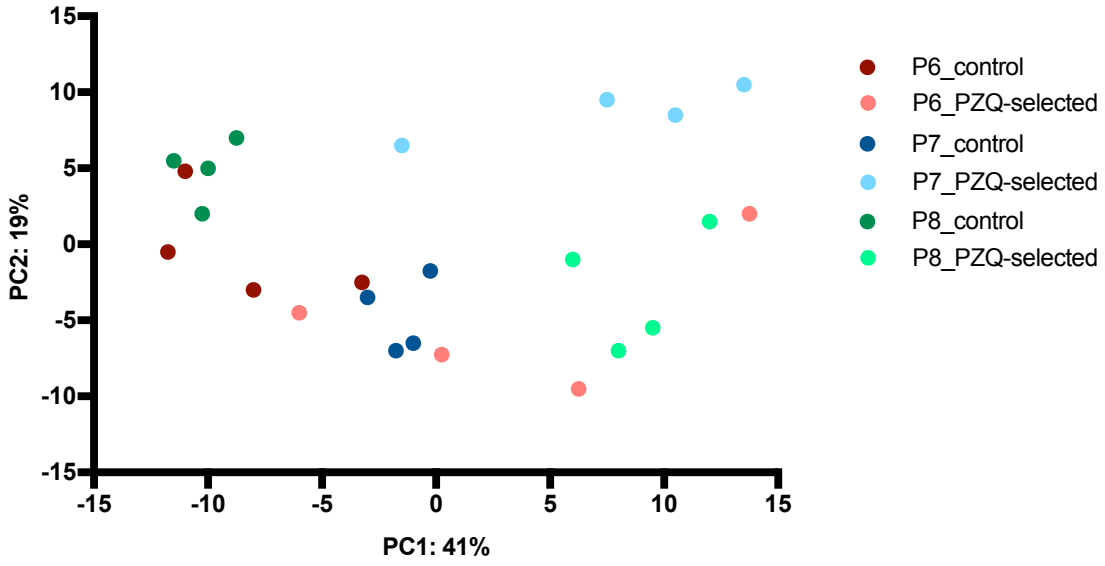
(Q < 20), a total of 315 million 150 base paired end reads were obtained. In each of the six groups the mean number of reads was between 11.5 (P8, PZQ-selected) and 17.8 million (P6, control), and a mean of 50.0% (P8, PZQ-selected) and 62.5% (P6 & P7, control) paired reads mapped to the *S. mansoni* genome (S3 Table). Each group contained 4 biological replicates/generation and the number of reads provided enough coverage for differential expression analysis [36].

S3 Table. Summary of Illumina read counts and mapped reads for each sequenced sample.

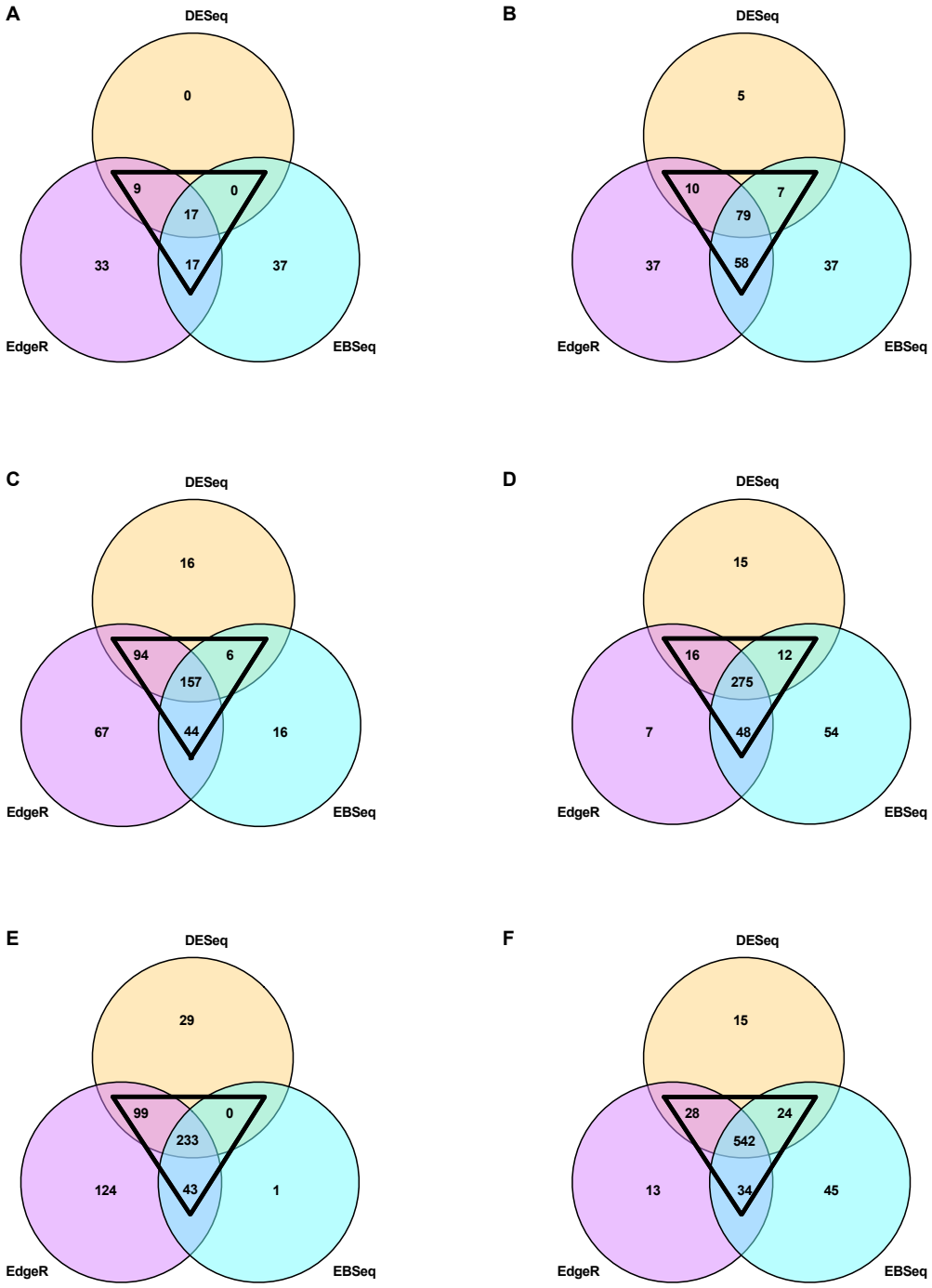
Sample	Total quality-trimmed paired reads (Q>20)	Percentage of *S. mansoni* mapped reads
P6_control_1	12339139	58.19
P6_control_2	15513283	60.32
P6_control_3	21353821	64.83
P6_control_4	21804032	66.45
P6_PZQ-selected_1	17696542	54.65
P6_PZQ-selected_2	17776418	53.11
P6_PZQ-selected_3	16234697	49.94
P6_PZQ-selected_4	18644407	56.74
P7_control_1	11574274	56.91
P7_control_2	12202288	60.08
P7_control_3	28374988	71.75
P7_control_4	15019608	61.18
P7_PZQ-selected_1	16431003	58.61
P7_PZQ-selected_2	11591609	59.04
P7_PZQ-selected_3	17144399	59.61
P7_PZQ-selected_4	15186746	58.48
P8_control_1	14402932	65.05
P8_control_2	13869791	62.85
P8_control_3	15254212	59.26
P8_control_4	16732065	62.06
P8_PZQ-selected_1	12841503	48.60
P8_PZQ-selected_2	11488047	49.26
P8_PZQ-selected_3	11443465	50.54

Sample	Total quality-trimmed paired reads (Q>20)	Percentage of *S. mansoni* mapped reads
P8_PZQ-selected_4	10289245	51.22

Principle Component Analysis (PCA) resulted in transcriptomes of the PZQ-selected and control groups segregating broadly into two distinct regions with individual transcriptomes generally clustering with others from their treatment/control group. P6 PZQ-selected samples were the most diverse with two of the 4 samples clustering more with the control selected samples than PZQ-selected (S2 Fig). All samples were retained for further analyses to maintain an *n* of 4 across all groups.



S2 Fig. Principal component analysis. Two-dimensional principal component (PC) analysis was used to characterize the scattering or clustering of replicates in each treatment groups.



S3 Fig. Venn diagrams displaying selection of differentially expressed genes in this study. Three programs (DESeq, EBSeq, and EdgeR) were employed to identify differentially expressed genes in PZQ-selected schistosomes compared to controls. Only differentially expressed genes (DEG) that were seen in at least two of these programs were analyzed further. Separated DEGs in this study are shown in each triangle, (A) P6 up-regulated, (B) P6 down-regulated, (C) P7 up-regulated, (D) P7 down-regulated, (E) P8 up-regulated, and (F) P8 down-regulated.

Three programs (DESeq, EBSeq, and EdgeR) were used to identify differential transcript expression and PZQ-selected samples were normalized to control samples for each generation. The number of DEGs observed using each program is shown in S3 Fig and transcripts that were identified by at least 2 of the programs were retained for further analyses. Both the number of up- and down-regulated DEGs increased with each successive generation (S4 Table) with a total of 197, 652 and 1003 DEG identified at P6, 7 and 8 respectively.

S4 Table. Number of all differentially expressed genes for passages (P) 6, 7, and 8.

Passage	No. of up-regulated genes	No. of down-regulated genes
P6	43	154
P7	301	351
P8	375	628

The number of DEGs present across all 3 generations was small in comparison to the total numbers with only 7 up-regulated and 13 down-regulated genes shared across P6-8 (Fig. 3).

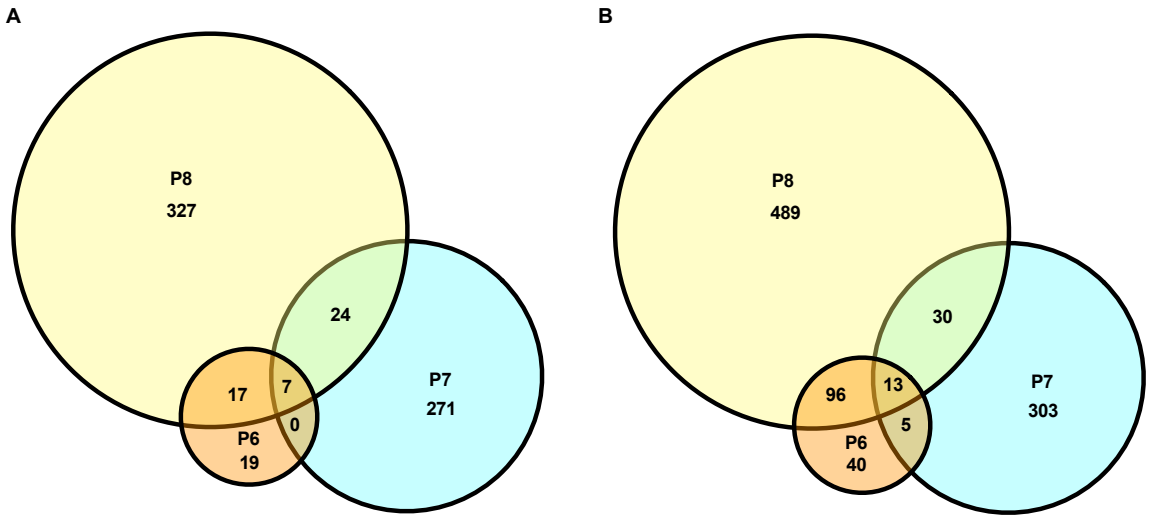
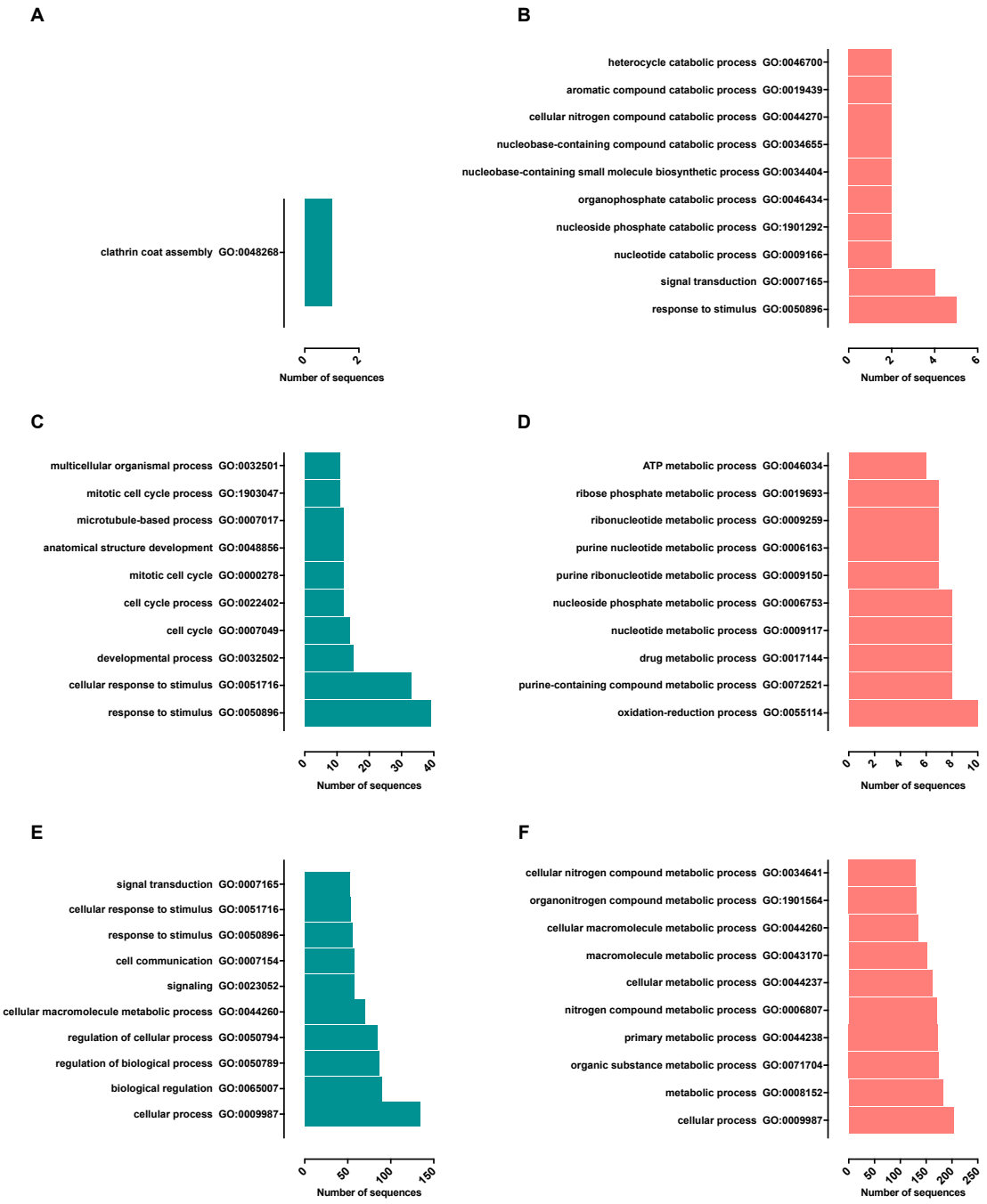


Fig. 3. Venn diagrams of differentially expressed genes. Indicated in the Venn diagrams are the numbers of (A) up-regulated and (B) down-regulated differentially expressed genes shared between or exclusive to *S. mansoni* derived from P6, P7 and P8. The diameter of each circle is proportional to the number of transcripts it represents.

Gene Ontology (GO) analysis was employed to provide a statistical assessment of over-represented GO Biological Process terms and *S. mansoni* reference sequences. The over-represented up-regulated sequences in PZQ-selected schistosomes were weighted towards cell cycling processes at P7 but had no other obvious discernable pattern (S4A, C and E Fig). The over-represented down-regulated sequences were overwhelmingly involved in metabolic processes across P6-8: (S4B, D and F Fig) with 8 transcripts designated as 'drug metabolic process' being amongst most overrepresented sequence at P7. On further examination 4 of these were hypothetical proteins while the remaining transcripts encoded putative pyruvate kinase (Smp_065610), adenosine kinase (Smp_008370), phosphatidylcholine-sterol acyltransferase (Smp_082120) and a kunitz-type protease inhibitor (Smp_180810). Overall, however, it was difficult to discern a significant pattern in the GO data related to selection for PZQ resistance.



S4 Fig. Functional classification of differentially regulated transcripts. Blast2GO was employed to categorize up- and down-regulated differentially expressed transcripts into Biological Process (BP) according to Gene Ontology (GO) terms. Enrichment analysis was performed with Fisher's Exact Test ($p<0.05$) to identify over-representation of sequences compared to reference annotation and results of the top 10 are shown in (A) P6 up- and (B) down-regulated transcripts, (C) P7 up- and (D) down-regulated transcripts, and (E) P8 up- and (F) down-regulated transcripts. Some sequences are present in multiple categories.

3.3. Expression analysis of transcripts related to PZQ-selection

Fig. 4. Expression of selected *S. mansoni* genes during passages (P) 6, 7 and 8. Heat map of the 50 most differentially expressed genes (25 up- and 25 down-regulated) in PZQ-selected parasites relative to controls. Genes not differentially expressed are colored gray and increased and decreased gene expression are teal and pink respectively. The color scale indicates $\log_2$ fold change for the average of four biological replicates.

A heat map was constructed to examine those genes that showed the greatest levels of differential regulation in the PZQ-selected versus control lines at P6-8 (Fig. 4). The top 25 up– and 25 down-regulated genes were selected based on their expression at any of these 3 passages. Twenty genes encoded 'hypothetical' or 'unnamed' proteins underlining the relative paucity of original annotation of Schistosoma *spp.* genes as well as the evolutionary distance between Schistosoma *spp.* and the classical model organisms. Only 4 genes showed expression in *S. mansoni* at more than one passage and only two (both hypothetical) showed expression across all three passages. Among the highly differentially regulated genes with functional annotation there were some interesting observations. Two of the putative gene products were similar to transcriptional regulator Homeobox proteins engrailed-like SMOX-2 (Smp_145200) and SMOX-4 (Smp_029620), two other products matched the putative DNA-binding Early Growth Response Protein (EGRP; Smp_094930 and Smp_134870) and two encoded homologs of RNA binding proteins (Smp_055740 and Smp_137080). While SMOX-2 and both RNA binding proteins were up-regulated in P7, both EGRP genes were down-regulated at this time and SMOX-4 was down-regulated at P8. These changes in expression of transcriptional and post-transcriptional regulators presumably reflect the increased transcriptional dynamics associated with the PZQ-selected line and are indirect consequences of drug selection. Other genes of note included the reproduction associated genes encoding female-specific protein 800 (Smp_000290) which is mooted to play a role in vitellogenesis [37], eggshell protein (Smp_191910) and trematode eggshell synthesis domain containing protein (Smp_112450), each of which were down- regulated only at P8, perhaps presaging the loss of the PZQ-selected line due to the lack of viable egg production.

Although the mechanism of action of PZQ and the molecular basis of juvenile resistance is not yet understood, previous studies have identified gene products that may play a role in both [18-20,38,39]. We have examined the differential regulation of a number of these genes previously and identified a number of stress related genes that are differentially regulated in response to PZQ exposure *in vitro* [19] and *in vivo* [20]. One of these, ferritin (Smp_047660), was highly up regulated in juvenile and sexually mature male *S. mansoni* after exposure to 4 x 250 mg/kg/day over 4 consecutive days but was among highly down-regulated genes at P7 (Fig. 4). In addition to ferritin, one other transcript encoding a previously identified PZQ induced stress protein transcript, thioredoxin peroxidase (TPX-1; Smp_059480), was also down- regulated at P7 ($\log_2$ change = -1.16, $p<0.05$). No other stress transcripts that have been identified previously as being differentially regulated in response to PZQ treatment were significantly altered though a number of other heat shock proteins that can also be responsive to stress (Smp_106930, Smp_072330 and Smp_049600) were significantly up-regulated at P7 or P8.

ABC superfamily members, especially those of the ABCB, ABCC and ABCG families have been shown repeatedly to excrete structurally unrelated drugs, potentially conferring multiple drug resistance on cells in which they are expressed [40]. At least 21 ABC transporter genes have been identified in *S. mansoni* [41] and we found four of these to be differentially regulated including 3 members of the ABCB and ABCC subfamilies. Pinto-Almeida et al. [15] examined a stable resistance phenotype to PZQ and found the relative expression of the ABCB1 homolog SmMDR2 (Smp_055780) increased in PZQ resistant strain males compared to susceptible strain while resistant strain female SmMDR2 levels were actually lower than the susceptible strains. We identified this ABCB1 homolog

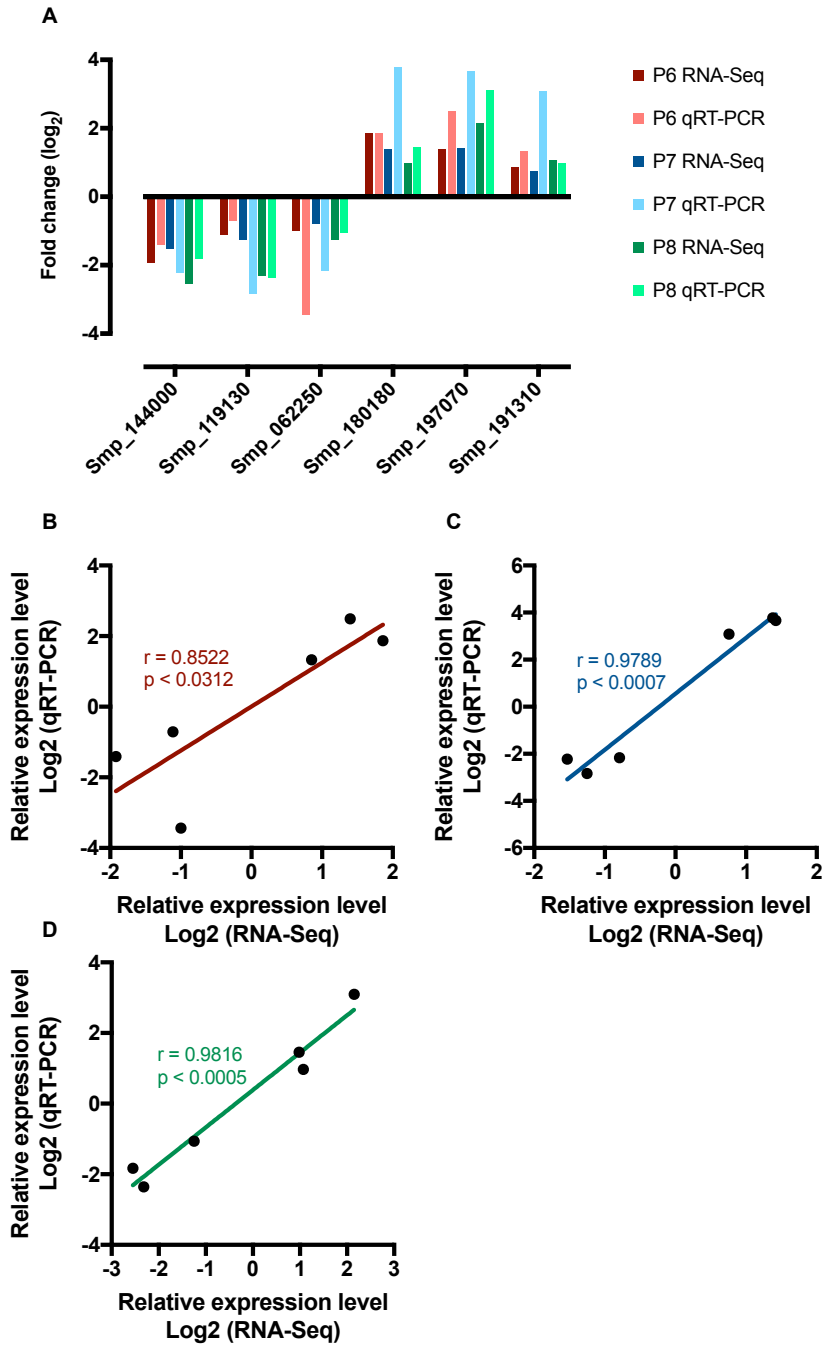
previously to be highly up-regulated in drug resistant mixed sex juveniles but not adult males [20]. In this study, Smp_055780 was not differentially regulated but this may be explained by the significantly higher number of females than males contributing to our data set. In addition to identifying an increase in the expression of Smp_055780, Sanchez et al. [20] also identified significant increases of a second ABCB1 homolog (Smp_089200) in mixed sex juveniles but not a third (Smp_170820). Here, a fourth ABCB1 homolog, Smp_137080, was up-regulated at P7 (1.94, $p<0.05$). *S. mansoni* subfamily homologs ABCC4 (Smp_167610) and ABCC10 (Smp_147250) were also differentially regulated at P7, however, while ABCC10 expression was decreased at P7 (-1.45, $p<0.5$), ABCC4 was increased (1.04, $p<0.05$). Therefore, while drug resistance in juvenile worms may be associated with increased expression of ABCB, ABCC and ABCG family members [20] there is only limited evidence from this data set that reduced susceptibility in P6-8 schistosomes is associated with the overexpression of ABC subfamily members. Each of these differences between previous and current data sets may, however, be due to the sampling of the transcriptome in this study having taken place 12 days after the last PZQ treatment rather that in the period immediately after the last administration of the drug allowing the protective stress and ABC transporter response induced acutely on drug exposure to dissipate as the PZQ is metabolized. If true, this would also infer that reduced susceptibility may be the result of changes in facultative rather than constitutive expression states.

Pinto-Almeida et al. [39] revealed proteins associated with *S. mansoni* PZQ resistance through comparative proteomics. PZQ resistance was generated in a fully susceptible isogenic *S. mansoni* strain treated with 0.3 μM PZQ and compared to a fully susceptible strain using high throughput LC-MS/MS identification of protein spots after 2-dimensional

gel electrophoresis. Of the eight proteins Pinto-Almeida and colleagues identified in the mixed sex PZQ-resistant strain, none were identified in our data set though one of the three proteins associated with PZQ-resistance exclusive to females, a receptor for activated Protein Kinase C was down-regulated in P8 (Smp_102040; -1.10, $p<0.05$).

3.4. RNA-Seq validation

Quantitative real-time PCR (qRT-PCR) was employed to validate results obtained by RNA-Seq analysis. Six transcripts encoding homologs of cytochrome oxidase C (Smp_144000), cercarial elastase (Smp_119130), myosin 2 light chain (Smp_062250), SIL 1 (Smp_180180), kinesin K1F6 (Smp_197070) and histone deacetylase (Smp_191310) were validated based on their differential expression across P6, 7 and 8 (S5A Fig**).** Patterns observed in the qRT-PCR data mirrored those obtained by the RNA-Seq data and showed significant correlation at P6 ($r = 0.85$, $p <0.0312$**;** S5B Fig), P7 ($r = 0.98$, $p <0.0007$; S5C Fig) and P8 ($r = 0.98$, $p <0.0005$; S5C Fig).



S5 Fig. Comparison and correlations between RNA-Seq and qRT-PCR analysis of six differentially expressed genes. (A) $\log_2$ fold changes of expression in 6 selected *S. mansoni* transcripts from PZQ-selected parasites relative to controls for each of six genes assessed by RNA-Seq and qRT-PCR at P6-8. qRT-PCR fold changes were normalized to GAPDH. Gene expression profiles at each point are the average of three biological replicates. Correlations between RNA-Seq and qRT-PCR transcription for the six genes in (B) P6, (C) P7, and (D) P8 were determined using Pearson's correlation.

4. Conclusions

The underlying basis for the reduction in sensitivity to PZQ seen in this study could have a number of explanations. For example, there may be one or more mutations in a single gene, such as that encoding the PZQ binding target, that causes a loss of function without a change in expression. Alternatively, a mutation or epigenetic change in a single gene may lead to a change in its expression, which can in turn alter the toxicity and/or half-life of a drug. While the transcriptomic analysis carried out here did provide insight into genes that are differentially regulated between PZQ-selected and sensitive lines it did not immediately suggest any single candidate gene or pathway as being responsible for reduced susceptibility. For example, while there were some differences in expression of ABC transporters that might enhance drug efflux, there was not enough consistency in expression for any single ABC gene to suggest that this superfamily alone played a significant role. William et al. [9] concluded the factors leading to selection and maintenance for PZQ resistance varied among isolates and a third drug resistance mechanism may be, as Roquis et al., [42] suggested, 'due to the epistatic interaction of multiple gene products rather than an (epi)mutation of a single locus'. For example, *S. mansoni* resistance to the anthelmintic drugs hycanthone and oxamniquine emerged readily in natural populations [43]. A single autosomal recessive locus was found to encode both oxamniquine and hycanthone resistance in two strains [44] and subsequently, non-synonymous coding mutations associated with a sulfotransferase gene were identified, thus establishing the mechanism of resistance for both of these compounds [45]. Subsequently, however, Roquis et al. [42] found laboratory generated hycanthone-resistant parasites that do not carry these mutation but instead carry a high number of epigenetic changes compared to controls. Changes in chromatin structure between resistant

and sensitive worms were identified and 5 genes involved in stress responses and one ABC transporter were among 69 genes associated with these modifications. Thus, while we also see changes to stress, ABC transporter, and Receptor for activated PKC gene expression, like Roquis et al. [42] and Pinto-Almeida et al. [39] we also see no obvious single gene or pathway behind the observed PZQ resistance in *S. mansoni* PR1 and therefore do not rule out the possibility of such an 'epistatic interaction of multiple gene products'. Identifying the true genetic actors in such interactions may need either more powerful transcriptomic or proteomic analyses or meta-analyses as well as a systems approach to understanding of the role of epigenetics in the generation of resistance. In addition, the mechanism by which resistance is generated may differ depending on whether relatively genetically homogeneous laboratory strains are targeted for selection as opposed to heterogeneous field populations in which the classic monogenic pattern of resistance can more easily arise.

Conflict of interest

The authors declare that there is no conflict of interest.

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CONCLUSIONS

Schistosomiasis significantly impacts the lives of more than 206 million individuals with more than half comprised of children (WHO, 2017). As we come upon more then fifteen years of mass drug administration (MDA) program implementation, the number of people treated for this diseases has increased greatly with 62 million individuals treated in 2014 compared to 89 million in 2016 (WHO, 2017), resulting in significant gains being achieved (Hotez and Aksoy, 2017). With the drug praziquantel (PZQ) being at the heart of control and currently no alternative options available, a better understanding of PZQ is needed in order to sustain progress and continue control and eventually eliminate the disease. The goal of this research was to apply transcriptomics to provide a better understanding of the effect PZQ on schistosomes.

The first goal for this dissertation was to provide a comprehensive study of how PZQ affects the host-parasite interaction. Therefore we analyzed livers from *S. mansoni* infected mice that were treated with PZQ or drug vehicle in order to provide transcriptomic profiles in the presence or absence of granulomas. The results revealed the presence of immune and fibrotic responses in both PZQ and vehicle treated mice, but severe pathology was present in the mice where PZQ was absent. Additionally the classic T helper 1 (Th1) response followed by a T helper 2 (Th2) upon increase in egg burden was seen in vehicle, even though the PZQ treated mice had significant reduction in the egg burden the immune response was similar, perhaps explained by antigens released from dying parasites.

Additionally we examined *S. mansoni's* response in the host-parasite interaction using quantitative real-time PCR to investigate the ATP-binding Cassette (ABC) transporter genes in both juvenile and adult parasites treated with PZQ *in vivo*. Results revealed that

members of the ABC-B, -C, and -G family transporter genes were transcriptionally enriched in juvenile parasites compared to adult. Prior research (Kasinathan et al., 2010; Kasinathan et al., 2014) has suggested that ABC transporters may play a role in the resistance of juveniles to PZQ and our study provided support for this hypothesis.

The second objective was the investigation of adult male *S. mansoni* exposed to the individual R- and S-PZQ enantiomers. Using transcriptomic analysis we found 101 differentially regulated transcripts in R-PZQ treated parasites compared to only 22 in S-PZQ treated parasites compared to controls. The enantiomers only shared a total of 11 differentially expressed transcripts, up-regulated = 4 and down-regulated = 7. Furthermore S-PZQ treated parasites only had one differentially expressed transcript (Smp_125510, putative cadherin) compared to 63 in R-PZQ treated parasites, with 26 labeled as hypothetical or uncharacterized proteins. We observed more transcripts (up- and down-regulated) in R-PZQ compared to S-PZQ, perhaps exposing the dissimilarity in the anthelmintic activity between the enantiomers. This analysis is still at an initial stage, however, and we may have to revise and ease the constraints for calling genes differentially regulated in order to gain further detail in the response profiles.

The last objective was to investigate the molecular basis for reduced PZQ sensitivity in *S. mansoni*. Selection pressure through increasing doses of PZQ was continuously applied to *S. mansoni* throughout nine passages, and resulted in a reduced susceptibility of miracidia after one passage and a significantly reduced susceptibility of sexually mature *S. mansoni* during passages five though eight. There was a marked shift in sex ratio starting at passage five and continuing through passage nine resulting in more females compared to males in the PZQ-selected line. Additionally the parasite's size was recorded throughout the study and

females had a significant reduction in length compared to control parasites. We lost the PZQ insensitive isolates after passage 9, an observation seen by Coeli et al (Coeli et al., 2013) after passage 11. Loss could also suggest parasites have reduced fitness associated with PZQ resistance (William et al., 2001), or the skewed sex ratio leads to a loss of fecundity (Coeli et al., 2013; Lamberton et al., 2017). Transcript analysis revealed reduced PZQ sensitivity was not associated with any specific gene or pathway profiles, perhaps suggesting that reduced PZQ sensitivity is a combination of epigenetic modifications in multiple gene products and a single loss of function mutation to a key gene in the drugs mechanistic pathway.

The research in this dissertation provides extensive, novel transcript profiles of *S. mansoni* and its murine host in the presence and absence of PZQ. Ultimately the three large data sets produced will provide a resource for future analyses and meta-analyses of the host-pathogen-drug interaction.

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Praziquantel sensitivity of Kenyan *Schistosoma mansoni* isolates and the generation of a laboratory strain with reduced susceptibility to the drug

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ABSTRACT

Schistosomiasis is a neglected tropical disease caused by blood-dwelling flukes of the genus *Schistosoma*. While the disease may affect as many as 249 million people, treatment largely relies on a single drug, praziquantel. The near exclusive use of this drug for such a prevalent disease has led to concerns regarding the potential for drug resistance to arise and the effect this would have on affected populations. In this study, we use an *in vitro* assay of drug sensitivity to test the effect of praziquantel on miracidia hatched from eggs obtained from fecal samples of Kenyan adult car washers and sand harvesters as well as school children. Whereas in a previous study we found the car washers and sand harvesters to harbor *Schistosoma mansoni* with reduced praziquantel sensitivity, we found no evidence for the presence of such strains in any of the groups tested here. Using miracidia derived from seven car washers to infect snails, we used the shed cercariae to establish a strain of *S. mansoni* with significantly reduced praziquantel sensitivity in mice. This was achieved within 5 generations by administering increasing doses of praziquantel to the infected mice until the parasites could withstand a normally lethal dose. This result indicates that while the threat of praziquantel resistance may have diminished in the Kenyan populations tested here, there is a strong likelihood it could return if sufficient praziquantel pressure is applied.

1. Introduction

Schistosomiasis is a water-borne parasitic disease that affects more than 249 million people (World Health Organization, 2014) with a global disease burden calculated at 24–56 million disability-adjusted life-years lost (King, 2010). Of the limited number of drugs available to treat schistosomiasis, praziquantel (PZQ) is the least expensive and easiest to use (Hagan et al., 2004) and, since PZQ is highly effective against all schistosome species that infect humans, its use in mass treatment campaigns has grown significantly. In 2006, approximately 12 million people were treated with PZQ and by 2012 this number reached approximately 42 million (World Health Organization, 2014). While the drug is highly effective against sexually mature forms of the parasite it is often unable to cure infections due to its inability to kill juvenile schistosomes at 2–4 weeks post-infection (Pica-Mattoccia and Cioli, 2004; Aragon et al., 2009). As PZQ is often administered with a significant time lapse measured in months or years between treatments this can leave a significant reservoir of schistosomes infecting people that are unaffected by the drug. This, combined with continuing exposure to the parasite, means the drug can often only provide short-term relief from infection. Despite this drawback, PZQ remains the only readily available treatment for schistosomiasis amid concern that as it becomes more widely dispensed, drug resistance traits may emerge thus removing the most effective, albeit flawed drug from the limited treatment options available.

There have been a number of *in vivo* and *in vitro* studies documenting differential sensitivity of *Schistosoma mansoni* isolates to PZQ. For example, a relatively low cure rate was reported during a study of PZQ efficacy and side effects in Senegal in 1991 (Stelma et al., 1995). A subsequent study of a field isolate derived from snails in the same geographical area suggested that, when compared with two isolates from Puerto Rico and Kenya, the Senegal isolate matured in mice at a significantly slower rate thus likely rendering it less susceptible to the drug at the times tested (Fallon et al., 1997). Ismail et al. (1999) generated 12 *S. mansoni*

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isolates from patients who had failed to be cured by 3 doses of PZQ that would normally prove effective. These isolates were maintained in mice and 8 were found to have a significantly higher ED_{50} than controls as well as a significantly diminished contractile response *in vitro*. In a related study, 3 of 6 isolates retained their decreased response to PZQ after several passages through the life cycle in the absence of PZQ (William et al., 2001). In addition, these isolates had an associated diminished reproductive fitness suggesting reduced PZQ sensitivity may have come with a significant biological cost. In a study using miracidial assays to determine PZQ sensitivity, Lamberton et al. (2010) noted that *S. mansoni* hatched from eggs obtained from the feces of children after two or more PZQ treatments were more likely to survive *in vitro* exposure to the drug compared with those from newly infected children. Although this may point to a variation in susceptibility of adult *S. mansoni* worm survival to repeated drug treatment, it may also reflect differential susceptibility to PZQ of eggs at different stages of maturity as they pass through the host. While treatment failure has been reported in travellers returning from areas endemic for *Schistosoma haematobium* (Mendonca da Silva et al., 2005; Alonso et al., 2006), a study of individuals infected with *S. haematobium* on Pemba Island, Tanzania found no indication of PZQ resistance as determined by egg counts and miracidial viability after 20 years of mass drug administration (Guidi et al., 2010).

Fallon and Doenhoff (1994) were able to induce PZQ resistance in the laboratory by exposing a pool of *S. mansoni* isolates from Puerto Rico, Brazil, Kenya and Egypt that had been maintained in the laboratory for up to 10 years to increasing sub-curative doses of PZQ. Mice infected with the 6th generation of selected cercariae were then treated with 3 × 300 mg/kg doses at days 28, 35 and 37 after infection, and showed only a 7% reduction in worm burden compared with an 88% reduction in mice infected with non-selected worms. Coeli et al. (2013) infected mice with *S. mansoni* (LE strain) and treated with PZQ following a protocol based on Fallon and Doenhoff (1994) to generate worms with similarly reduced drug sensitivity. This led to a decrease in genetic heterogeneity suggesting that multi-generational PZQ exposure resulted in reduced population diversity. When schistosomes of the drug-selected strain were bred with those of an unselected strain, the F1 offspring were found to have intermediate PZQ sensitivity suggesting the reduced susceptibility trait was co-dominant (Pica-Mattoccia et al., 2009). Couto et al. (2011) were able to generate *S. mansoni* with reduced PZQ susceptibility within a single life cycle by feeding PZQ to snails harboring the parasite.

Clearly, while studies of PZQ resistance have to take into account confounding factors such as the rate of schistosome maturity as well as individual variations in drug metabolism and immune competency, the development of reduced susceptibility to PZQ is a real possibility. Here, we examine the *in vitro* susceptibility of miracidia hatched from eggs derived from fecal samples of patients living in two regions of Kenya endemic for schistosomiasis. In addition, we used some of these miracidia to establish an *S. mansoni* laboratory isolate with significantly reduced susceptibility to PZQ.

2. Materials and methods

2.1. S. mansoni egg collection from fecal samples of infected individuals to obtain miracidia

Isolates of *S. mansoni* were recovered from eggs in fecal samples of adults working as car washers or sand harvesters in Kisumu, western Kenya. Both groups spend significant periods of time in the water of Lake Victoria or the surrounding streams as part of their occupation. All adults enrolled for this study came from one of these two groups and all had previously been treated with PZQ with the year of last treatment being between 2006 and 2013.

From 2004 to 2007, collaboration between the Kenya Medical Research Institute (KEMRI) and Japan International Cooperation Agency administered a school-based schistosomiasis and soil-transmitted helminth control project in Mwea, central Kenya. The project dispensed annual doses of dewormers including PZQ to all school-aged children in the region regardless of their infection status. Thereafter, the National Deworming Program took over the control activities, and has continued with annual treatment of children since 2012 to date. In this study, enrolled students were between 5 and 16 years of age and were from three primary schools: Thiba, MbuiNjeru and Mukou. Children were sampled in June 2013 or February 2014 with the date of last treatment within 1 year of sample collection.

Fecal samples were tested for *S. mansoni* eggs using the modified Kato Katz technique. Miracidia obtained from eggs in positive fecal samples from individual patients were used to estimate their *in vitro* sensitivity to PZQ and to establish a line of *S. mansoni* with reduced sensitivity to PZQ.

The KEMRI Scientific and Ethical Committees and the Institutional Review Board of the University of New Mexico approved this study. All adults and the parents or guardians of the children involved provided informed consent.

2.2. PZQ susceptibility assay of miracidia derived from fecal samples

Miracidial sensitivity to PZQ was tested *in vitro* using a modified version of the technique developed by Liang et al. (2001). Freshly hatched miracidia derived from stools of sand harvesters ($n = 24$), car washers ($n = 14$) or school children ($n = 34$) were placed in each well (4–6 miracidia per well) of a 96-well microtitre plate in 40 µl aged tap water. Each row represented a single group of miracidia and received either 0, 10^{-6} or 10^{-5} M PZQ. PZQ was prepared as a stock solution of 10^{-4} M in 1% DMSO and the final concentration of DMSO was 0.1% in all wells including the control. The mean number of groups of miracidia used per patient per concentration of PZQ was 19.2 (range: 6–42). This was dependent on the number of miracidia obtained from a fecal sample. Miracidia were observed with a dissecting microscope prior to (0 min) and 20 min after the addition of PZQ. An independent observer, who had no knowledge of the miracidial source or PZQ concentration used, recorded the number of dead miracidia. Miracidia were assumed dead if they remained immobile. The percentage of miracidia mortality after treatment for 20 min with 10^{-6} and 10^{-5} M PZQ was calculated as follows:

$$\frac{(\text{Mean No. live miracidia from control group}) - (\text{Mean No. live miracidia from treated group}) \times 100}{\text{Mean No. live miracidia from control group}}$$

2.3. Snail sampling and parasite propagation in mice

In addition to testing PZQ sensitivity of *S. mansoni* miracidia from parasite eggs of naturally infected individuals, miracidia derived from cercariae shed from naturally infected, field-collected *Biomphalaria snails* were also tested after passage through laboratory mice. *Biomphalaria* spp. were collected from sites where

schistosomiasis is known to be endemic including Lake Victoria shores at Kisumu and Asao stream, both in western Kenya as well as Kibwezi stream in southern Kenya and the Mukou and Nice Rice irrigation ditches at Mwea, in central Kenya. Snails were washed and separated into 24-well plates with aged tap water for up to 3 days with intermittent inspection for the appearance of *S. mansoni* cercariae using a dissecting microscope. Shedding snails were pooled and approximately 100 cercariae were used to infect each of 2–3 outbred mice. At 49 days post-infection, *S. mansoni* eggs were obtained from the mouse livers and hatched in aged tap water. PZQ sensitivity of hatched miracidia was assessed using the assay outlined above.

All animal experimentation complied with the policies, regulations and guidelines mandated by the Institutional Animal Care and Use Committees of the University of New Mexico and KEMRI.

2.4. Generation of an *S. mansoni* laboratory strain with reduced PZQ susceptibility

Miracidia were hatched from eggs retrieved from fecal samples obtained from 7 car washers and combined. This pool was used to infect *Biomphalaria sudanica* and the cercariae shed subsequently used to infect outbred mice. An isolate with reduced sensitivity to PZQ was established after 5 generations with exposure to increasing doses of PZQ essentially as described by Fallon and Doenhoff (1994) with the following differences. Briefly, 10 outbred mice were infected with approximately 100 cercariae and randomly distributed into two groups and treated with either 100 mg/kg/day PZQ in 2.5% Cremophor EL (Sigma, USA) ($n = 5$) or an equivalent volume of 2.5% Cremophor EL vehicle alone ($n = 5$) on each of days 28 and 35 post infection. 3 weeks after the final dose of PZQ or vehicle, mice were perfused with RPMI medium and the number of worms counted. Statistical analysis of differences in worm yield from PZQ treated and vehicle treated mice was calculated using an unpaired Student's *t*-test assuming equal variance.

For each passage, eggs from the liver that survived PZQ treatment (selected) or vehicle treatment (non-selected) were used to infect *B. sudanica* and the cercariae subsequently used to infect the next generation of mice. This protocol was repeated for the second passage with the dose of PZQ being increased to 200 and 250 mg/kg/day for the third and fourth passages. Mice in the 5th passage received 300 mg/kg/day on days 28, 35 and 37 post-infection. In addition, mice infected with non-selected *S. mansoni* received 2 × 200 and 2 × 250 mg/kg PZQ on days 28 and 35 post-infection (passages 3 and 4) and 3 × 300 mg/kg PZQ on days 28, 35 and 37 post-infection (passage 5) to establish lethality of the drug at these concentrations for the isolates being selected upon.

PZQ sensitivity of hatched miracidia obtained after each passage was assessed using the protocol described above. Approximately 6 drug-selected miracidia were placed in each of 48 wells of a 96 well microtitre plate. Twenty four wells were treated with 10^{-5} M PZQ and an additional 24 with the same volume of PZQ vehicle and observed after 0 (pretreatment control), 10 and 20 min by an independent observer. Non-selected miracidia were treated identically. The percentage of drug selected or non-selected miracidia surviving at each time point after treatment was calculated as follows:

$$\frac{\text{Mean No. live miracidia from PZQ treated group}}{\text{Mean No. live miracidia from vehicle treated group}} \times 100$$

Statistical analysis of miracidial survival was performed using an unpaired Student's *t*-test assuming unequal variance.

3. Results and discussion

In 2009, we (Melman et al., 2009) published a study measuring PZQ sensitivity of *S. mansoni* miracidia hatched from eggs derived from feces of adult Kenyan car washers and sand harvesters and discovered there was a 2.42-fold increase in the chance that miracidia would survive PZQ exposure if they were from individuals previously treated with PZQ compared to untreated. Miracidia derived from patients who had had between 4 and 20 PZQ treatments showed mortality that ranged from 30 to over 80% when exposed to 10^{-5} M PZQ *in vitro*, while the untreated cohort showed mortality ranging from 60% to 100%. For this study we returned to these occupational groups as well as a cohort of Kenyan school children undergoing PZQ therapy to determine if there is significant variability in PZQ sensitivity of the *S. mansoni* population infecting these individuals. In addition, we examined variation in PZQ sensitivity of *S. mansoni* miracidia obtained from mice infected with cercariae derived from naturally infected snail populations.

Fecal samples were obtained from a total of 72 individuals with a history of treatment with PZQ. For the purpose of a direct control, it was not possible to identify individuals within our patient groups who had not received PZQ previously. Irrespective of the patient group the mean miracidial mortality of *S. mansoni* at 10^{-5} M PZQ was between 82.1% and 84.6% with the lowest observed value of miracidia from a single patient being 72.7% (Table 1). As these data are comparable with the sensitivity of miracidia derived from eggs of the untreated cohort in our 2009 study, it suggests there is no evidence of diminished PZQ sensitivity in *S. mansoni* infecting these populations. For the car washers and sand harvesters, the last treatment dates were between 1 and 9 years before the current study and thus, it is perhaps not surprising that, without sustained PZQ treatment, a population of *S. mansoni* with reduced responsiveness to PZQ has failed to materialize. In addition, there was no indication of reduced miracidial susceptibility to PZQ among samples obtained from the 34 school children that previously had between one and five PZQ treatments

Table 1
In vitro susceptibility to PZQ of *S. mansoni* miracidia derived from eggs in patient fecal samples.

Patient group and location	No. of patients sampled	Mean No. of PZQ treatments per patient (range)	Mean% miracidial mortality (range)	
			10^{-6} M PZQ	10^{-5} M PZQ
Car washers Kisumu	14	8.4 (3–20)	26.1 ± 5.5 (17.7–33.3)	83.2 ± 4.2 (74.4–88.0)
Sand harvesters Kisumu	24	4.7 (1–11)	21.4 ± 3.1 (17.2–25.3)	81.5 ± 4.8 (72.7–88.8)
School children Mwea–MbuiNjera	11	3.8 (1–5)	27.4 ± 5.0 (19.4–32.1)	84.6 ± 4.8 (76.3–91.6)
School children Mwea–Mukou	18	3.3 (1–5)	27.0 ± 5.4 (19.4–39.8)	83.8 ± 4.5 (76.5–90.7)
School children Mwea–Thiba	5	3.8 (2–5)	25.4 ± 4.7 (21.0–32.5)	82.1 ± 3.7 (78.0 ± 82.6)

Data shown as mean ± 1 standard deviation.

Table 2
Worms recovered from infected mice after treatment with PZQ during 5 passages of *S. mansoni*.

Passage No.	PZQ treatment mg/kg	No. of worms Mean ± SD	Male:female ratio
1	2 × Vehicle	48.0 ± 5.9*	1.8
	2 × 100	37.2 ± 5.2	3.0
2	2 × Vehicle	43.8 ± 7.7	2.6
	2 × 100	41.0 ± 7.6	3.1
3	2 × Vehicle	43.2 ± 4.6	1.5
	2 × 200	36.4 ± 8.6	1.4
4	2 × Vehicle	53.4 ± 4.2	1.5
	2 × 250	49.6 ± 4.6	1.5
5	2 × Vehicle	51.4 ± 1.7	1.3
	3 × 300	50.2 ± 7.8	1.6

SD: 1 standard deviation.
* $p < 0.05$.

(one treatment per year) as part of the Kenyan National Deworming Program. Similarly, when miracidia derived from mice infected with cercariae from naturally infected snails collected in Kisumu, Mwea and Kibwezi areas were tested, no evidence of reduced susceptibility to PZQ was found (mortality at 10^{-5} M PZQ = 82.3 ± 4.9%; range = 75.8–90.3%). Thus, using miracidia as an indicator of *S. mansoni* sensitivity to PZQ in the definitive host our data implies there is, as yet, no evidence to suggest that resistance or even reduced sensitivity is an immediate threat in the areas surveyed.

Administration of one round of PZQ treatment to school children in Tanzania resulted in a significant reduction in genetic diversity of *S. mansoni* populations within the children (Norton et al., 2010; French et al., 2013). While there are many reasons to account for such genetic 'bottlenecking' after PZQ treatment, one concern is it may lead to a greater likelihood of development of PZQ resistant parasite strains. Clearly, at least in the case of *S. mansoni* infecting the school children who took part in this study this has not happened. A recent analysis of genetic variability of *S. mansoni* in this cohort suggests there has been no reduction in schistosome burden and genetic diversity actually increased after 4 years of mass drug administration (data not shown). This latter observation would be more in agreement with Huyse et al. (2013) who reported that regular treatment with PZQ did not affect the genetic diversity of *S. mansoni* in Senegal.

In recent years, PZQ treatment of car washers and sand harvesters in Kisumu has become intermittent with the result that only a small proportion of the infected population is undergoing treatment at any one time. This would leave a significant reservoir of parasites unaffected by the drug and likely allow any *S. mansoni* strain with reduced susceptibility to be lost from patients, especially if, as has been reported, an ability to withstand PZQ treatment also carries a cost to reproductive fitness (William et al., 2001; Coeli et al., 2013). In order to determine if this population still harbored the potential to generate *S. mansoni* with reduced PZQ sensitivity, we used a mouse infection model to study the impact of increasing amounts of PZQ on a population of parasites derived from 7 car washers. During the first and second passages, mice were treated with 100 mg/kg PZQ on days 28 and 35 after infection. After the first passage there was a small but significant fall in the number of worms recovered after treatment with 100 mg/kg PZQ compared to vehicle treated controls and an increase in the male to female ratio from 1.8 to 3.0 (Table 2). During passage 5, 3 × 300 mg/kg PZQ was administered to mice on days 28, 35 and 37 after infection with no effect on worm numbers or sex ratio compared with vehicle treated controls. In contrast, treatment of mice infected with non-PZQ selected *S. mansoni* during passages 3, 4 and 5 with 2 × 200, 2 × 250 and 3 × 300 mg/kg PZQ respectively resulted in 36, 66 and 86% reductions in worm numbers compared to vehicle treated mice. This data suggests that we were able to generate a PZQ isolate with low susceptibility to a normally effective dose of PZQ and is in close accordance with that of Fallon and Doenhoff (1994) who used a number of laboratory strains of *S. mansoni* from geographically diverse regions as the source of their genetic material. Sexually mature female worms isolated from bisexual infections in mice have been shown to be less sensitive to PZQ *in vitro* than mature male worms
(Pica-Mattoccia et al., 2004) while Delgado et al. (1992) showed a preferential killing of female worms *in vivo*. Despite some initial selection for males in the first round of PZQ treatment in the experiment reported here we saw no subsequent evidence for the selection of either sex. Interestingly, in a similar experiment using *S. mansoni* LE strain, Coeli et al. (2013) were also able to generate an isolate that was able to withstand 3 × 300 mg/kg PZQ after 6 generations, but were unable to maintain the strain beyond the 11th generation under PZQ pressure due to a change in the male: female ratio from 2.5 in treated, non-selected worms to 8.7 in treated, PZQ selected worms suggesting that female worms of the LE strain are more susceptible to PZQ after repeated exposure and selection. We will continue to passage our selected *S. mansoni* strain to determine if heightened female sensitivity to PZQ reported by Coeli et al. (2013) is due to the use of a laboratory strain as the founder population or whether a more genetically diverse founder population leads to a more stable long-term sex ratio.

Miracidia hatched from livers of PZQ and vehicle treated mice during each passage were assayed for their ability to survive PZQ treatment *in vitro* (Fig. 1). Our data suggests that miracidia produced by drug-selected *S. mansoni* are also less susceptible to the drug, especially during the 4th and 5th passage suggesting that acquired resistance may be a heritable trait.

We have shown that a cohort of 72 adults and children living in endemic areas of western and central Kenya who have undergone recent or historical treatment with PZQ do not harbor *S. mansoni* with reduced PZQ susceptibility. Nonetheless, while schistosomes with a 'resistant' phenotype may not be problematic within these populations, we have also shown there is a significant potential for the emergence of such a phenotype should sufficient PZQ pressure be applied. It is fortuitous that with perhaps only approximately 15% of people with schistosomiasis being treated with PZQ together with the intermittent nature of much of that treatment, a large *refugium* for drug sensitive parasites will continue to exist. This, together with apparent fitness costs associated with PZQ resistance (William et al., 2001; Coeli et al., 2013), may well prevent the near-term establishment of drug resistant strains in the human population. The far-term prospects for keeping resistance at bay are more worrisome. In 2012, the WHO announced a 'roadmap' for the elimination of 17 neglected tropical diseases (NTD) (World Health Organization, 2012), one of which was schistosomiasis. It was proposed that the disease could be eliminated as a public health problem in multiple African countries by 2020 and globally by 2025. This in turn inspired a global alliance of 22 partners including the WHO, The Bill and Melinda Gates Foundation, World Bank and major pharmaceutical companies to announce through the 2012 'London Declaration' a sustained program to 'control' schistosomiasis by 2020 (http://unitingtocombatntds.org). While 42 million PZQ tablets were dispensed in 2012 (World Health Organization, 2014) this number is likely to increase greatly in the near future to meet the immediate goal of disease control. Merck KgaA will make 250 million PZQ tablets per year freely available in the medium-term and, with other manufacturers expected to contribute tablets to help bridge the expected shortfall in supply, there are significant grounds for concern that

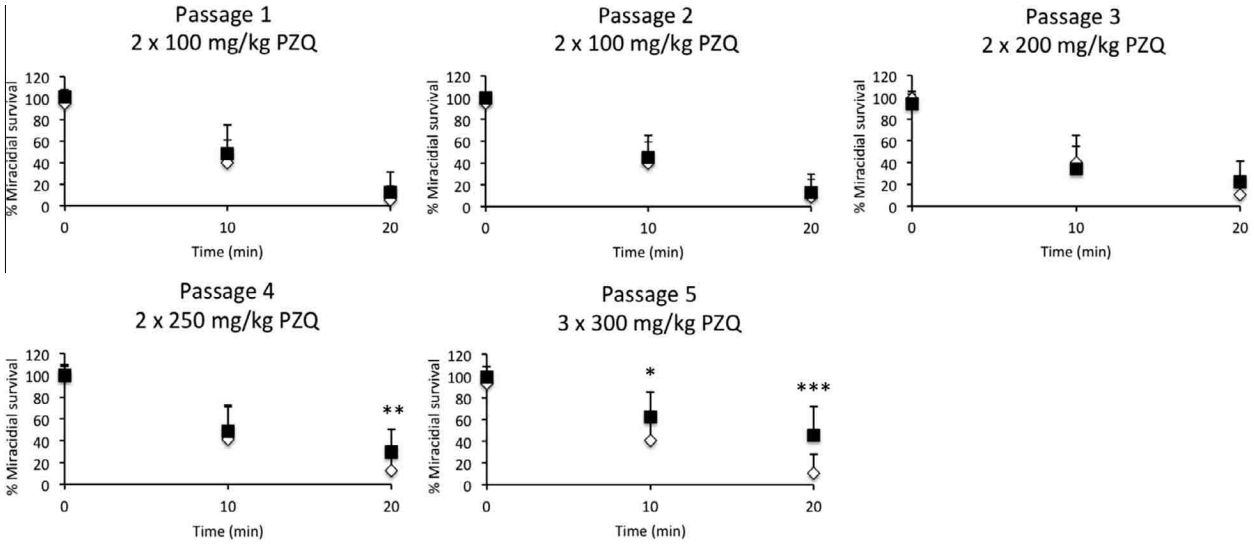

Fig. 1. *In vitro* efficacy of 10^{-5} M PZQ in killing PZQ selected and non-selected *S. mansoni* miracidia. Mice infected with *S. mansoni* were treated with the indicated doses of PZQ over 5 passages (selected) or PZQ vehicle alone (non-selected). The survival of the selected (■) and non-selected (◇) miracidia treated with 10^{-5} M PZQ was calculated as a percentage of vehicle treated controls at each time point. Data shown as mean + 1 SD ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

drug pressure will increase significantly in the years ahead. Clearly, close monitoring of drug efficacy should have an important role to play as control efforts are ramped up in the coming years.

Conflict of interest

The authors declared that there is no conflict of interest.

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