Marina de Moraes Mourão

Silenciamento gênico por interferência de RNA (RNAi) de transcritos de *Schistosoma mansoni*

Tese apresentada ao Curso de Pós-graduação em Bioquímica e Imunologia da Universidade Federal de Minas Gerais como requisito parcial para obtenção do título de Doutor em Ciências.
Área de concentração: Bioquímica.

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ATA DA DEFESA DA TESE DE DOUTORADO DE MARINA DE MORAES MOURÃO. **Aos seis dias do mês de agosto de 2009 às 10:00 horas, reuniu-se no Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, a Comissão Examinadora da tese de Doutorado, indicada *ad referendum* do Colegiado do Curso, para julgar, em exame final, o trabalho intitulado "Silenciamento Gênico por Interferência de RNA (RNAi) de Transcritos de *Schistosoma mansoni*", requisito final para a obtenção do grau de Doutor em Ciências: Biologia Molecular. Abrindo a sessão a Presidente da Comissão, Profa. Glória Regina Franco da Universidade Federal de Minas Gerais, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra a candidata para apresentação de seu trabalho. Seguiu-se a argüição pelos examinadores, com a respectiva defesa da candidata. Logo após a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição do resultado final. Foram atribuídas as seguintes indicações: Dra. Iscia Lopes Cendes da Universidade de Campinas, aprovada; Dr. Guilherme Corrêa Oliveira do Centro de Pesquisas René Rachou-Fiocruz, aprovada; Dr. Sérgio Costa Oliveira da Universidade Federal de Minas Gerais, aprovada; Dr. Marcos Horácio Pereira da Universidade Federal de Minas Gerais, aprovada; Dra. Glória Regina Franco, orientadora, da Universidade Federal de Minas Gerais, aprovada. Pelas indicações a candidata foi considerada APROVADA. O resultado final foi comunicado publicamente a candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente da Comissão encerrou a reunião e lavrou a presente Ata que será assinada por todos os membros participantes da Comissão Examinadora. Belo Horizonte, 6 de agosto de 2009.**

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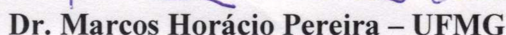

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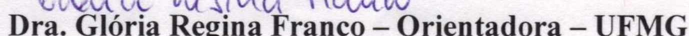

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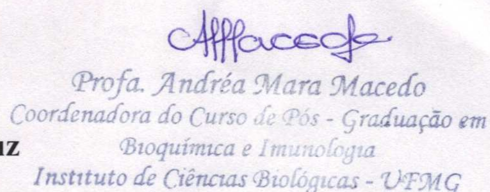

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Instituto de Ciências Biológicas - UFMG

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Lista de Abreviaturas

·OH- radical hidroxil

$^1O_2$- oxigênio singlete

Acc. No. – *accession number*

AGO2- Argonauta

ANOVA- Análise de Variância

BACs- *Bacterial Artificial Chromosomes*

BCIP- 5-bromo-4-cloro-3-indolil fosfato

DAG- Diacil glicerol

DHHC- Putativo Domínio Zinc Finger

dsRNA- RNA de dupla fita

EF1α- Fator de elongação 1 alfa

EST- Etiquetas de Seqüências Transcritas

GAPDH- Gliceraldeído-3- fosfato- desidrogenase

GFP- proteína fluorescente verde

GPx- Glutationa Peroxidase

GR- Glutationa Redutase

GST- Glutationa-S-Transferase

H_2O_2- Peróxido de hidrogênio

HEXBP- *Hexamer binding protein*

HOCl- ácido hipoclórico

IP3- 3 fosfato inositol

LD- Lactato desidrogenase

LDL - *Low density lipoprotein*

miRNA- micro RNA

mRNA- RNA mensageiro

NBT- nitro-azul tetrazolio

NRS- Espécies reativas de nitrogênio

PBS- Solução salina fosfatada

PCR- Reação em cadeia da polimerase

PFA- paraformaldeído

PKC- Proteína quinase C

PTGS- *Post Transcriptional Gene Silencing*

q-PCR- Reação em Cadeia da Polimerase quantitativa

RISC- *RNA-Induced Silencing Complex*

RNAi- técnica de interferência por RNA

Rod- rodamina

ROS- Espécies reativas de oxigênio

RT-PCR- Reação da Transcriptase Reversa

SAGE – Análise Serial de Expressão Gênica

shRNA- *short hairpin RNA*

siRNA- *short interfering RNA*

SmRbx- proteína Ring Box de *Schistosoma mansoni*

SmZF1- Proteína Zinc Finger de *Schistosoma mansoni*

SOD- Superoxido dismutase

SRB- *Scavenger Receptor Binding*

TGR- Tiorredoxina Glutationa Redutase

TPx- Tiorredoxina Peroxidase

TRx- Tiorredoxina Redutase

WGS- *Whole Genome Shot gun*

Resumo

Atualmente, interferência por RNA (RNAi) é a única metodologia de genética reversa disponível em *Schistosoma sp.*, pois as técnicas de nocaute e super-expressão gênica ainda não são possíveis de serem realizadas nesse parasita. Apesar do silenciamento gênico por RNAi estar sendo mais comumente utilizado, existe muito que se aprimorar para sua aplicação em larga escala em parasitas helmintos. No presente estudo, 33 genes foram selecionados para silenciamento, incluindo fatores de transcrição, moléculas de sinalização, enzimas metabólicas e anti-oxidantes. Estes alvos foram escolhidos, em sua maioria, devido a sua alta expressão na fase larval do parasita, e/ou pelo seu possível envolvimento no processo de desenvolvimento do organismo, como é o caso das enzimas anti-oxidantes, que parecem estar envolvidas na manutenção do equilíbrio redox celular em *Schistosoma mansoni,* contribuindo para a sobrevivência do parasita no hospedeiro intermediário *Biomphalaria glabrata*.

Neste estudo miracídios de *S. mansoni* foram transformados *in vitro* e expostos a dsRNAs dos genes alvos por sete dias, durante os quais, mudanças no fenótipo foram observadas, dentre estas: (1) falha ou atraso na transformação, (2) perda de mobilidade, (3) alteração de tamanho e (4) viabilidade. Dentre os fenótipos avaliados, apenas redução de tamanho dos indivíduos foi consistentemente detectado e observado em 11 de 34 tratamentos com dsRNA dos genes SOD, Smad1, RHO2, Smad2, Cav2A, ring box, GST26, calcineurina B, Smad4, lactato desidrogenase e EF1α. Após sete dias de incubação com dsRNA, apenas 6 tratamentos demonstraram consistente e significante diminuição nos níveis de transcritos medidos por qRT-PCR. Inesperadamente, a expressão de um dos genes cujos parasitas exibiram um fenótipo tratamento-associado, o gene SOD, foi altamente induzida (~1600 vezes). Variações no nível dos transcritos em conseqüência do tratamento com dsRNA, também foi evidente em grupos de esporocistos sem fenótipo aparente. Níveis de transcritos de 14 dos 23 grupos tratados com dsRNAs (que não apresentaram fenótipo aparente) foram analisados e, destes, 7 genes exibiram consistente redução no nível de expressão. Resultados demonstraram que a eficácia da utilização da técnica de RNAi é altamente dependente do alvo a ser silenciado, da sequência de dsRNA utilizada e do momento da análise.

Adicionalmente, na tentativa de avaliar a função anti-oxidativa endógena de esporocistos de *S. mansoni*, foi feita a caracterização funcional de algumas enzimas anti-oxidantes do parasita

(GST26, GST28, GPx, TPx1/2 e SOD). Foi mostrado através de *Western blot* que tratamentos com dsRNA para EF1α, GST26 ou TPx1/2 resultaram em significativa diminuição dos níveis proteicos (80%, 90% e 50%, respectivamente), dados corroborados por experimentos de imunolocalização. Experimentos *in vitro* foram conduzidos para determinar o efeito na sobrevivência dos parasitas silenciados para as enzimas anti-oxidantes na presença de concentrações subletais de H_2O_2. Maior susceptibilidade (60-80% de mortalidade após 48 h) ao estresse oxidativo pode ser claramente observado para parasitas silenciados para GST26, GST28, TPx1/2 e GPx, comparado ao controle GFP (~15 % mortalidade). Co-culturas dos parasitos silenciados para as enzimas anti-oxidantes com hemócitos de *B. glabrata* susceptível à infecção pelo *S. mansoni* permitiram avaliar a hipótese de que a redução da habilidade anti-oxidante dos esporocistos aumentaria a sua vulnerabilidade aos níveis sub-letais de ROS normalmente produzidos pelos hemócitos durante o processo de encapsulação. Assim, foi demonstrado que a sobrevivência de esporocistos silenciados para GST26, GST28 e TPx1/2 foi afetada após 24 horas de co-cultura, mostrando um papel significativo de TPxs e GSTs de proteção para sobrevivência do parasita no seu hospedeiro intermediário.

Apesar de RNAi prometer grandes avanços como ferramenta de genômica funcional em estágios larvais de esquistossomas, observamos que tratamentos com dsRNA podem gerar eficiências de silenciamento variáveis, indicando uma necessidade de padronização da técnica de forma gene-específica, como parte essencial do desenho experimental. Adicionalmente, experimentos funcionais com enzimas anti-oxidantes suportam fortemente a hipótese de que a regulação desta classe de enzimas em esporocistos tem uma função direta na proteção contra o estresse oxidativo e contra o ataque citotóxico das células de defesa do hospedeiro.

Abstract

RNA interference (RNAi) represents the only reverse genetic method currently available for manipulating gene-specific expression in *Schistosoma* spp., since knockout and super-expression induction are not feasible in this parasite. Lately, RNAi has been widely used for gene silencing, but its application as a functional genomic profiling tool in helmints has yet to be explored. In the present study 33 genes, including transcription factors, cell signaling molecules, metabolic enzymes and antioxidants, were selected to determine if gene knockdown by RNAi was associated with morphologically definable phenotypic changes in early intramolluscan larval development. Transcript selection was based on their high expression in *in vitro* cultured *S. mansoni* primary sporocysts and/or their potential involvement in developmental processes, such as the anti-oxidants enzymes which are produced by the parasite *Schistosoma mansoni* and are believed to be involved in the maintenance of cellular redox balance, thus contributing to larval survival in their intermediate snail host, *Biomphalaria glabrata*.

At the present study, miracidia were allowed to transform to sporocysts in the presence of synthesized double-stranded RNAs (dsRNAs) and cultivated for 7 days, during which time developing larvae were closely observed for phenotypic changes including failure/delay in transformation, loss of motility, altered growth and death. Of the phenotypes evaluated, only one was consistently detected; namely a reduction in sporocyst size based on length measurements. The size-reducing phenotype was observed in 11 of the 34 dsRNA treatment groups, and of these, 11 phenotype-associated genes (SOD, Smad1, RHO2, Smad2, Cav2A, ring box, GST26, calcineurin B, Smad4, lactate dehydrogenase and EF1α). Após sete dias de incubação com dsRNA, apenas 6 tratamentos demonstraram consistente e significante diminuição nos níveis de transcritos medidos por qRT-PCR. After seven days of incubation, only 6 treatments demonstrated a significant and consistent knockdown of specific transcript expression was detected by q-PCR. Unexpectedly one phenotype-linked gene, SOD, was highly induced (~1600-fold) upon dsRNA exposure. Variation in dsRNA-mediated silencing effects also was evident in the group of sporocysts that lacked any definable phenotype. Out of 23 nonphenotype-expressing dsRNA treatments, 14 were assessed for the transcript levels. Of those, 7 genes exhibited consistent reductions in steady-state transcript levels, while expression level for the rest remained unchanged. Results demonstrate that the efficacy of dsRNA-treatment in producing phenotypic changes and/or

altered gene expression levels in *S. mansoni* sporocysts is highly dependent on the selected gene (or the specific dsRNA sequence used) and the timing after treatment.

Aditionally, we have focused on the functional characterization of specific anti-oxidant enzymes, including GST26, GST28, GPx, TPx1/2 and SOD, known to be involved in cellular redox reactions, in an attempt to evaluate their endogenous anti-oxidant function in the early-developing primary sporocyst stage of *S. mansoni*. Further, we show that treatment of sporocysts with dsRNA of EF1α, GST26 or TPx1/2, resulted in a significant decrease (80%, 90% and 50%, respectively) using Western blot analysis compared to dsRNA-GFP control treatment. These results were further confirmed by immunocytochemistry. Experiments were then conducted to determine if anti-oxidant RNAi-induced protein knockdown had a modulating effect on *in vitro* parasite survival in presence of a sublethal concentration of H_2O_2. Results clearly demonstrated a significantly higher susceptibility of GST26, GST28, TPx1/2 and GPx –dsRNA-treated larvae to H_2O_2 oxidative stress (60-80% mortalities at 48 hr) compared to GFP dsRNA controls (~15 % mortality). Co-culture of hemocytes and sporocyts allowed evaluating the hypothesis that reducing the antioxidant ability of sporocysts during hemocyte encapsulation reactions would increase their vulnerability to sublethal levels of ROS normally produced by susceptible snail hemocytes. Hence, we demonstrate that GST26, GST28 and TPx1/2-modified sporocysts survival was affected after 24 hours of co-culture, showing the significant protective role of TPxs and GSTs in sporocysts during susceptible intermediate host *B. glabrata* interaction

Although RNAi holds great promise as a functional genomics tool for larval schistosomes, our finding of variable efficiencies in specific gene knockdown indicate a critical need for gene-specific testing and optimization as an essential part of experimental design, execution, and data interpretation. Moreover, anti-oxidant functional experiments strongly support the hypothesis that endogenous expression and regulation of larval anti-oxidant enzymes serve a direct role in protection against external oxidative stress, including immune-mediated cytotoxic reactions.

1. Introdução

1.1-Organismo de estudo – *Schistosoma mansoni*

Parasitos trematódeos do gênero *Schistosoma* infectam cronicamente mais de 200 milhões de pessoas em países em desenvolvimento e mais 600 milhões de pessoas estão em risco de contaminação (WHO: www.who.int/tdr/diseases/schisto/direction.htm). Entre as sete doenças tropicais negligenciadas causadas por helmintos, seis são devidas à infecções por nematódeos e apenas uma, a esquistosomosse, é causada por trematódeos (Hotez *et al*; 2006).

A esquistossomose é uma das parasitoses humanas mais comuns, sendo endêmica em 76 países, principalmente naqueles em desenvolvimento (Engels *et al*., 2002) (Figura 1). A doença é um dos mais sérios problemas de saúde pública, sendo considerada a segunda doença tropical mais prevalente e com alta morbidade. Esta persiste, devido às precárias condições de vida, ao fornecimento inadequado de água e pela inadequação das instalações sanitárias, nas quais a população está inserida.

A esquistossomose é causada por organismos do filo Platelminto, classe Trematodea, ordem Digenea, da família Schistosomatidae e do gênero *Schistosoma*, que compreende espécies como *Schistosoma mansoni* (África e América Latina), *S. haematobium* (África e Oriente Médio), *S. intercalatum* (10 países do cinturão de florestas equatoriais da África), *S. japonicum* (restrito à região do Pacífico, incluindo a China, e nas Filipinas) e *S. mekongi* (limitado a áreas do Laos e Camboja) (WHO:http://www.who.int/tdr/diseases/schisto/diseaseinfo.htm). No Brasil, a esquistossomose é causada pela espécie *S. mansoni* (Figura 2) e seu hospedeiro intermediário é o molusco da especie *Biomphalaria glabrata*.

A doença é determinada, principalmente, pela distribuição do hospedeiro intermediário. O primeiro alvo para erradicação da esquistossomose deve ser impedir a infecção e segundo reduzir a morbidade. Novos focos surgem principalmente em locais onde foram realizados projetos de direcionamento da água para indústria e agricultura. Este desenvolvimento ampliou o número e a distribuição de áreas alagadas e, portanto habitats dos

hospedeiros intermediários. O posterior aumento da concentração de pessoas nestas áreas favoreceu que a esquistossomose se espalhasse (Sturrock, 2001).

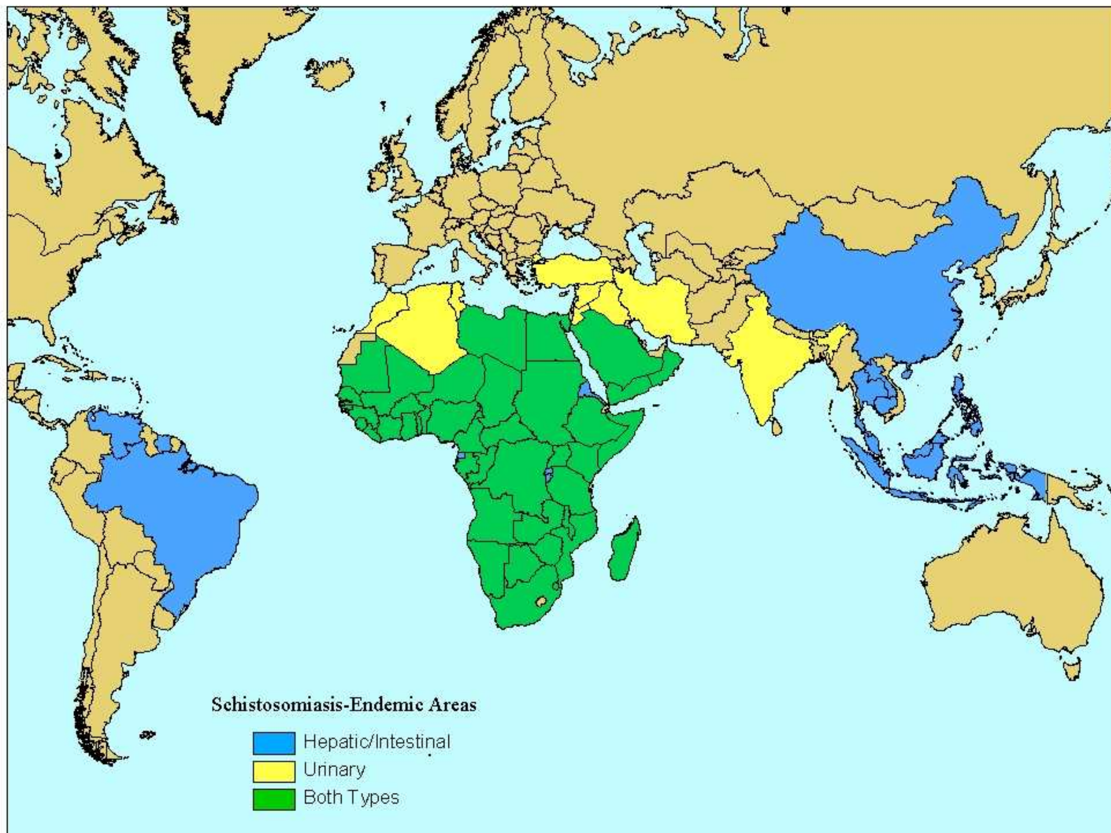


Figura 1. Áreas endêmicas da esquistossomose no mundo. O mapa mostra áreas endêmicas de esquistossomose no mundo, categorizadas por áreas de ocorrência da esquistossomose hepática/intestinal, urinária e onde ambas as formas da doença ocorrem. Fonte: http://www.cdc.gov/travel/diseases/maps/schisto_map.htm.

Em alguns países, programas nacionais de controle da esquistossomose foram bem sucedidos. Porém, nos países africanos abaixo do Saara (compreendendo 80% das pessoas infectadas do mundo), o controle é praticamente inexistente.

Apesar de ser uma doença muito antiga, o estudo científico da esquistossomose começou apenas há 150 anos. O surgimento de drogas contra a esquistossomose no início dos anos 80, dentre elas o praziquantel, que é efetivo e seguro contra todos os esquistossomas, instaurou a intenção de erradicação da doença (http://www.who.int/tdr/diseases/schisto/). No entanto, não há prevenção contra reinfecções, o que invariavelmente ocorre, já que a doença é dificilmente controlada. Adicionalmente, já foram relatados casos de resistência do parasito ao praziquantel (Hagan *et al.*, 2004). Quimiogenômica tem sido utilizada para a identificação de alvos para o desenvolvimento de novas drogas, como relatado no estudo de Caffrey e colaboradores (2009), onde se detectou 18 potenciais proteínas alvos com estruturas tridimensionais conhecidas assim como seus ligantes, sendo este um grande avanço farmacogenômico para o desenvolvimento de drogas contra este parasita. A enzima Tiorredoxina Glutationa Redutase (TGR) foi também recentemente identificada como um importante alvo para o desenvolvimento de drogas, tendo sido testado como alvo para esta enzima o promissor composto 4-fenil-1,2,5-oxadiazol-3-carbonitrila-2-oxido (Sayed *et al.*, 2008).

Vários esforços têm sido dedicados para controle e tratamento da esquistossomose. Contudo, apesar da aplicação de medidas sanitárias, supressão do hospedeiro intermediário e tratamentos quimioterápicos, a prevalência da doença não mostrou redução significativa (Engels *et al.*, 2002). Os programas de controle de maior sucesso foram aqueles que incluíram algum método de interrupção da transmissão através do uso de moluscidas, métodos esses que controlam o crescimento do hospedeiro intermediário, caramujos do gênero *Biomphalaria* (Webbe & El Hak, 1990). Pesquisas de desenvolvimento de vacinas contra o parasito também não obtiveram ainda resultados favoráveis, embora hajam alguns antígenos candidatos em testes clínicos. As tentativas de desenvolver vacinas utilizando antígenos do parasito em desafios ao sistema imune de roedores e primatas foram relatadas, no entanto, a extensão destes resultados a humanos ainda não foi demonstrada. (Taylor *et al.*, 1984; James & Colley, 2001; Lebens *et al.*, 2004; Wilson *et al.*, 2004; Bergquist *et al.*, 2008).

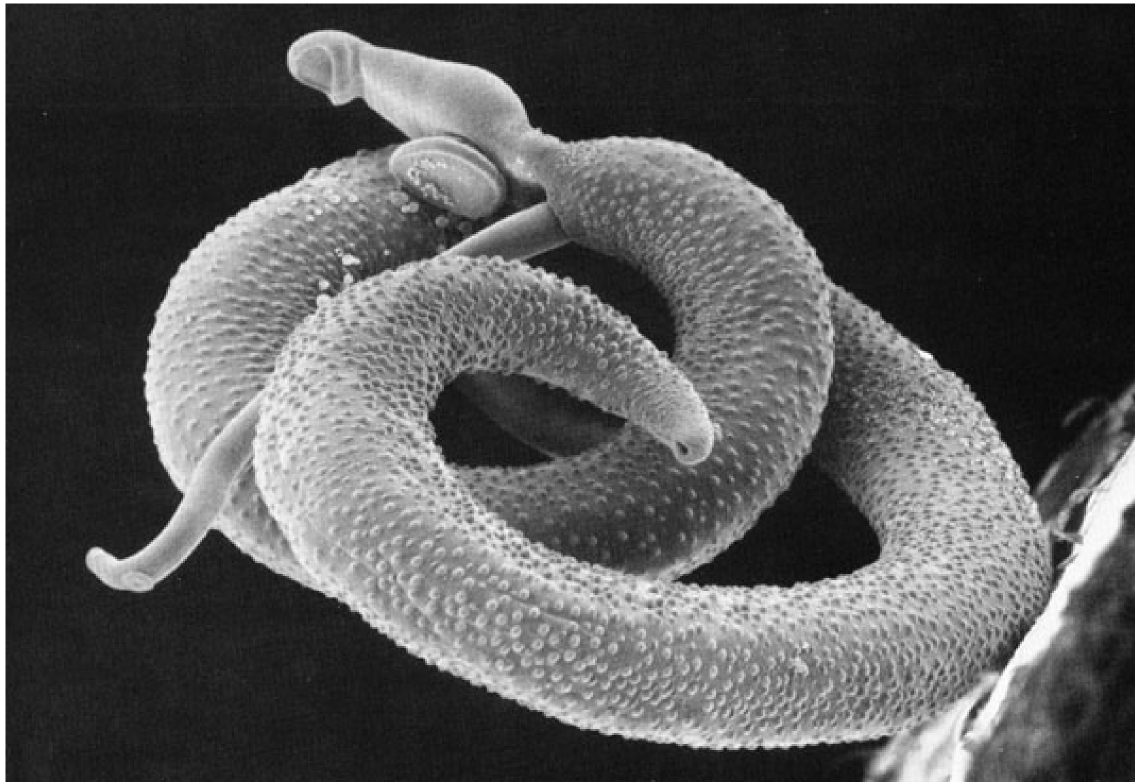


Figura 2: Vermes adultos de *Schistosoma mansoni.* Fonte: www.usuhs.mil/mic/Davies/Research.html

1.2 O ciclo de vida do *Schistosoma*

O ciclo biológico (Figura 3) foi descrito, na mesma época, por Lutz no Brasil e Lerper no Egito. O *S. mansoni* apresenta ciclo de vida complexo, envolvendo hospedeiros intermediários (moluscos), onde se desenvolve sua fase assexuada, e hospedeiros definitivos (mamíferos), onde desenvolve-se a fase adulta sexuada do parasita (Pessoa & Martins, 1982).

Os ovos são eliminados na água através das fezes de hospedeiros infectados. Em condições ótimas, estes eclodem liberando o miracídio que nada ao encontro do caramujo específico, penetrando-o e, assim, passa a ser seu hospedeiro intermediário. O estágio no caramujo inclui a transformação em duas gerações de esporocistos (primário e secundário) e posteriormente em cercária (fase assexuada). Um miracídio pode gerar até 300.000 cercárias. Após serem liberadas, as cercárias vão para a água nadando e penetram ativamente em seu hospedeiro definitivo, comumente o homem. Durante sua penetração, a cercária perde sua cauda bifurcada e glicocálice, transformando-se em esquistossômulo. Estes migram através do sistema sangüíneo e linfático para os pulmões, até se instalarem no sistema porta hepático, onde atingem a maturidade sexual. Os vermes adultos - dióicos, com ventosa oral e ovíparos - vivem acasalados no plexo venoso mesentérico e portal e, para ovoposição, migram para as veias mesentéricas inferiores (Lichtenberg, 1987). As fêmeas colocam em média 400 ovos por dia. Estes ovos atravessam para o lúmem do intestino sendo, então, eliminados nas fezes. Alguns ovos ficam alojados na mucosa intestinal ou nos capilares do sistema porta, tornando-se circundados por células características de resposta inflamatória granulomatosa do hospedeiro (revisado por Pessoa & Martins, 1982).

Neste trabalho foi estudada a fase larval de esporocisto de *S. mansoni*, transformados e cultivados *in vitro* apartir de miracídios obtidos de ovos extraídos de fígado de camundongos infectados.

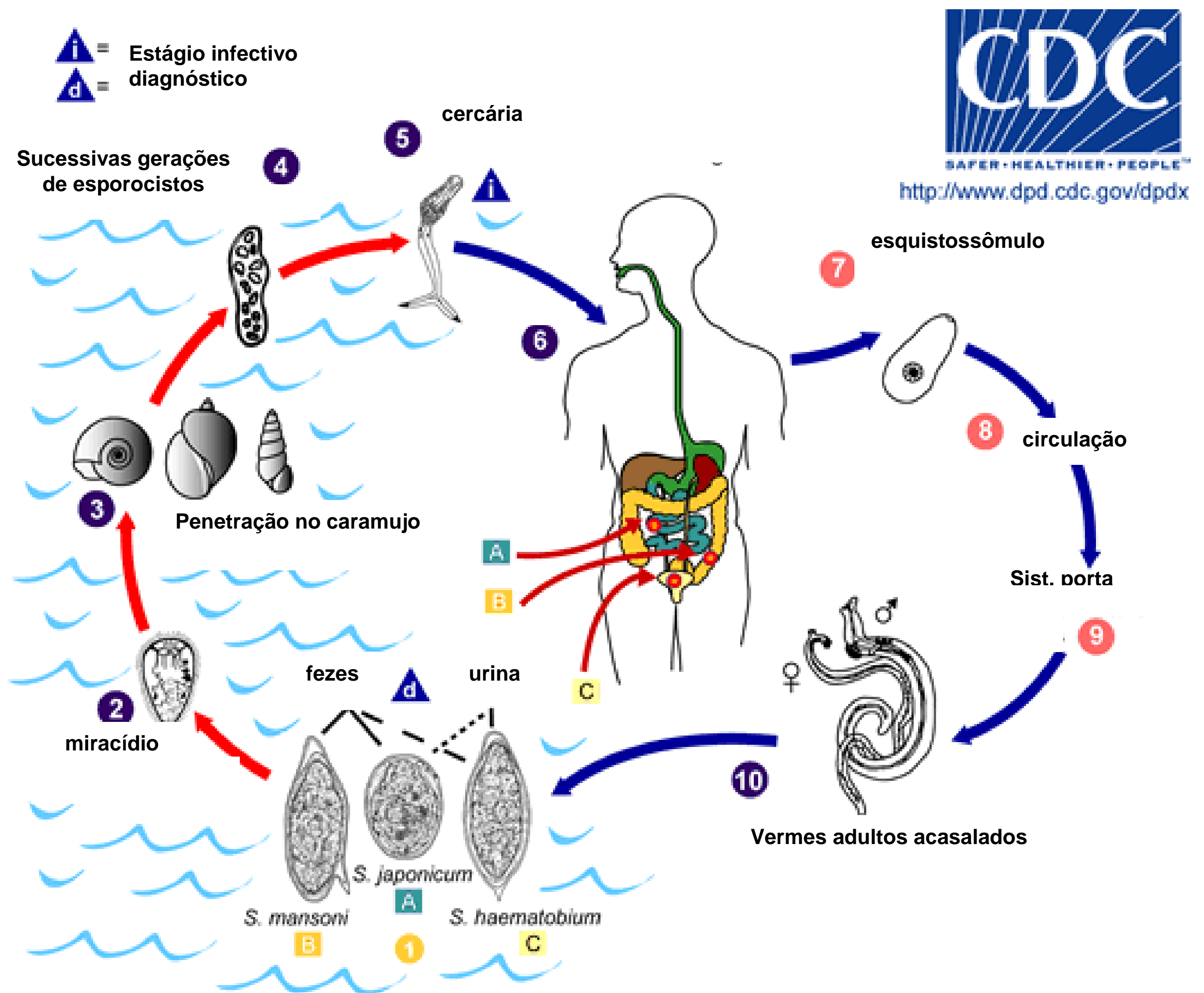


Figura 3: Ciclo de vida do verme *Schistosoma*. Fonte: Adaptado de http://www.dpd.cdc.gov/dpdx/HTML/Schistosomiasis.htm.

1.3 O Genoma do *Schistosoma*

O estudo do genoma do parasita é uma forma de se obter informações que podem ser traduzidas em novas ferramentas de controle da doença, além disso, genomas de parasitas oferecem melhores perspectivas para identificação de novos alvos para drogas, vacinas, diagnósticos e bases biológicas para o entendimento da interação parasita-hospedeiro. O estudo do genoma do *Schistosoma*, pode também ajudar a elucidar questões mais fundamentais sobre este organismo e sobre a evolução dos animais, já que este é representante do nível mais simples dos animais que apresentam simetria bilateral com cromossomos heterogaméticos podendo produzir, assim, respostas às questões sobre alguns dos genes mais primitivos para tais funções evolutivas.

Estudos iniciais do genoma de *S. mansoni* revelaram um genoma diplóide com a presença de sete pares de cromossomos autossomos e um par de cromossomos sexuais. Neste organismo o sexo homogamético é o macho (ZZ) e a fêmea o heterogamético (ZW) (Short & Grossman, 1981; Short, 1983).

O genoma haplóide estimado deste organismo, compreende ~270 Mb, sendo composto de aproximadamente 40% de sequências altamente repetitivas, incluindo aqui os genes ribossomais que estão arranjados seguidamente, podendo ter de 500 a 1000 cópias no genoma e 60% do genoma representa seqüências de cópias únicas ou pequenas famílias gênicas (Simpson *et al*., 1982). Estima-se que esse organismo possua entre 15.000 a 25.000 genes (Franco *et al*., 2000, Verjovski-Almeida *et al*., 2003), e 66% de seu genoma seja composto de adenina e timina.

Dado o sucesso limitado dos métodos tradicionais utilizados no controle da esquistossomose, outro enfoque para se obter informações que pudessem ser traduzidas em novas ferramentas de controle da doença, produção de novas drogas e uma vacina eficaz se faz ainda necessário. As pesquisas em genômica, transcriptômica e proteômica têm como objetivo preencher esta lacuna.

O projeto de descoberta gênica do *Schistosoma* foi iniciado em 1992 no Brasil, e a partir de 1995 passou a contar com o suporte da Organização Mundial de Saúde (United Nations Development Programme e World Bank Special Program for Reserch and Training in Tropical Diseases – UNDP/World Bank/TDR). O projeto visava a descoberta e caracterização de novos genes de *S. mansoni* e S. *japonicum* e seqüenciamento e análise do

genoma mitocondrial. Franco e colaboradores (1995), inauguraram a era de descoberta gênica em *S. mansoni* utilizando a estratégia de ESTs ou Etiquetas de Seqüências Transcritas (Adams *et al*., 1993). Após esta data, houve um enorme crescimento do banco de dados de seqüências deste organismo (Franco *et al*., 1995, 2000; Oliveira *et al*., 2008), visto que em 1993 haviam 600 ESTs depositadas e hoje temos 205.892 depositadas em banco de dados públicos (dbEST release 052209, data 22/05/09).

O sequenciamento completo do genoma do *S. mansoni,* e de outras espécies do gênero, iniciou-se, em 2002, inicialmente adotando como estratégia o sequenciamento de *Bacterial Artificial Chromosomes* (BACs) e de *whole genome shot gun* (WGS) e vem sendo desenvolvido no The Institute for Genomic Research (agora J. Craig Venter Institute) e pelo Wellcome Trust Sanger Institute e sendo financiado pelo Wellcome Trust e o National Institutes of Health (NIH). Este projeto está em fase final de anotação e será publicado em breve (LoVerde *et al*., 2004; Wilson *et al*, 2007).

Enquanto isso, projetos de transcriptômica utilizando ESTs e ORESTES (pequenas seqüências de cDNAs, em geral representando a parte central dos transcritos), resultaram na produção de um número enorme de seqüências de genes dos parasitos do gênero *Schistosoma*, que vêem sendo armazenadas em bancos de dados de domínio público (Franco *et al*., 1997; Santos *et al*., 1999; Franco *et al*., 2000; Oliveira & Johnston, 2001; Verjovski-Almeida *et al*., 2003; Hu *et al*. 2003; Oliveira *et al*., 2004; LoVerde *et al*., 2004; El-Sayed *et al*., 2004; Liu *et al*. 2006). Mais recentemente, tem havido uma grande contribuição na descoberta gênica e de padrões de expressão através de projetos utilizando Analise Serial de Expressão Gênica (SAGE) (Williams *et al.,* 2007; Taft *et al*., 2009), plataformas de microarranjos (Ojopi *et al*., 2007; Fitzpatrick *et al*., 2005, 2008, Verjovski-Almeida *et al*., 2007, Waisberg *et al.,* 2007, 2008; Gobert *et al*., 2009) e proteômica (Liu *et al*., 2006; Wu *et al*., 2008; Guillou *et al*., 2007; Gourbal *et al*., 2008; Roger *et al*., 2008).

1.4 Era pós-genômica

Atualmente, seis anos depois da emergência desse enorme volume de informação genética, poucas soluções foram encontradas no que se diz respeito à identificação de genes alvos, desenvolvimento de drogas e diagnóstico para o controle da esquistossomose. Entramos na era da genômica funcional, onde entendemos que para decifrar o código

genético é necessário o desenvolvimento de métodos confiáveis de genética reversa e novas ferramentas de manipulação gênica (Pearce & Freitas, 2008). Neste ponto, o estudo dos nematódeos parasitas tem se beneficiado amplamente da aplicabilidade das informações geradas no estudo do nematódeo de vida livre, o *Caenorhabditis elegans*. Porém, a inexistência de um modelo análogo em esquistossomas tem atrasado bastante a experimentação de novas técnicas para o conhecimento da biologia do organismo.

Estudos funcionais de genes envolvem passos experimentais *in vivo, in vitro* e *in silico*, não tão facilmente automatizáveis quanto o sequenciamento. A pesquisa em biologia de esquistossomos ainda sofre com várias dificuldades experimentais. Em primeiro lugar, esquistossomos são parasitos com ciclo de vida complexo, que envolve dois hospedeiros distintos e muitos estágios diferentes. Segundo, ainda não foi desenvolvida nenhuma técnica para multiplicar esquistossomos *in vitro*. Assim, parasitos devem ser isolados a partir do sacrifício de animais de laboratório infectados, o que limita muito o número de parasitos que podem ser usados em estudos experimentais. Terceiro, esquistossomos são parasitos eucarióticos multicelulares que apresentam tecidos distintos, como órgãos reprodutores e glândulas secretoras, cada um contendo um conjunto específico de diversas células para as quais ainda não existe uma linhagem contínua que pode ser cultivada *in vitro* (Wilson *et al.*, 2006). Quarto: ainda não foi possível a produção de organismos transgênicos, apesar de técnicas como: vetores retrovirais (Kines *et al.*, 2008) e metodologias de transformação da linhagem germinal de *S. mansoni* (Beckmann *et al.*, 2007) estarem sendo desenvolvidas para esse fim.

Até recentemente, ferramentas moleculares para manipulação da expressão gênica em *Schistosoma* não existiam, nos impedindo de revelar mecanismos moleculares nestes importantes parasitas. No entanto, nos últimos anos, estamos alcançando um importante estágio no estudo da biologia destes trematódeos. Conquistamos a habilidade de utilizar genética reversa nestes organismos, através da técnica de interferência por RNA. Através desta ferramenta se faz possível aprofundar no estudo das funções gênicas, enriquecendo o estudo da relação parasita-hospedeiro e revelando o(s) suposto(s) papel(is) de cada proteína, o que até hoje tem sido feito, principalmente, através de estudos de similaridade com genes de diferentes organismos, depositados em bancos de dados. Muitas vezes, as simples análises de ortologia gênica podem resultar em informações equivocadas, uma vez que, cada organismo é único e uma proteína pode assumir diversas tarefas. Como exemplo, temos o caso da Glutationa Redutase (GR) e da Tiorredoxina Redutase (TR), que em *S. mansoni* estão

ausentes e suas funções de detoxificação são desempenhadas por uma só enzima multifuncional, a Tiorredoxina Glutationa Redutase (TGR) (Kuntz *et al*. 2007).

1.4.1 Interferência por RNA (RNAi)

O termo interferência por RNA (RNAi) se refere a um processo celular no qual RNAs de fita dupla (dsRNA), inibem específicamente a expressão de um dado gene. Inicialmente, este processo foi chamado de *post-transcriptional gene silencing* (PTGS) e foi observado em petunias por Napoli e colaboradores (1990). O mecanismo de silenciamento foi elucidado no organismo *C. elegans*, em 1998, por Andrew Fire e Craig Mello e passou a se chamar interferência por RNA. O mecanismo de RNAi involve inúmeras proteínas celulares e estas são altamente conservadas nos organismos eucariotos. Presumivelmente, RNAi faz parte de um mecanismo de defesa celular contra infecção por vírus RNA dupla fita e/ou elementos móveis, como retrotransposons, conservando a estabilidade genética. Este mecanismo tornou-se uma poderosa técnica, utilizada *in vivo* e *in vitro,* para o silenciamento pós-transcricional de genes de interesse (revisado por Kurreck, 2009). Com promessas de sua aplicabilidade terapêutica e com a grande carência de técnicas de descoberta das funções gênicas em todo tipo de organismo em pesquisa básica, RNAi se tornou a ferramenta molecular de escolha, e hoje, juntamente com a proteômica, desponta como o campo mais dinâmico da biotecnologia.

Resumidamente, o mecanismo de RNAi envolve um complexo protéico, chamado DICER, que possui atividade de ribonuclease III e cliva os RNAs dupla fita longos (dsRNA) que entram nas células, produzindo pequenos fragmentos de 21 a 23 pb de RNA dupla fita. Estes pequenos RNAs, conhecidos como *short interfering RNAs* (siRNAs), contém dois nucleotídeos de cada um dos lados despareados. Os siRNAs se ligam a outro complexo chamado RISC (*RNA-induced silencing complex*), sendo posteriormente selecionada uma das fitas do siRNA (de preferência a fita antisenso) para permanecer ligada a esse complexo. O complexo é guiado ao mRNA de seqüência complementar ao siRNA ligado à RISC e uma endonuclease, chamada Argonauta2 (Ago2), presente neste complexo cliva o mRNA em um ponto interno central às fitas pareadas siRNA/mRNA e os fragmentos liberados dessa clivagem são posteriormente degradados devido à perda de elementos essenciais à estabilidade dos mRNAs, tais como o cap e cauda poliA, suprimindo-se, assim a tradução do mRNA alvo do silenciamento (Hamilton & Baulcombe, 1999; Zamore *et al*, 2000). Gera-se como resultado, fenótipos de perda de função (Figura 4).

Logo após a descoberta de que os dsRNAs eram os efetores de RNAi, foi descoberta a existência de uma classe de moléculas de RNA endógenas que continham o mesmo tamanho em vários organismos, desde de plantas até humanos, e estes foram chamados microRNAs (miRNA). Os miRNAs são processados de precursores em forma de grampo de cabelo, maiores, usando parte da maquinaria de RNAi e podem regular a expressão gênica por degradação dos mRNAs ou supressão da tradução, exercendo, assim, regulação gênica (Murchison *et al*., 2004).

A eficácia da técnica interferêcia por RNA é transiente e a degradação do mRNA alvo inicia-se imediatamente após a entrada do dsRNA na célula;organismo. No entanto, o decréscimo na quantidade de proteína dependerá da estabilidade da proteína-alvo, do organismo no qual a técnica está sendo empregada, na forma de distribuição do RNA de dupla fita e da sequência utilizada para a síntese do dsRNA/siRNA. Sabe-se que a eficiência do siRNA dependerá de sua estabilidade termodinâmica e acessibilidade para ligar-se ao transcrito alvo. Existem várias formas de utilização da técnica de RNAi: o silenciamento pode ser disparado através da utilização de longos dsRNAs, de siRNA (modificados ou não), ou de vetores transcrevendo *short hairpin* RNA (shRNA). Finalmente, a técnica de RNAi é caracterizada, na maioria das vezes, por apenas um decréscimo na quantidade de transcritos, por isso o nome dado ao efeito ser *knockdown* e não *knockout* (revisado por Kurreck, 2009).

1.4.2. Interferência por RNAi em parasitas

Apesar da técnica de RNAi estar sendo aplicada em esquistossomas desde 2003 (Boyle *et al*; 2003; Skelly *et al*; 2003), muitos mecanismos intrínsecos a esta ferramenta continuam por ser desvendados neste organismo, desafiando e impondo barreiras à sua utilização em helmintos parasitas (Gelhof *et al*; 2007). Acreditamos que por esta razão, até hoje, apenas um reduzido número de genes foram silenciados em *S. mansoni* explorando esta nova técnica. Na sua grande maioria, esta ferramenta foi utilizada no silenciamento das Catepsinas e genes relacionados a esta família de proteínas, ou seja, focando na elucidação de proteínas relacionadas à digestão de hemoglobina pelos parasitas e abrangendo tipicamente vermes adultos como alvos (Skelly *et al*., 2003; Correnti *et al*., 2005; Krautz-Peterson & Skelly, 2008; Morales *et al*. 2008) e em alguns poucos trabalhos, a técnica de RNAi foi utilizada em vermes adultos e ovos do parasita na identificação funcional de genes individuais (Nabhan *et al*., 2007, Pereira *et al*., 2008, Rinaldi *et al*., 2009). Apenas três genes

foram silenciados na fase larval deste parasita: GAPDH, transportador de glicose SGTP1 e o receptor tipo *scavenger* (SR) para ligação de LDL modificado, denominado SmSRB (Boyle *et al*., 2003; Correnti *et al*., 2005; Dinguirard & Yoshino, 2006).

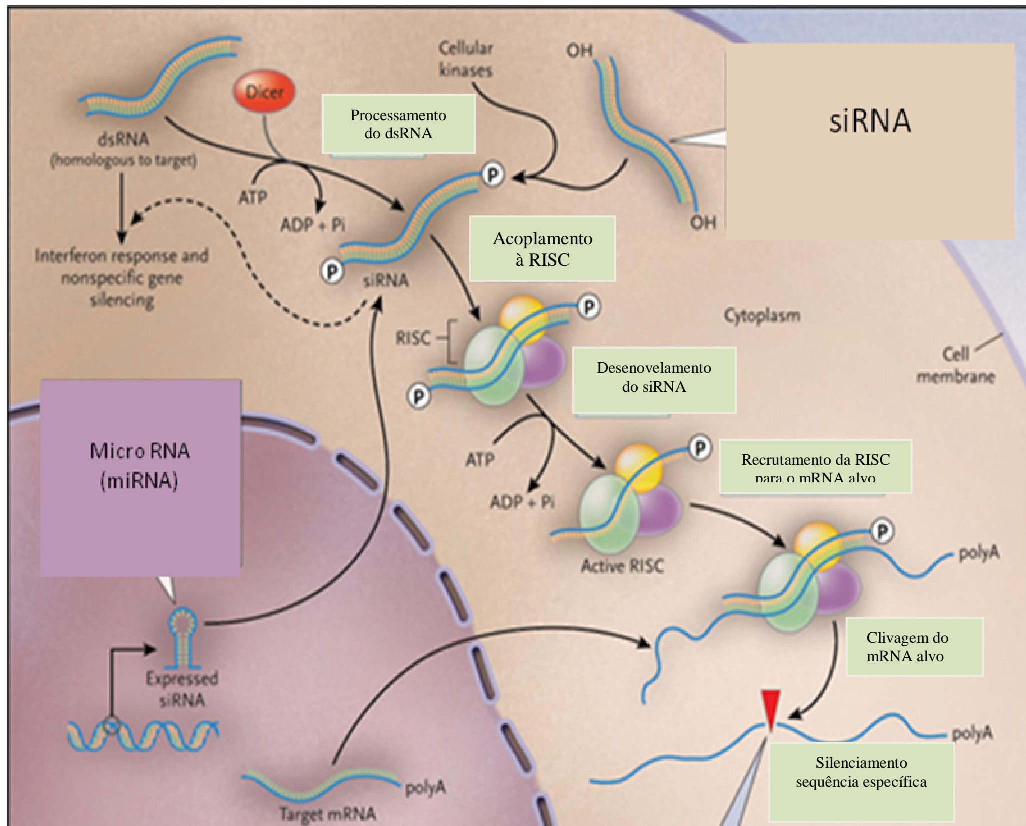


Figura 4: Ilustração do mecanismo de RNAi por diferentes vias:- dsRNA, siRNA e miRNA. Modificado de http://www.gghjournal.com/volume21/1/ab04.cfm.

Em *C. elegans*, o silenciamento gênico por RNAi tem revolucionado a biologia experimental, sendo aplicado até mesmo em escala genômica para caracterização de seus genes (Lenz, 2005). Como recentemente discutido, por Gelhof e colaboradores, 2007, o mesmo não vem acontecendo em parasitos helmintos. Apesar das publicações utilizando RNAi nestes organismos concluírem que esta técnica poderia ser muito útil se aplicada em larga escala, estudos recentes com um número grande de genes mostraram uma não reprodutibilidade entre experimentos. Entre onze genes alvos testados, apenas dois foram silenciados no nematódeo *Haemonchus contortus* (Gelhof *et al.,* 2006). É altamente discutível como a técnica tem sido utilizada e relatada pela comunidade científica. Muitas das publicações nesta área apenas demonstram um efeito indireto do silenciamento nos níveis de proteína ou atividade enzimática, outras relatam um silenciamento inespecífico também nos controles e algumas reportam que os métodos de introdução do dsRNA nas células muitas vezes levam os organismos tratados à morte. Além do mais, há indicações de que apenas os genes alvo onde foi obtido um silenciamento efetivo foram divulgados. No entanto, não se sabe verdadeiramente quantos genes foram analisados para cada organismo em pauta.

Buscando achar respostas para muitas destas questões, neste trabalho 33 genes foram selecionados para silenciamento utilizando RNAi. Muitos destes foram escolhidos baseado em sua super expressão gênica diferencial na fase de esporocistos de *S. mansoni*, utilizando um banco de dados de SAGE (OrganismDB) (Williams *et al.,* 2007). Dentre os alvos selecionados estão moléculas envolvidas em vias de sinalização intracelular (Smads e proteínas que se ligam a cálcio), possíveis fatores de transcrição (*Zinc Fingers*), enzimas metabólicas clássicas, entre outras que apresentam um elevado nível de transcritos nesta fase larval do parasito. Outra parte das moléculas selecionadas são enzimas envolvidas em proteção do organismo contra danos oxidativos.

1.5 O papel das enzimas anti-oxidantes no parasita *S. mansoni*

Durante seu complexo ciclo de vida, o *Schistosoma* vive em ambientes diversos no interior de seus hospedeiros, sobrevivem e se desenvolvem na presença de ataques pelas células do sistema de defesa destes hospedeiros. Portanto, é vital um efetivo mecanismo para manutenção do equilíbrio redox celular, tendo em vista o seu papel-chave na inativação de

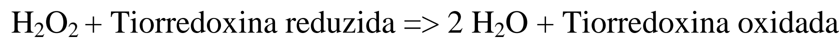
espécies reativas de oxigênio geradas pela resposta imune dos hospedeiros e em seu próprio organismo aeróbico.

A penetração do miracídio no seu hospedeiro intermediário representa um período de transição crítico entre um estágio larval de vida livre e um posterior confronto com um ambiente hostil no interior do hospedeiro, onde o parasita necessita estabelecer uma infecção viável (Bayne *et al*., 2001). Durante este período de transformação, as larvas de *S. mansoni* se encontram especialmente vulneráveis ao estresse oxidativo gerado no plasma (Bender *et al*., 2002), ou pelo oxigênio reativo (ROS) e espécies de nitrogênio (RNS) resultantes das respostas mediadas por hemócitos da *B. glabrata* (Hahn *et al*., 2000; 2001a; 2001b; Zelck & Von Janowsky, 2004). Portanto, é vital que o parasita possua a capacidade de manter um equilíbrio redox para o sucesso da infecção no caramujo.

Na maioria dos eucariotos existem duas principais formas de detoxificação de espécies reativas de oxigênio, através da transferência de espécies reativas à glutationa (GSH), ou utilizando a pequena proteína tioredoxina como espécie receptora (Kuntz *et al.*, 2007). Estudos recentes mostraram que esporocistos de *S. mansoni* possuem numerosas enzimas envolvidas no metabolismo de ROS e na detoxicação de produtos oxidativos (Guillou *et al*., 2007; Roger *et al*., 2008; Vermeire and Yoshino, 2007; Williams *et al*., 2007; Wu *et al*., 2008). Interessantemente, estes parasitas não possuem Catalase, a principal enzima que neutraliza H_2O_2 em muitos organismos. Além disso, sua Glutationa Peroxidase (GPx) pertence a uma classe que possui baixa reatividade contra H_2O_2 (Sayed & Williams, 2004). Atualmente, sabemos que este organismo tem uma gama de enzimas que provavelmente se complementam no combate aos superóxidos, dentre estas estão:

- Glutationa-S-Transferases (GST26 e GST28), que tem função de neutralizar potenciais danos de membrana através da utilização da glutationa reduzida (GSH) por reações de detoxificação envolvendo conjugação-tiol dos xenobióticos (Yan *et al*., 2008) (Figura 5);
- Tiorredoxina Peroxidases (TPx 1 e TPx 2), estão envolvidas na manutenção do equilíbrio redox, através da redução do peróxido de hidrogênio (H_2O_2) usando a tiorredoxina como doador de elétrons (Kwatia *et al.*; 2000). Os altos níveis de atividade das enzimas TPx encontradas em vermes adultos de *S. mansoni* parecem indicar que estas sejam as principais contribuintes na manutenção da atividade antioxidante neste organismo (Sayed *et al*., 2006) (Figura 6);

Reação:



H_2O_2 + Tiorredoxina reduzida => 2 H_2O + Tiorredoxina oxidada

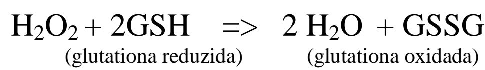
- Superóxido dismutases (SOD), são metaloenzimas responsáveis pelo mecanismo de defesa contra a toxicidade do oxigênio através da catálise da dismutação do radical superóxido à oxigênio e peróxido de hidrogênio (Sirmuda, *et al*; 1988) (Figura 6);

Reação:

$^1O_2 + {}^1O_2 + 2H^+ \Rightarrow O_2 + H_2O_2$

- Glutationa peroxidase (GPx), enzima que metaboliza H_2O_2, protegendo as membranas de danos por peroxidação de fosfolipideos (Kwatia *et al.*, 2000; Williams *et al.*, 1992) (Figura 5 e 6);

Reação:

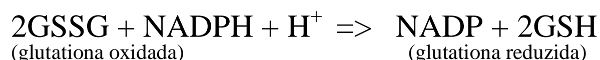


H_2O_2 + 2GSH (glutationa reduzida) => 2 H_2O + GSSG (glutationa oxidada)

- Tiorredoxina Glutationa Redutase, enzima essencial, específicamente presente em *S. mansoni* e que parece assumir a função das Glutationa Redutase (GR) e das Tiorredoxinas Redutase (Trx) existentes em mamíferos, como já dito anteriormente, sendo esta responsável pela redução de dissulfetos de glutationa (GSSH) e de tiorredoxina. Esta foi identificada como mais uma promissora enzima candidata ao desenvolvimento de drogas anti-esquistosomóticas (Kuntz *et al.*, 2007) (Figura 6).

Reação:

Ex: Glutationa Redutase (GR)



2GSSG (glutationa oxidada) + NADPH + H^+ => NADP + 2GSH (glutationa reduzida)

O conhecimento deste sistema redox, potencialmente poderá nos guiar a promissores candidatos ao desenvolvimento de quimioterápicos. A importância destas proteínas com função anti-oxidante no parasita foi recentemente ressaltada por Kuntz e colaboradores, 2007.

Estudos anteriores demonstraram que esporocistos de *S. mansoni* são muito sensíveis à toxicidade do peróxido de hidrogênio (Hahn *et al*., 2001) e que a exposição *in vitro* de esporocistos à H_2O_2, dispara a superexpressão dos genes que codificam proteínas envolvidas no combate à oxidação (Zelck & Von Janowsky, 2004; Vermeire & Yoshino, 2007). Estes dados corroboram com a hipótese de que esporocistos são capazes de reagir contra o dano causado pelas espécies reativas de oxigênio através da atividade de um sistema anti-oxidante endógeno (Bayne *et al*., 2001). No entanto, até hoje, um sistema capaz de combater e proteger esporocistos de ROS produzido externamente, ainda não foi demonstrado. Devido ao exposto, selecionamos algumas destas enzimas (GST26, GST28, TPx1, TPx2, SOD e GPx) para serem silenciadas e possivelmente desvendar as consequências da mudança na expressão destes genes e especialmente as funções exercidas por estes na relação parasito-hospedeiro intermediário.

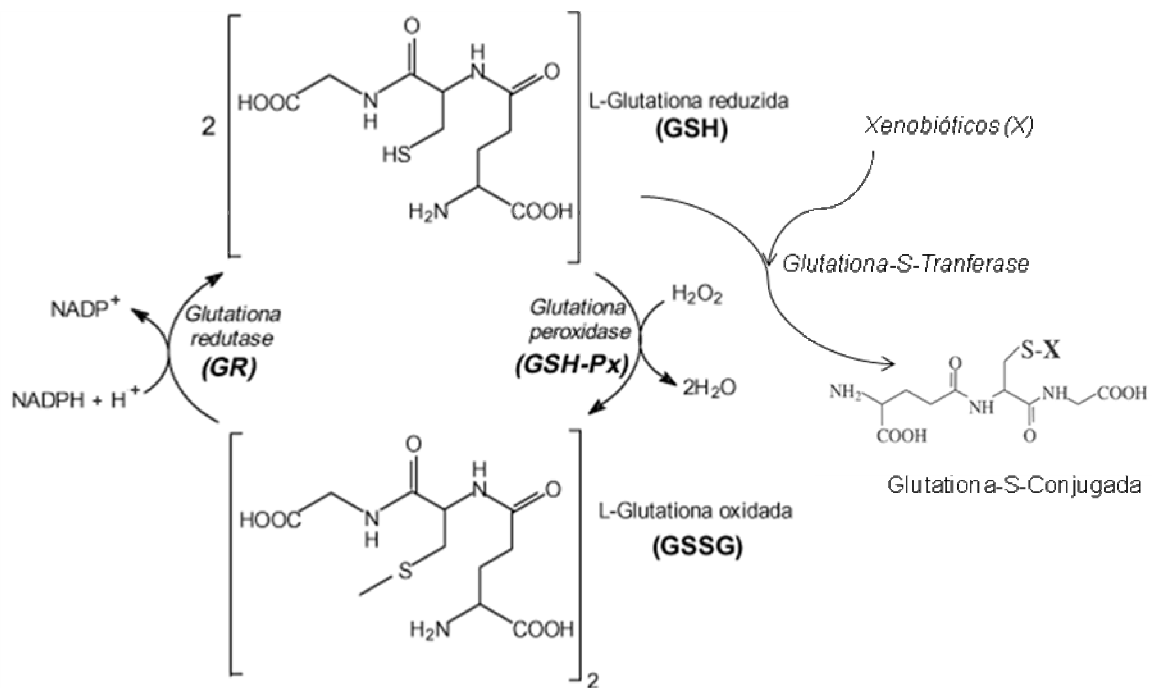


Figura 5: Interconversão de glutationa nas suas formas reduzida (GSH), oxidada (GSSG) e conjugada (GSX) pela ação das enzimas glutationa redutase, glutationa peroxidase e glutationa-S-transferase (modificado de Rover Júnior *et al*., 2001).

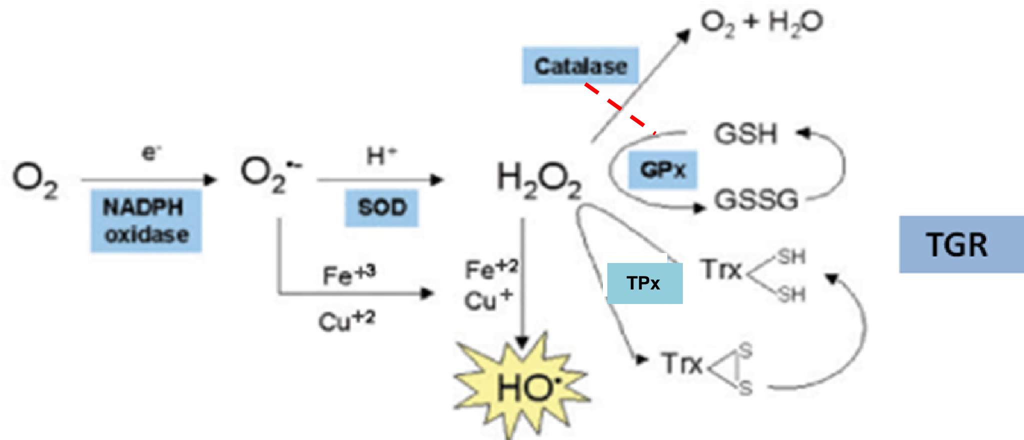


Figura 6: Esquema das reações e das respectivas enzimas (em azul) envolvidas na transformação das espécies reativas de oxigênio. SOD- superóxido dismutase, TPx- Tiorredoxina peroxidase, GPx- glutationa peroxidase, TGR- Tiorredoxina Glutationa redutase, enzima específica de *S. mansoni* . GSH e GSSG, glutationa nas formas reduzidas e oxidadas, respectivamente. A enzima catalase não existe em *S. mansoni* (Modificado de Barbosa *et al*., 2006).

2- Objetivos

Silenciamento gênico utilizando a técnica de interferência por RNA para estudo funcional de 33 genes alvos em *Schistosoma mansoni.*

2.1 - Objetivos específicos

- Padronização e aperfeiçoamento da técnica de RNAi para a fase de esporocisto.

- Silenciamento de 33 genes alvos (e 34 tratamentos) selecionados de acordo com seu nível de expressão aumentado nos esporocistos, em relação a outras fases do ciclo de vida do organismo.

- Estudo funcional do mecanismo de defesa de *S. mansoni* contra o estresse oxidativo através do silenciamento dos transcritos de enzimas anti-oxidantes do parasita, para a investigação de sua importância na relação parasito-hospedeiro.

3-Materiais e Métodos

3.1 Parasitos

Parasitas *S. mansoni* da cepa NMRI foram usados em todos os experimentos. Fígados de camundongos infectados por 7-8 semanas foram extraídos e homogeneizados em solução salina 1,2% com estreptomicina 50µg/ml e penicilina 60µg/ml. Ovos de parasitas eclodiram em água salina, *Pond water*, (66,7µM $CaCO_3$, 7,9µM $MgCO_3$, 11,4µM NaCl, 1,8µM KCl) contendo penicilina e estreptomicina preparada em laboratório. Miracídios foram deixados por 20 minutos em gelo para diminuição de mobilidade e decantação dos parasitos. Estes foram lavados em água salina e centrifugados a 1600 rpm por 2 minutos a 4ºC. Este processo foi repetido duas vezes.

A transformação de miracídios em esporocistos ocorreu *in vitro* cultivando os miracídios em solução salina balanceada Chernin, CBSS, (NaCl 47,9 mM, KCl 2 mM, Na_2HPO_4 0,5 mM, $MgSO_4$ 1,8 mM, $CaCl_2$ 3,6 mM e $NaHCO_3$ 0,6 mM), suplementado com 1 g/L de glicose e trealose (CBSS+), pH 7,2 (Chernin, 1963). Foram adicionados 60 µg/ml de penicilina e 50µg/ml de streptomicina. CBSS+ mimetiza a composição salina da hemolinfa do hospedeiro intermediário do *S. mansoni*, *Biomphalaria glabrata*, no qual 90% dos miracídios perdem placas ciliares e se tornam esporocistos primários desenvolvidos dentro de 48 horas de cultivo (Ivanchenko *et al*., 1999; Yoshino & Laursen, 1995).

Na tentativa de observar longevidade da cultura tratada e fenótipos na segunda geração, foi adicionado nas culturas de esporocistos meio Bge suplementado com 10% de soro fetal bovino inativado por aquecimento (Sigma) e antibióticos como descrito por Yoshino & Laursen, 1995. Células do hospedeiro foram mantidas rotineiramente no meio Bge a 26º C. Sobrenadante com produtos secretados das células foram adicionados nas culturas.

3.2 Genes alvos

Na triagem utilizando a técnica de interferência por RNA foram escolhidos 33 genes alvos de *S. mansoni* para serem silenciados. Estes foram, em sua maioria, escolhidos baseado em sua aparente expressão diferencial nas fases de miracídios e esporocistos, quando comparados com vermes adultos. Para isto, foi utilizado o banco de dados de SAGE OrganismDB (Williams *et al*; 2007), procurando por perfis de expressão gênica diferencial nas fases larvais, principalmente em esporocisto (http://gmod.mbl.edu/perl/site/s_mansoniest). Os genes selecionados foram:

Calcineurina B (GeneBank Acc. No. AJ276885.1),

Lactato Desidrogenase (GeneBank Acc. No. U87629.1.),

Smad4 (GeneBank Acc. No. AY371484.1.),

Smad2 (GeneBank Acc. No. AF232025.1),

Smad1 (GeneBank Acc. No. AF215933.1),

Glutationa-S-Transferase 26 (GeneBank Acc. No. M73624.1),

Glutationa-S-Transferase 28 (GeneBank Acc. No. S71584.1),

14.3.3 (GeneBank Acc. No. U24281.1),

Scmeg (receptor de fator de crescimento epidermal) (GeneBank Acc. No. M86399.1),

Fosfoenolpiruvato Carboxiquinase (PepcK) (GeneBank Acc. No. AF120929.1),

Calpaina (GeneBank Acc. No. M74233.1),

Putativa Proteína Ligadora de Hexâmero (HEXBP)

Putativa Fibrilarina

Fator de Elongação 1α (GeneBank Acc. No. Y08487.1),

RHO 1 GTPase (GeneBank Acc. No. AY158212.1),

RHO 2 GTPase (GeneBank Acc. No. AY158214.1),

Calcio ATPase 2 (SMA2) (GeneBank Acc. No. AF074400.1),

SPO1 (GeneBank Acc. No. AF109181),

PKC B (GeneBank Acc. No. AY337620.1),

PKC receptor (GeneBank Acc. No. AF422164.1),

Putativo Domínio Zinc Finger DHHC,

Miosina-cadeia leve (GeneBank Acc. No. AF071011.1),

Calreticulina (GeneBank Acc. No. L24159.1),

Canal de cálcio ativado por alta voltagem (Cav2A) (GeneBank Acc. No. AF361883.),

Canal de cálcio ativado por alta voltagem subunidade beta 2 (Calcium Channel) (GeneBank Acc. No. AY277532.1),

Glicoproteína K 5 (GeneBank Acc. No. AY903301.1)

Zinc Finger 1 (SmZF1) (GeneBank Acc. No. AF316827.1.),

Ring Box (SmRbx) (GeneBank Acc. No. DQ466078.1.),

Glutationa Peroxidase (GeneBank Acc. No. M86510.1)

Tiorredoxina Peroxidase 1 (TPx-1) (GeneBank Acc. No. AF121199.1)

Tiorredoxina Peroxidase 2 (TPx-2) (GeneBank Acc. No. AF157561.1)

Superoxido Dismutase (SOD) (GeneBank Acc. No. M27529.1)

Calmodulina1

As descrições destes genes se encontram a seguir:

- ❖ Calcineurina B (GeneBank Acc. No. AJ276885.1) é uma proteína fosfatase também conhecida como proteína fosfatase 2B, estimulada por Ca^{2+} e pela Calmodulina, sendo responsável por ativar a transcrição de interleucina 2, que estimula o crescimento e diferenciação de células T. (Lin *et al.*,1999).

- ❖ Lactato Desidrogenase (LD) (GeneBank Acc. No. U87629.1.). Sabe-se que estágios do parasita dependem largamente de energia do metabolismo anaeróbico. Além disso, enzimas glicolíticas são essenciais e são vistas como potenciais alvos para ataques quimioterápicos. Lactato Desidrogenase é a enzima que catalisa a interconversão de piruvato à lactato com concomitante interconversão de NADH e NAD. Esta enzima é reportada como sendo o possível alvo de drogas anti-esquistossomóticas, como Pranziquantel (Lu *et al.*, 2006)

- Smad 4 (GeneBank Acc. No. AY371484.1.). Liga-se a SMADs reguladas por receptor (SMAD1 e SMAD2), facilitando a translocação do complexo heterodimérico para o núcleo, onde este se liga a promotores e interage com ativadores transcricionais. Está envolvida em muitas funções celulares como, diferenciação, apoptose, gastrulação, desenvolvimento embrionário e ciclo celular. É uma Co-Smad (Whitman, 1998; Attisano & Wrana, 2000).

- Smad 2 (GeneBank Acc. No. AF232025.1). Esta proteína participa da transmissão de sinais extracelulares para o núcleo celular advindos da ligação de fatores de crescimento aos seus receptores, neste caso TGFβ. Quando os receptores extracelulares são ativados, Smad 2 é fosforilada em seu motivo Serina-Serina-Metionina-Serina, formando um complexo com Smad 4. Este complexo, então, é deslocado para o núcleo participando na regulação da expressão gênica (Whitman, 1998; Attisano & Wrana, 2000).

- Smad 1 (GeneBank Acc. No. AF215933.1) Pertencente à família SMAD de proteínas, mas é ativada por receptores BMP (*Bone Morphogenic Protein*). O ligante induz multidimerização, autofosforilação e ativação dos receptores, e estes, por sua vez, fosforilam Smad 1, Smad 5 e Smad 8. As Smads fosforiladas se ligam a co-Smad 4 e translocam-se para o núcleo, estimulando transcrição. Como outros moduladores desta família, Smad1 está envolvida em sinalização celular relacionada com proliferação, diferenciação e apoptose (Whitman, 1998; Attisano & Wrana, 2000).

- Glutationa-S-Transferase 26, Sm26 (GeneBank Acc. No. M73624.1) e Glutationa-S-Transferase 28, Sm28 (GeneBank Acc. No. S71584.1). Estas pertencem a um grupo de isoenzimas que catalisam a detoxificação por tio-conjugação com moléculas lipofílicas utilizando glutationa reduzida (GSH). Em *S. mansoni*, uma função direta encontrada para GSTs foi a de proteção contra toxinas. As GSTs parecem proteger a superfície do parasita neutralizando danos em sua membrana decorrentes da resposta imune do hospedeiro. Vários estudos demonstraram a importância deste grupo de proteínas como alvo para vacinas. Imunização com GST-26 recombinante resulta em diminuição de vermes e ovos de *S. japonicum* (Bergquist, 1995; Gobert *et al.*, 1998).

- 14-3-3 (GeneBank Acc. No. U24281.1). São proteínas que interagem com uma infinidade de moléculas de sinalização como quinases, fosfatases e receptores transmembrana. No entanto, pouco é sabido sobre estas interações. Sabe-se apenas que estas parecem estar envolvidas no controle do ciclo celular, crescimento, diferenciação, sobrevivência, apoptose e migração celular. Pertencem a uma família de proteínas altamente conservadas (Mhawech, 2005). É também mais um importante alvo indicado para o desenvolvimento de vacinas contra esquistossomose (Siles-Lucas *et al*., 2007).

- Scmeg- Homóloga ao Receptor do Fator de Crescimento Epidérmico - SER (GeneBank Acc. No. M86399.1). Scmeg é capaz de fazer autofosforilação e parece participar na transdução de sinais em esquistossomas. Talvez esteja relacionada com o desenvolvimento muscular. Foi mostrado que este receptor é ativado por fator de crescimento epidérmico e é um importante fator na diferenciação de esporocistos (Vicogne *et al*., 2004).

- Fosfoenolpiruvato Carboxiquinase (PepcK) (GeneBank Acc. No. AF120929.1). Enzima que catalisa a conversão do oxaloacetato e ATP a fosfoenolpiruvato, CO2 e ADP. É encontrada na forma citosólica e mitocondrial. Khayath e colaboradores (2006) descreveram em esquistossomas um processo chamado gliceroneogênese, no qual glicerol é produzido a partir de precursores como glutamina. PEPCK é massivamente expressa nos estágios larvais e sua inibição reduz o efeito da glutamina no crescimento dos parasitos, sugerindo uma importância do glicerol formado, mediado por PECK, na adaptação destes helmintos ao ambiente osmótico e energético do hospedeiro.

- Calpaína (GeneBank Acc. No. M74233.1). Pertencente à família de proteínas cálcio dependente. É uma cisteína protease, expressa ubiquamente na maioria dos organismos. Ativa proteína quinase C (PKC) e está envolvida em degradação do citoesqueleto. Calpaína, em esquistossomas, pode estar envolvida na sinalização para o *turnover* da membrana e tem sido intensamente estudada como alvo para vacinas anti-esquistossomóticas (Hota-Mitchell *et al*., 1997).

❖ Proteína Putativa Ligante de Hexâmero (HEXBP) (OrganismDB: Tag 623). Proteína dedo de zinco com motivo CCHC, que se liga a hexâmeros de DNA.

❖ Putativa Fibrilarina (SchistoDB: Smp_044280.1). Este produto gênico é um componente de uma pequena ribonucleoproteína nucleolar (snRNP) que parece participar no processamento de RNA pre-ribossomal. Foi demonstrado por Amim e colaboradores (2007) que seu silenciamento em células HeLa interferia com a proliferação e crescimento celular, podendo estar envolvida com biossíntese ribossomal.

❖ Fator Elongação 1α (EF1α) (GeneBank Acc. No. Y08487.1). É um componente essencial do aparato de tradução eucariótico e uma proteína ligante a GTP que catalisa a ligação dos aminoacil-tRNAs no ribossomo (Tatsuka *et al.*, 1992).

❖ RHO 1 GTPase (GeneBank Acc. No. AY158212.1). Pertencente à família das RHO GTPases pequenas envolvidas em transdução de sinais, que regulam o citoesqueleto de actina. Estão envolvidas em uma ampla variedade de funções celulares como polarização celular, reorganização de citoesqueleto e migração celular. Estas estão inativas quando ligadas a GDP (Spencer *et al.,* 2001; Hoffmann *et al*., 2001). Apesar de RHO de *S. mansoni* apresentar alto grau de similaridade com seus ortólogos em diferentes espécies, pequenas diferenças na sua seqüência podem determinar interações e regulações diferenciais no parasita (Aguiar *et al*., 2006).

❖ RHO 2 GTPase (GeneBank Acc. No. AY158214.1). Apresenta funções similares às funções descritas previamente para RHO 1.

❖ Cálcio ATPase 2 (SMA2) (GeneBank Acc. No. AF074400.1). Isoforma da bomba de cálcio ATPase 2+ que preenche o retículo sarcoplasmático com cálcio, utilizando a energia proveniente da quebra de ATP. O cálcio é necessário para manter a

homeostase e mobilidade celulares e para a sinalização. Até hoje foram caracterizadas três SMAs em *Schistosoma*. (Da'dara *et al*., 2001; Talla *et al*., 1998).

- SPO1 (GeneBank Acc. No. AF109181). Gene também chamado Sm16, preferencialmente expresso em estágios larvais do parasito. Parece fazer parte do mecanismo de imunorregulação na interação parasita-hospedeiro (Rao & Ramaswamy, 2000).

- PKCβ (GeneBank Acc. No. AY337620.1). Bahia e colaboradores (2006) mostraram que miracídios e esporocistos apresentam os maiores níveis de mRNA e de proteína de PKCβ, respectivamente. Essa proteína, dependente de cálcio, é ativada pela quebra de fosfatidil-inositol 4,5-bisfosfato pela fosfolipase C, gerando DAG e IP3. DAG se liga em um dos domínios da proteína aumentando sua afinidade por fosfolipídios, liberando seu domínio catalítico e possibilitando esta de fosforilar seus substratos, desencadeando uma série de sinais que controlam os mais diversos processos celulares.

- Receptor de PKC (GeneBank Acc. No. AF422164.1). São dímeros que são estimulados por uma grande gama de ligantes, fazendo assim, trans-autofosforilação e sendo responsáveis por ativação de diversas cascatas de transdução de sinais. (Bahia *et al*., 2006)

- Putativo *Zinc Finger* com Domínio DHHC (DHHC) (SchistoDB: Smp_179300.3). São proteínas altamente conservadas, que possuem dedos de zinco com domínios ricos em cisteína. Parecem transferir o lipídeo palmitato para diferentes proteínas, estando envolvidas com espermatogênese em *C. elegans* (Gleason *et al*., 2006).

- Miosina-cadeia leve (GeneBank Acc. No. AF071011.1). Parte da proteína miosina responsável pela mobilidade baseada em actina, transporte vesicular e contração vacuolar.

- Calreticulina (GeneBank Acc. No. L24159.1). Muitas funções têm sido designadas para esta proteína. Esta é uma conhecida proteína ligante de cálcio, participando na manutenção da homeostase celular. Além disso, age como chaperona, se ligando à proteínas erroneamente enoveladas e prevenindo que elas sejam exportadas do retículo endoplasmático para o complexo de golgi. É também uma lectina extracelular, entre muitas outras funções. Dificilmente uma única proteína desempenha tamanha complexidade de funções (Coppolino & Dedhar, 1998).

- Canal de cálcio ativado por alta voltagem, subunidade alfa (Cav2A) (GeneBank Acc. No. AF361883.). Canais de cálcio contribuem para propagação de impulso em células excitáveis e regulam os níveis de cálcio intracelular. São proteínas de membrana heterodiméricas e esta subunidade é a unidade formadora do poro (Kohn, 2001).

- Canal de cálcio ativado por alta voltagem, subunidade beta 2 (GeneBank Acc. No. AY277532.1). A subunidade beta do canal de cálcio é moduladora da unidade formadora do poro, a subunidade alfa (Kohn, 2001).

Outros genes foram inseridos na triagem de acordo com interesses específicos dos laboratórios envolvidos. Os produtos protéicos dos genes descritos a seguir estão sendo caracterizados por nosso grupo:

- Glicoproteína K5 (GeneBank Acc. No. AY903301.1). É uma glicoproteína secretada do ovo de *S. mansoni*. Acredita-se ser um alvo potencial para imunodiagnóstico (Hamilton *et al*., 1999).

- Zinc Finger 1 (SmZF1) (GeneBank Acc. No. AF316827.1.). Em 2004, Calzavara-Silva e colaboradores demonstraram que SmZF1 (uma proteína contendo três dedos de zinco) liga-se ao DNA de maneira específica. Esta proteína também foi capaz de se ligar ao RNA, mas com uma afinidade aproximadamente 10 vezes menor.

Posteriormente, SmZF1 foi visualizada e detectada principalmente no núcleo de células do parasita. Em ensaios de ativação da expressão gênica foi demonstrado que SmZF1 é capaz de duplicar a expressão do gene que codifica para luciferase em células COS-7 (Calzavara-Silva *et al*, manuscrito em preparação). Tais resultados sugerem fortemente que SmZF1 possa atuar como um fator de transcrição em *S. mansoni.*

❖ SmRing Box (SmRbx) (GeneBank Acc. No. DQ466078.1.). Santos e colaboradores (2007) demonstraram que esta proteína de *S. mansoni* interage com a proteína culina 1 de levedura e complementa a função deste gene em leveduras nocauteadas para o ortólogo de SmRbx. Esta é, provavelmente, parte de um complexo que catalisa a ubiquitinação de proteínas e as direciona para degradação pelo proteassomo 26S.

❖ Glutationa Peroxidase (GPx) (GeneBank Acc. No. M86510.1). GPx é uma enzima que protege estruturas celulares contra danos oxidativos. Esta proteína catalisa a redução do nível de peróxido de hidrogênio, mantendo a membrana celular e o DNA íntegros. Funciona em tetrâmeros, onde cada um carrega um átomo de selênio. A atividade de GPx em *S. mansoni* aumenta significantemente nas fases intra-hospedeiros e é positivamente correlacionada à resistência a antioxidantes (Williams *et al*., 1991). Esta enzima é outro possível alvo para o desenvolvimento de vacinas anti-esquistossomóticas.

❖ Tiorredoxina Peroxidase 1 (TPx-1) (GeneBank Acc. No. AF121199.1) e Tiorredoxina Peroxidase 2 (TPx-2) (GeneBank Acc. No. AF157561.1). Enzimas envolvidas no balanceamento e sinalização redox, afetando fosforilação protéica, regulação transcricional e apoptose. Esta enzima tem a propriedade de utilizar tiorredoxina como doador de elétrons (Kwatia *et al.*, 2000). Em *S. mansoni*, a tiorredoxina é mantida no seu estado reduzido através da enzima dependente NADPH, Trx-GSH redutase (TGR) (Sayed & Williams, 2004).

- Superóxido Dismutase (SOD) (GeneBank Acc. No. M27529.1). É uma metaloprotease que catalisa a conversão de dois radicais superóxidos a peróxido de hidrogênio e oxigênio molecular, como defesa contra a toxicidade do ânion superóxido. SOD pode ocorrer em três formas: citossólica, mitocondrial e extracelular (Sirmuda *et al*., 1988). Foi testada como alvo para vacinação e promoveu uma redução de 54% no número de vermes (Cook *et al*., 2004).

- Calmodulina 1 é conhecida por ativar uma série de processos essenciais em eucariotos, incluindo formação de microtúbulos, secreção e metabolismo de nucleotídeos cíclicos (Thompson *et al*., 1986).

Como controle inespecífico foi sintetizado dsRNA com 500 pares de base para a proteína fluorescente verde (GFP). Este foi amplificado a partir do vetor pAcGFP (Clontech). Moléculas de RNA dupla fita foram sintetizadas usando iniciadores gene-específicos desenhados para conter a inserção do promotor da RNA polimerase T7 em cada ponta 5’, como descrito por Clemens e colaboradores 2000. Estes foram, então, utilizados para amplificar cada um dos genes por PCR (como descrito abaixo), utilizando como molde bibliotecas de cDNA de esporocistos ou apenas, o cDNA deste organismo (já disponível no laboratório). Os iniciadores foram desenhados para amplificar fragmentos de aproximadamente 500 pares de bases, com exceção de transcritos menores.

As seqüências dos iniciadores utilizados estão descritos abaixo (Tabela 1) e as reações de amplificação foram feitas da seguinte forma: Adicionou-se 1µl de cada iniciador por reação de PCR (concentração estoque 10pmol/µl), 0,5µl de cDNA ou 3µl de biblioteca de cDNA de esporocistos, 15µl de PCR Master Mix 2x (Promega), num volume total de 30µl de reação. Os moldes foram desnaturados a 96ºC por 5 minutos e posteriormente realizados 40 ciclos de amplificação, da seguinte forma: - Desnaturação a 96ºC por 30 segundos, anelamento dos iniciadores a 52ºC por 45 segundos, e extensão a 72ºC por 45 segundos. Para a otimização e amplificação de alguns transcritos a reação foi modificada com a adição de 0,4µl de GoTaq DNA polimerase (Promega) e modificações nas temperaturas de anelamento. Ao final dos ciclos, os produtos foram estendidos por 7 minutos a 72ºC.

Tabela 1: Seqüência dos iniciadores com cauda do promotor T7, utilizados para a amplificação de transcritos e síntese de dsRNAs

Gene	Forward 5'-3'	Reverse 5'-3'
GFP-control	taatacgactcactatagggGTGCCCATCCTGGTCGAG	taatacgactcactatagggCCGTTCTTCTGCTTGTCGG
Smad1 (gb: AF215933.1)	taatacgactcactatagggTTGGTTGGAAACAAGGAGACG	taatacgactcactatagggTTGCTGAGTCTGTCATCGGG
Smad2 (gb: AF232025.1)	taatacgactcactatagggTGACTTCAGTAGCTTATCAAGAACCTG	taatacgactcactatagggTTTGTCGGCGATAATCAGCA
PKC Receptor (gb: AF422164.1)	taatacgactcactatagggATGCTACCCAGCCGGATCTT	taatacgactcactatagggGTCCGTAATGTGTTGTGCGC
Calcineurina B (gb: AJ276885.1)	taatacgactcactatagggAATTTGCGTTTAAAATATACGACATGG	taatacgactcactatagggCCGGGAGAGGAGAGACCTTG
RHO1(gb: AY158212)	taatacgactcactatagggTTGGAGATGGTGCATGCG	taatacgactcactatagggCGGCTCGAGTAGCTGCTACG
RHO2 (gb: AY158214.1)	taatacgactcactatagggTTATTCGTCGTGTACTTCATGCATTT	taatacgactcactatagggCGAGAATATAACATTTAGCCGAACAA A
Proteína Kinase C Beta (PKCβ) (gb: AY337620.1)	taatacgactcactatagggTTGGATAAGTATATTGGTGTTAATTATTT CACA	taatacgactcactatagggTTGATGATCAAGTTAACGAGCAGATA
Smad4 –forward (gb: AY371484.1)	taatacgactcactatagggTTGGATCAACAGGTCGGTGA	taatacgactcactatagggTGTGCCAGGTAAAGATCCTGG
Canal de Cálcio (gb:	taatacgactcactatagggATCAACTTCCAGATGCTTGTGAAC	taatacgactcactatagggCCAACAGATGTAGGATTTATGCGA

AY277532)		
SCMEG (gb: M86399.1)	taatacgactcactatagggAAAGCCTGTCGTGTTTGGGA	taatacgactcactatagggAATATCACAACCGGCGTGAGA
14-3-3 (gb: U24281.1)	taatacgactcactatagggGGAAGCGAAATGAAAGCGG	taatacgactcactatagggGTCACTGGCCCATAACGTCA
Calpaína (gb: M74233.1)	taatacgactcactatagggCACTGCAGATGGAGAATTTTGG	taatacgactcactatagggTCGAATGTACTCGGAATAACAACATAT
Calreticulina (gb: L24159.1)	taatacgactcactatagggAATTTCACGGCGAATCACCTTA	taatacgactcactatagggGGACGTTCCCACTCACCATC
Sma2 (gb: AF074400.1)	taatacgactcactatagggCACTTTATGCCATGGAGACTTATGTTA	taatacgactcactatagggCCTTATATTCGAACACTCTTTCATGAA
Cav2a (gb: AF361883.1)	taatacgactcactatagggCAGATAAAAGCAGAAGAGGAATGGA	taatacgactcactatagggAAACCGTTGGACTATCTCGTTGAT
Pepck (gb: AF120929.1)	taatacgactcactatagggCATGTGAATTGGTTCCGTTTAAATAA	taatacgactcactatagggATTAATGTCTACGAACAACAATGAAAGAA
Fator de Elongação 1α (EF1α) (gb: Y08487.1)	taatacgactcactatagggGTTATTGATGCACCTGGGCA	taatacgactcactatagggTTGCAAGGGAATTCTGAGTGG
Cadeia leve de Miosina (gb: AF071011.1)	taatacgactcactatagggCGAGCTTTCTTACTTAAATAACATCATGAG	taatacgactcactatagggTCTTTTACTTGGAAGGACCAGCC
HEXBPzinc	taatacgactcactatagggACGTCCCGGTCATTATGCTC	taatacgactcactatagggCATAACCATGGCACTTATAGCACTG
DHHCzinc	taatacgactcactatagggGGAAAAGATTCAATGCCCAATTC	taatacgactcactatagggGGAACAGCCAATGCTTGATGT
Lactato Desidrogenase	taatacgactcactatagggCCAACAGATATCGAGCCTCGTT	taatacgactcactatagggAGGTAAGGGCGACACCCAA

(gb:U87629.1)		
SmRing Box (gb: DQ466078.1)	taatacgactcactatagggAAAGGTCTTCAGCGCGC	taatacgactcactatagggTTATCTGAACAGGTACAACCGTAAC
Fibrilarina	taatacgactcactatagggGCCAAAGGCAAAGAAGACTG	taatacgactcactatagggATGGATATAACGGCGTGACC
Glutationa-S-transferase 26, (SmGST26) (gb: M73624.1)	taatacgactcactatagggGTGTGGCGAAACGAAAAGTT	taatacgactcactatagggCCAACCTTGTAGAGGCCATT
Glutationa peroxidase (GPx) (gb: M86510.1)	taatacgactcactatagggCCGTGGTCACGTTTGTCTAA	taatacgactcactatagggCAAATGGCAACCAATGAACA
Glutationa-S-transferase 28 (SmGST 28) (gb: S71584.1)	taatacgactcactatagggATTGGCCAAAAATCAAACCA	taatacgactcactatagggTTTCCTGTCGACCCTTTCAG
Tiorredoxina Peroxidase 2 (TPx-2) (gb: AF157561.1)	taatacgactcactatagggCCTGCTCCTGATTTTGAAGG	taatacgactcactatagggCCAGTTCGCTGGACAAACTT
Tiorredoxina peroxidase (TPx-1) (gb: AF121199.1)	taatacgactcactatagggCCTGCACCAGAATTCAAAGG	taatacgactcactatagggCTTCACCATGCTTCTCCACA
Superoxido Dismutase (SOD) (gb: M27529.1)	taatacgactcactatagggTTTGATCCGGCTATTGCTTC	taatacgactcactatagggTGGTACGTCCAACAAAAATCA
SPO1 (gb: AF109180.1)	taatacgactcactatagggTCTTCCAGTAAAATGAAAGTGACG	taatacgactcactatagggTTTTCATCAATCTTTATTATTTGCTCA

Calmodulina 1	taatacgactcactatagggATGGCTGATCAGTTGACCGA	taatacgactcactatagggAGGGTTGGGAAGGAGCACCCCC
K5 (gb:AY903301.1)	taatacgactcactatagggCCTTCTAATAAACGGAGTCACTTTACTG	taatacgactcactatagggAACCATCGAATTGTCATCAAATCTAAT

Após amplificação, os produtos da reação de PCR foram aplicados em gel de agarose 1% e purificados utilizando QIAquick Gel Extraction kit (Qiagen), segundo instruções do fabricante. Visando confirmar a autenticidade dos produtos de PCR obtidos, estes foram sequenciados no centro de Biotecnologia (University of Wisconsin-Madison) no aparelho Applied Biosystems 3730XL automated DNA analyzer (Applied Biosystems), utilizando os mesmos iniciadores descritos anteriormente, na quantidade final de 1pmol. Para a reação de sequenciamento 0,5µl do produto de PCR purificado foi adicionado a 6µl de água, 1µl de Bigdye (Applied Biosystems) e 1,5µl de Bigdye Buffer (Applied Biosystems). Os moldes foram desnaturados a 96ºC por 3 minutos e posteriormente realizados 35 ciclos de amplificação, da seguinte forma: - Desnaturação a 96ºC por 10 segundos, anelamento dos iniciadores a 50ºC por 15 segundos, e extensão a 60ºC por 2 minutos. Ao final dos ciclos, os produtos foram estendidos por 7 minutos a 72ºC. Para a precipitação foram adicionados aos produtos: 10µl de CleanSeq beads (Agencourt Bioscience) e 80µl de etanol 80%. Os tubos foram colocados em placa magnética por 2-3 minutos e o líquido retirado. Após este procedimento, 200µl de etanol 80% foi adicionado e da mesma maneira retirado. Os tubos foram removidos da placa magnética e adicionado 50µl de água. As seqüências obtidas foram submetidas à pesquisa de similaridade em bancos de dados usando a ferramenta *Basic Local Alignment Search Tool* (http://blast.ncbi.nlm.nih.gov/Blast.cgi), para confirmação de que os produtos do sequenciamento se tratavam realmente dos genes escolhidos.

Os dsRNAs foram então sintetizados utilizando o kit T7 RiboMAX Express RNAi (Promega) seguindo protocolo do fornecedor. As amostras foram deixadas durante 16 horas a 37ºC com o intuito de aumentar a concentração de dsRNA. Os dsRNAs foram tratados com DNAse por 30 minutos a 37ºC, purificados com fenol-clorofórmio e precipitados com isopropanol. Os produtos da reação foram dissolvidos em água tratada com dietilpirocarbonato (DEPC). Posteriormente à síntese, os dsRNAs foram aquecidos a 70ºC e resfriados em temperatura ambiente para se anelar corretamente em fita dupla. A densidade óptica OD260/280 nm foi medida utilizando Nanodrop Spectometer ND-1000 (Nanodrop) para estimativa da concentração e a integridade verificada em gel de agarose 1% não desnaturante. Os dsRNAs foram armazenados a -20ºC.

3.3 - Exposição dos parasitos aos dsRNAs

Para o tratamento dos parasitos com dsRNA, soluções contendo 100 nM de dsRNA foram preparadas em CBSS+ e adicionadas em placas (Costar) de 96 (100µl) ou 24 (250µl) poços. Os parasitas foram lavados com água de lagoa estéril e, após a última lavagem, os miracídios decantados foram ressuspendidos em CBSS+ contendo os antibióticos penicilina 60µg/ml e estreptomicina 50µg/ml e quantificados por contagem em microscópio invertido. Foram adicionados aproximadamente 500 miracídios em 200µl de CBSS+ (volume final) por poço nas placas de 96 poços (primeira etapa-observação de fenótipos) e 7000 parasitos em 500µl de CBSS+ nas placas de 24 poços (segunda etapa - quantificação dos transcritos por PCR quantitativo). A concentração final de dsRNA foi de 50 nM após a adição dos parasitas. Cada amostra foi ensaiada em duplicata. Ao quarto dia de incubação, mais 10nM de dsRNA foi adicionado em cada poço.

Em geral, os parasitos foram cultivados por sete dias, como descrito por Boyle *et al.*, 2003, pois este seria o tempo em que o silenciamento estaria maximizado nos esporocistos. Após sete dias de exposição aos tratamentos com dsRNAs, os parasitos foram observados em microscópio invertido Nikon Eclipse TE 300. Câmera Nikon (Nikon Corporation) e o software Metamorph versão 7.0 (Meta Imaging series, Molecular Devices) foram utilizados para obtenção de fotos e medidas de tamanho. Estes também foram cultivados por um período maior (14 dias) para a observação da possibilidade de modificações nos esporocistos primários, efeitos na sua transformação, ou na progênie originária de parasitos tratados.

3.4 - Captação de dsRNA pelos parasitas

Para observar e certificar que os RNAs dupla fita estavam sendo captados pelos esporocistos, dsRNA para os genes Smad 4, GFP e Lactato Desidrogenase (LD) foram marcados com rodamina, utilizando o kit Label IT, CX-Rhodamine labeling Kit (Mirrus), de acordo com as especificações do fabricante. Após sete dias de exposição aos dsRNAs marcados, os parasitos foram observados em microscópio invertido Nikon

Eclipse TE 300 excitados com laser no comprimento de onda 572nm. Câmera Nikon (Nikon Corporation) e o software Metamorph versão 7.0 (Meta Imaging series, Molecular Devices) foram utilizados para obtenção de fotos e medidas de tamanho. O número de parasitas fluorescentes foi contado para cada tratamento. Após as medidas, foram feitas as seguintes comparações: (1) comprimentos dos esporocistos tratados com dsRNAs (GFP, Smad4 e LD) marcados e não marcados e (2) comprimento dos parasitos tratados com dsRNA (Smad4 e LD) marcados comparados aos parasitas tratados com dsRNA-GFP marcado e Branco. A análise de significância estatística foi feita utilizando o teste de Mann-Whitney (Wilcoxon-Sum of Ranks, $p<0,05$).

Testamos a absorção de dsRNA pelos esporocistos utilizando o reagente de transfecção GeneSilencer (Genlantis). Para isto, foram feitas duas preparações: (A) 1µl de reagente adicionado a 25µl de CBSS+; (B) 2,5µl de diluente adicionado a 15µl de CBSS+ e dsRNA 2 vezes concentrado. A preparação B foi incubada por 5 minutos e, então, misturada à preparação A e deixada incubando por 30 minutos. O reagente de transfecção contendo o dsRNA foi dividido em dois poços e o volume completado para 100µl. Este experimento foi repetido 3 vezes.

3.5 - Triagem de fenótipos

Na triagem dos fenótipos, procuramos observar mudanças no tamanho e forma, lise do tegumento e sobrevivência, perda ou aumento de movimento, falha/atraso na transformação dos parasitas e possivelmente, a ausência de indivíduos se transformando em esporocisto primário ou progênie (esporocisto secundário).

A triagem foi conduzida em duas etapas: para uma primeira observação dos possíveis fenótipos após silenciamento, os parasitos foram transformados e cultivados em placas de 96 poços. Todos os tratamentos foram feitos em duplicatas e os parasitos observados e fotografados com câmera Nikon (Nikon Corporation) todos os dias até o sétimo dia, ou até o décimo quarto dia (especificamente para a observação de esporocistos primário ou progênie). As imagens obtidas no sétimo dia foram medidas utilizando o software Metamorph versão 7.0 (Meta Imaging series, Molecular Devices). Os tamanhos dos esporocistos tratados foram comparados com parasitos tratados com os controles GFP e Branco. Análises estatísticas foram feitas utilizando o teste de

Mann-Whitney (Wilcoxon-Sum of Ranks, $p<0,05$, $N\geq3$), pois as amostras não exibiam uma distribuição normal. Posteriormente, os parasitos passaram a ser cultivados em números maiores e em placas de 24 poços para a extração de RNA e proteínas.

3.6 - Estudo do silenciamento em nível de transcrição

Os níveis dos transcritos dos genes silenciados nos esporocistos foram avaliados por Real Time PCR (q-PCR). Para o experimento de PCR quantitativo os RNAs de aproximadamente 7000 parasitos (por amostra) foram extraídos com Trizol Reagent (Invitrogen), segundo protocolo do fabricante. Amostras foram ressuspendidas em 6µl de água DEPC e tratadas com DNAse por 30 minutos a 37ºC, utilizando o kit DNA Free (Ambion), segundo manual do fabricante. Posteriormente, a densidade óptica OD260/280 do RNA tratado foi medida utilizando Nanodrop Spectometer ND-1000.

Para a síntese de cDNA, foram utilizadas as mesmas quantidades de RNA total de cada amostra dentro de um mesmo experimento. No entanto, haviam variações de quantidade entre diferentes experimentos (~0,5µg-1,5µg). Os cDNAs foram sintetizados em um volume total de 20µl por reação usando o Kit Superscript III First-strand Synthesis System RT-PCR (Invitrogen). Os produtos foram tratados com RNAse H e incubados a 37º C por 20 minutos, após a síntese. Controles das reações, com RNA das amostras sem adição de transcriptase reversa foram feitos para observação de contaminação das amostras com DNA genômico.

Os iniciadores para amplificação dos transcritos foram desenhados, sempre que possível, para as regiões externas àquelas utilizadas no desenho dos iniciadores para produção dos dsRNAs, seja na porção 5' ou 3' do transcrito. Os amplicons possuíam, em média, 100 pb. Os genes Gliceraldeído 3-fosfato Desidrogenase (GAPDH) e alfa-Tubulina foram utilizados como controle endógeno normalizador. Para se estabelecer a concentração em que os iniciadores seriam mais eficientes na amplificação, os mesmos foram testados em diferentes combinações de concentrações como mostrado na tabela 2 a seguir:

Tabela 2: Concentrações de iniciadores testadas para otimização da reação de q-PCR.

Iniciadores forward concentração (nM)	Iniciadores reverse concentração (nM)			
	50	300	600	900
50	50/50 nM	50/300 nM	----------------	50/900 nM
300	300/50 nM	300/300 nM	----------------	300/900 nM
600	----------------	-----------------	600/600 nM	-----------------
900	900/50 nM	900/300 nM	----------------	900/900 nM

As concentrações ideais encontradas foram de 600nM ou 900nM para todos os pares de primers forward e reverse. As reações de Real Time PCR foram realizadas no aparelho Applied Biosystems 7500 Real Time PCR System (Applied Biosystems), utilizando 12,5µl de SYBR GREEN PCR Master Mix (Applied Biosystems), 2,5µl de cDNA (~0,5µg-1,5µg) e 10µl de iniciadores forward e reverso (240 nM e 360nM concentração final por reação), em um volume final de 25µl por reação, em placas de 96 poços (96-Well Optical Reaction Plate, ABI PRISM, Applied Biosystems). Para a análise dos níveis do transcrito de um determinado gene em estudo foram utilizadas as seguintes amostras: cDNA de vermes tratados com dsRNA do gene em questão, cDNA de vermes tratados com dsRNA-GFP (controle 1) e cDNA de vermes tratados com um dsRNA não relacionado, correspondendo a um gene qualquer do parasita (controle 2). Cada amplificação foi feita em triplicata. Na mesma placa de reação, triplicatas de todas as amostras acima relacionadas foram amplificadas com iniciadores para os genes GAPDH e alfa-Tubulina (controle para normalização). Em cada placa havia um controle negativo (contendo todos os reagentes para a síntese de cDNA e para a amplificação, exceto o RNA) e controle de DNA genômico das amostras (onde todos os reagentes foram adicionados, com exceção da transcriptase reversa).

Os resultados foram normalizados e os níveis de expressão de cada gene em parasitos silenciados foi comparado com o nível de expressão de transcritos do mesmo gene em parasitas tratados com dsGFP (controle). Os experimentos de q-PCR foram repetidos pelo menos três vezes ($N \geq 3$) para cada tratamento de dsRNA. A significância dos resultados foi analisada pelo teste estatístico Wilcoxon-Sum of Ranks (Mann-Whitney, significância $p<0,05$) utilizando os ΔΔCTs obtidos, ou seja, a diferença no nível de transcritos após a normalização dos dados (Livak & Schmittgen, 2001).

As seqüências dos iniciadores utilizados para o q-PCR se encontram listados a seguir (Tabela 3):

Tabela 3: Seqüência e posição dos iniciadores utilizados nas reações de q-PCR

Genes	Forward 5'-3'	Posição	Reverse 5'-3'	Posição
Alpha-tubulina	CAAATGGGAAATGCTTGTTG	43	TGAACGAGTCATCACCACCT	148
GAPDH	TCGTTGAGTCTACTGGAGTCTTTACG	605	AATATGAGCCTGAGCTTTATCAATGG	670
Calcineurina B	TTGCAAGGGTTATCGAGATTT	187	TTGGCTTCTTTCTCACCCTT	295
PKC B	CTTGGATGTGGTTCAGATGG	327	TCACGTGGTGATAAAGTAACTGG	454
SmZF1	ACTTCTCTCAGAAATCCAGCCT	2	TGGAGAGGATTATACAATCTGGTT	98
SmRing Box	GGCATCCCTCAGTGAGAATAA	55	TCCGGCAGATAGCACAATTA	158
Cadeia leve de Miosina	CCGTGTCCTTCTTTCTATGTTG	2	AGGATTCATGGAATTGTGAAAA	100
SPO1	CCGAGTGAAAAAGACATGGA	50	TGACCGTTTTATTTCGTTGC	142
Glutationa-S-Transferase (GST26)	TCAAAGGCCTTGTACAACCA	52	CGTCATTGCGATCATACAAA	135
Smad1	CCGGAAACAGTGGAACTTCT	54	CTTGATTGGGTTGAGTTTGG	155
Smad2	TTTAGCTGCCCTCCTAGCTC	726	TATACGCGTCAAGTGCATCA	832

RHO2	CTTCTGTCCAAATGTTCCGA	316	ATTCCTGACGAACTTTGGCT	413
Fator de Elongação 1 A	ATGGCGATGCAGCTAACATA	432	TCATATCTCGAACGGCAAAG	533
Lactato Desidrogenase	CCACTTGAACTTGGTGACGAT	901	CATCAGCAACCATGAGTCGT	994
Tiorredoxina Peroxidase (TPx-1)	GTGAAGTGTGTCCGGTGAAC	19	CGGTGATCAATGAAGAACGA	108
Tiorredoxina Peroxidase (TPx-2)	TCTTTGAGAAACATGGCGAA	38	GAGAGAAGCAACAGGATCAGG	120
Glutationa-S-Transferase (GST-28)	CTGGCAAGTATCCTGAGATCC	90	TCGTTACACCGAGCTTTCTG	219
Glutationa Peroxidase (GPx)	TCTCGCTATATGACGATGGC	35	GCAATCAGGTGCACACAAA	113
Superoxido Dismutase (SOD)	GATCCGGCTATTGCTTCATT	106	CTGCCACGCTTCCATTAAC	202
PEPCK	AAGTTGGTCGAATTGGGTTC	250	GGCACATTTGGCACAGTATC	376
Fibrilarina	AGAGAGGCGGGTTTAGAGGT	131	CTCGTGGTGTTCCTCTGTTG	241
Calpaina	TCACTGGGTGGAAGTGCTTA	784	TCCAACAGAGCTGACCAGAA	894
14-3-3	TTCATATCGCCAAACTTGCT	56	CGTTCTTCGTTTCCTAAATTCC	158

K5	TTGCGAAGCTCAGTCTCCTA	66	TATTCGGACTCAACGCCATA	187
Cav2A	AACATCACAGGGCAGGTTTA	346	AAGGCACTAGCGCTTGGAT	436
Calmodulina 1b	GCGATGGGACCATCACCACC	----------	AAGGAGCTGGGCACTGTGATA	-----------
Calmodulina 99	ATAGGAGATCATCGAGGCCTTC	----------	CGAGGCTGACATCGACGGTG	-----------

3.7 - *Western Blot*

Para verificar o decréscimo do nível protéico em parasitos silenciados com dsRNA de GST26, EF1-α, TPx-1 e TPx-2 utilizamos anticorpos comerciais compatíveis com as proteínas do organismo (GST(91G1) #2625X, 1:1000; e eEF1A #2551, 1:500; Cell Signaling Technology), ou anticorpos monoclonais gentilmente cedidos pelo Dr. David Williams (TPx 1 e 2, 1:30) e fizemos *Western blot* utilizando protocolo descrito em Sambrook *et al*; 1989, com modificações. As proteínas totais de parasitos tratados com dsRNA foram extraídas utilizando Trizol Reagent (Invitrogen), segundo protocolo do fabricante. O precipitado de proteínas foi dissolvido em 20μl de solução tampão Protein Soluble Buffer (3M Ureia, 2% CHAPS, 40mM Tris). As quantidades de proteínas obtidas após cada extração foram dosadas por *Dot blot*, sendo que 1μl de diluições seriadas (1:2) em PBS da proteína foram aplicadas em membrana de nitrocelulose 0,2μm Trans-Blot Transfer Medium (Bio-Rad). Como padrão, BSA comercial (Pierce) foi aplicada na membrana nas concentrações de 2μg/μl, 1μg/μl, 0,5μg/μl, 0,25μg/μl e 0,125μg/μl. A membrana foi corada com MemCode™ Reversible Protein Stain Kit for nitrocelulose membranes (Thermo Scientific) e, posteriormente, lavada com água destilada.

Estabelecidas as concentrações de proteína total de cada amostra, aproximadamente 8μg destas foram colocadas em tampão de corrida 4x (Bio-Rad) e fervidas por 10 minutos. A seguir, as amostras foram separadas por SDS/PAGE 12,5%, usando equipamento Mini-Protean II (Bio-Rad). Como padrão de peso molecular foi aplicado 7μl de Precision Plus Dual Color (Bio-Rad). Após separação, as amostras foram transferidas para membrana de nitrocelulose por sistema semi-seco, Hoefer TE 70 semi-dry transfer unit (Amersham Biosciences). Para transferência, papel Whatmann, membrana e gel foram imersos em tampão de transferência (25mM Tris Base, 0,2M Glicina, 20% Metanol, pH:8,5) e submetidos à eletrotransferência a 100 mA por 1 hora e 15 minutos. Posteriormente, a membrana foi corada com MemCode™ Reversible Protein Stain Kit for nitrocelulose membranes (Thermo Scientific), para que pudéssemos detectar se a transferência havia ocorrido satisfatoriamente e se tínhamos quantidades similares de amostras em todos as canaletas. A membrana foi descorada

utilizando o mesmo kit que contém soluções de descoloração e em seguida bloqueada com albumina 5% em TBS (2,42g Tris base, 8g NaCl, pH:7,6) por 16 horas a 4ºC.

A membrana, após bloqueio, foi incubada com os anticorpos primários e como controle de concentração das amostras e normalização foram utilizados anticorpos anti-alfa-Tubulina (05-829, Millipore) ou anti-GST26. Os anticorpos primários foram diluídos 1:1000 em tampão de bloqueio e deixados por 16 horas a 4ºC. Após extensiva lavagem com TBS-T (2,42g Tris base, 8g NaCl, pH:7.6, 0,1% Tween), as membranas foram incubadas com anticorpos secundários IgG de carneiro anti-coelho ou anti-camundongo, dependendo do anticorpo primário utilizado, conjugados com fosfatase alcalina (diluído 1:10.000). A detecção colorimétrica da imunoreatividade foi feita usando 5-bromo-4-cloro-3-indolil fosfato (BCIP) e nitro-azul tetrazolio (NBT), diluídos em tampão AP (0.1M Tris, 0.1M NaCl, 0.05M $MgCl_2$, pH:9.5, filtrado).

3.8 – Imunolocalização

Utilizamos imunolocalização para compararmos os níveis de proteínas GST26, TPx 1 e 2 e Fator de Elongação 1α *in situ* em esporocistos silenciados para estes genes e em parasitas controle. Para o experimento de imunofluorescência utilizamos o seguinte protocolo, estabelecido laboratório do Dr. Yoshino. Os parasitas foram lavados 5 vezes em CBSS+ (suplementado com penicilina e estreptomicina) nas placas onde foram cultivados, para retirada das placas ciliares ou *plate cells*, perdidas pelos miracídios durante a transformação em esporocistos. Parasitas lavados foram transferidos para tubos siliconizados e centrifugados por 2 minutos a 1600 rpm. O sobrenadante foi retirado e 600µl de tampão PT (Tampão T: 2% Triton-X100 diluído em sPBS, Tampão P: 4% de paraformaldeído (PFA) estoque, combinados 1:1 para uma concentração final de 2% PFA, 1% Triton-X100/sPBS) foi adicionado a cada amostra. Estas foram incubadas por 5 minutos, sob agitação, em temperatura ambiente. Os tubos foram centrifugados a 1800 rpm por 2 minutos. O sobrenadante foi então removido e 700µl de tampão PT adicionado. As amostras foram incubadas por 16 horas a 4ºC, sob agitação.

No segundo dia, os parasitas foram centrifugados a 1600rpm por 2 minutos e lavados por 5 vezes com PBS com intervalos de 10 minutos por lavagem; após as quais, foram centrifugados a 2000rpm por 2 minutos. Posteriormente a lavagem final, o

sobrenadante foi retirado e 700µl de tampão de bloqueio adicionado (5% de soro de carneiro, 0,02% de azida diluídos em PBS). Após incubar por 20 minutos, as amostras foram centrifugadas por 2 minutos a 1800 rpm, o sobrenadante retirado e 1,2ml de tampão de bloqueio adicionado. Os tubos foram, novamente, deixados por 16 horas a 4ºC, sob agitação. Os parasitas foram centrifugados a 1600 rpm por 2 minutos, o sobrenadante removido e 200µl de tampão de bloqueio contendo anticorpos primários de GST26 (1:200), TPx 1 e 2 (1:30), EF1α (1:25) foi adicionado e incubado por 16 horas a 4ºC, sob agitação.

Novamente os parasitas foram centrifugados por 2 minutos a 1800 rpm. Lavados com PBS por 5 vezes, com intervalos de 10 minutos, e após cada lavagem, estes foram centrifugados por 2 minutos a 1800 rpm. Posterior a última lavagem, os esporocistos foram ressuspendidos em 600µl de Solução de Detecção (1,15µl de anticorpo conjugado com Alexa Fluor 488, 3µl de faloidina e 3µl de DAPI em 600µl de tampão de bloqueio). As amostras foram incubadas em agitador a 4ºC, por 16 horas. No dia seguinte, os esporocistos foram lavados por 5 vezes com PBS e centrifugados por 2 minutos a 3000 rpm. Após a última lavagem o líquido foi reduzido a 40µl e 10µl da preparação colocada em lâmina de microscópio contendo um gota de *anti-fade*. A lamínula foi colocada e a amostra vedada com esmalte e guardada em ambiente escuro. As amostras foram analisadas em aumento de 60x e fotografadas no microscópio de sistema confocal com base Nikon Eclipse TE2000 (Nikon) e sistema multifoton Bio-Rad Radiance 2100 MP Rainbow Confocal/Multiphoton System (Bio-Rad) no laboratório W.M. Keck for Biological Imaging da Universidade de Wisconsin-Madison

3.9 - Avaliação do efeito do estresse oxidativo em parasitos silenciados

Para observarmos o efeito funcional do silenciamento de enzimas anti-oxidantes nos parasitos silenciados para estes genes, expusemos os esporocistos a estresse oxidativo adicionando peróxido de hidrogênio ao meio de cultura.

3.9.1 - Curva de sobrevivência na presença de H_2O_2

Primeiramente, fizemos uma curva de sobrevivência, para estipularmos a concentração limite na qual esporocistos controle eram capazes de sobreviver. Para isso, parasitas expostos a dsRNA-GFP (controle) foram colocados em placas de 96 poços em duplicatas para cada concentração de água oxigenada testada por 24h. Testamos 5mM, 100µM, 50µM, 25µM, 10µM e 5µM de peróxido de hidrogênio (Sigma) em cada poço, em um volume final de 100µl de CBSS+. Foi estipulado que a concentração de H_2O_2 limite para sobrevivência seria 50µM. Para quantificarmos os parasitas mortos antes de serem expostos a H_2O_2, foi adicionado 5µg/ml de iodeto de propídio aos poços, que cora apenas os esporocistos mortos. Ao microscópio, os parasitas totais de todos os poços foram contados e, posteriormente, com o laser no comprimento de onda de 572nm, os organismos mortos foram identificados e contabilizados. Apenas esporocistos totalmente corados eram contados como organismos mortos.

3.9.2 - Experimento de estresse oxidativo

Parasitas cultivados por oito dias em placas de 24 poços foram lavados três vezes com CBSS+, para remoção das placas ciliares. Esporocistos tratados com dsRNA para GPx, GST26, GST28, TPx1/2, SOD e GFP (controle) foram transferidos para placa de 96 poços em triplicatas. Para quantificarmos os parasitas mortos antes de serem expostos a H_2O_2, foi adicionado 5µg/ml de iodeto de propídio aos poços. Ao microscópio, os parasitas totais de todos os poços foram contados e, posteriormente, com o laser no comprimento de onda de 572nm, os organismos mortos foram identificados e contabilizados. Esta contagem indicava a percentagem de parasitos mortos por outra forma que não pela adição de peróxido de hidrogênio. Após a contagem, peróxido de hidrogênio na concentração de 50µM foi adicionado a dois dos três poços contendo parasitos de cada tratamento e os poços restantes foram mantidos com CBSS+ (branco). A placa foi incubada a 26ºC por 4h, 24h e 48h e os parasitas mortos contados a cada um destes intervalos de tempo. Apenas esporocistos totalmente corados eram contabilizados como organismos mortos.

Para que fosse possível estabelecer uma correlação entre estresse oxidativo e a morte dos parasitas, subtraímos o número de organismos mortos antes da exposição ao peróxido de hidrogênio do número total de esporocistos por poço, sendo o resultado, o

número total de organismos vivos antes da exposição a H_2O_2. O número inicial de esporocistos mortos foi subtraído do número de parasitos mortos observados após cada contagem e a porcentagem de mortos calculada em relação ao número total de organismos vivos presentes inicialmente. Os resultados foram analisados por Two-way ANOVA e quando os resultados se mostraram significativos, aplicou-se a correção de Bonferroni, comparando-se cada tempo de análise do tratamento com seus respectivos tempos em esporocistos tratados com GFP (controle) (significância $p<0,05$, N=6).

3.9.3 - Neutralização do estresse oxidativo

Para nos certificarmos que era o peróxido de hidrogênio o único fator oxidante no meio, esporocistos tratados com dsRNA foram expostos a 100µM e 50µM de H_2O_2 e a mesma concentração de H_2O_2 contendo 0,1%, 0,05%, 0,025%, 0,0125% de enzima Catalase bovina (Sigma). As mesmas concentrações da enzima sem a presença de peróxido de hidrogênio foram utilizadas como controle. Estabelecemos que 0,025% da enzima catalase era a concentração ideal para inativação dos efeitos do peróxido de hidrogênio. Os parasitas foram incubados a 26ºC, sendo a sua integridade observada através da adição de 5µg/ml de iodeto de propídio e os organismos fluorescentes contados após cada intervalo de tempo (0h, 4h, 24h e 48h). A contagem foi feita como explicado no item 3.9.2. A sobrevivência dos parasitas foi comparada após a exposição a H_2O_2 nos tempos indicados. Os resultados foram analisados por Two-way ANOVA e quando os resultados se mostraram significativos, aplicou-se a correção de Bonferroni, comparando-se cada tempo de análise do tratamento com seus respectivos tempos em esporocistos tratados com GFP (controle) (significância $p<0,05$, N=6).

3.10 - Experimento utilizando hemócitos de *Biomphalaria glabrata* - *Killing Assay*

Com o objetivo de observarmos o efeito que os hemócitos de *B. glabrata* (cepa susceptível NMRI) teriam sobre esporocistos deficientes em enzimas anti-oxidantes, expusemos parasitos silenciados para GPx, TPx1/2, GST26, GST28, SOD e GFP

(controle) a estas células. Nosso interesse foi, nesse caso, avaliar o papel das enzimas anti-oxidantes para proteção do parasita ao ataque do sistema de defesa do hospedeiro.

Para o experimento, esporocistos silenciados, oito dias após transformação, foram lavados com CBSS+ três vezes para a remoção das placas ciliares e 100µl, contendo em média 150 parasitos, foram transferidos em quadruplicatas para lâminas de microscópio contendo 16 poços CultureWell™ Chambered Covereglass for cell culture (Molecular Probes/Grace Bio-Labs). Simultaneamente, dezoito caramujos *B. glabrata* da cepa NMRI (susceptível a infecção) foram desinfetados com álcool 70%, e imersos por trinta minutos em solução de água de lagoa contendo 25µg/ml anfotericina B, 60µg/ml penicilina e 50µg/ml estreptomicina, segundo Hahn e colaboradores (2001). Estes caramujos foram então secos e a hemolinfa obtida através da massa cefalopodal, segundo Sminia & Barendsen, (1978). A hemolinfa foi diluída 1:1 em CBSS e colocada em gelo por 15 minutos. Para a separação do plasma, a parte superior do líquido foi transferida para microtubos siliconizados contendo 50µl de agarose 0,1% previamente solidificada ao fundo e 100µl de ficoll 5% diluído em iBge (24% Schneider`s *Drosophila* medium – Invitrogen, 0,5% hidrolisado enzimático de lactoalbumina - Sigma Aldrich, 7,2mM galactose). Os tubos foram centrifugados a 20g por 17 minutos a 4ºC. As células foram recuperadas, homogeneizadas em um único tubo e o mesmo volume adicionado nos poços da lâmina contendo parasitas. Os hemócitos foram adicionados em dois poços contendo parasitos silenciados para cada tratamento com um volume final de 200µl. Como controle, nos outros dois poços de parasitas restantes, apenas CBSS+ foi adicionado. Para que as células se assentassem, estas foram incubadas por uma hora a 26ºC. 5µg de iodeto de propídio foi adicionado ao meio e os parasitas inicialmente mortos foram contados em microscópio invertido com o laser no comprimento de onda de 572nm. Contagens foram feitas novamente depois de 4 e 24 horas. Os resultados foram analisados por Student's Ttest (Ttest, unicaudal "one tail", significância $p<0,05$, N=4). Cada um dos tratamentos foram comparados com parasitas tratados com dsGFP (controle).

4- Resultados e Discussão

A poderosa técnica de RNA interferência vem sendo amplamente utilizada para gerar fenótipos de perda de função em uma variedade de organismos. Combinada com informações de anotações funcionais de seqüências do genoma, quase publicado, do *Schistosoma mansoni*, RNAi tem aberto novas portas para a caracterização da função de cada gene anotado no genoma. Um importante desafio agora é a integração de predições gênicas à informação funcional advinda de cada gene testado por RNAi, ou por outros métodos de caracterização (Fuchs & Boutros, 2006).

A triagem por RNAi em abrangência genômica está disponível para *C. elegans* (Fraser *et al.*, 2000; Sonnichen *et al.*, 2005) e *D. melanogaster* (Clemens *et al.*, 2000), onde muitas bibliotecas de RNAi foram construídas para o estudo fenotípico de quase todos os genes. No entanto, muito ainda tem que ser entendido para o melhoramento da técnica de RNAi em esquistossomas, já que esta é a única ferramenta de genética reversa viável neste parasita, sendo utilizada como via para o entendimento sobre funções específicas de cada gene. No entanto, como descrito por Geldhof e colaboradores (2007), os artigos produzidos em que se utiliza essa técnica em parasitas helmintos carecem de evidências, tanto do efeito direto nos níveis dos transcritos, quanto da demonstração da diminuição da quantidade de proteínas relativas aos alvos silenciados. Também há falta de controles que demonstrem a especificidade do silenciamento, ou a sensibilidade da transfecção e o número de parasitas silenciados. Adicionalmente, os autores comentam sobre a falta de relatos sobre os insucessos com essa técnica, ou quantos genes foram triados para se obter um mínimo de um gene silenciado com sucesso.

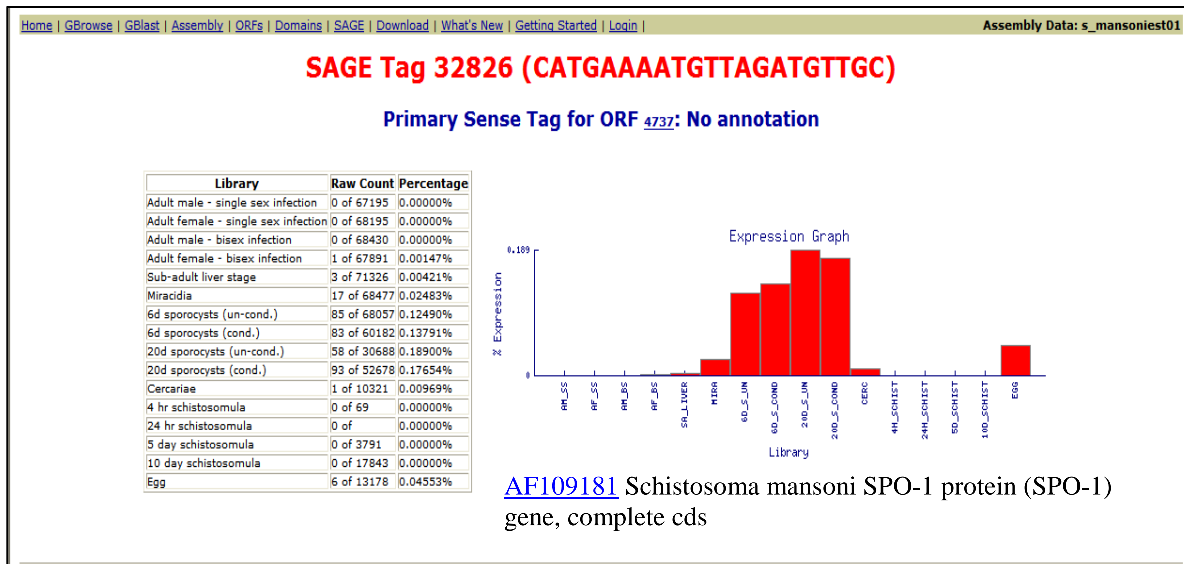
Esse estudo foi desenvolvido visando: (1) um melhor entendimento das funções de alguns dos genes que têm expressão aumentada nas fases larvais do *S. mansoni,* iniciando um estudo em larga escala neste parasito, (2) entender as peculiaridades da técnica de interferência por RNA no parasito e compreender os motivos que torna a técnica de RNAi menos operacional neste parasita quando comparada a outros organismos em que esta vem sendo amplamente utilizada, (3) uma melhor compreensão do mecanismo de defesa de *S. mansoni* contra o estresse oxidativo através do

silenciamento dos transcritos de enzimas anti-oxidantes do parasita para a investigação de sua importância na relação parasito-hospedeiro.

4.1 Escolha dos genes alvos

Na triagem utilizando a técnica de interferência por RNA foram escolhidos 33 genes alvos de *S. mansoni* para serem silenciados. Além dos tratamentos com dsRNA para Tiorredoxina peroxidase 1 e 2 terem sido feitos individualmente, esses dsRNAs foram combinados em um dos tratamentos, originando 34 tratamentos. Na sua maioria, os genes do parasito foram escolhidos baseado em sua aparente expressão diferencial nas fases de miracídios e esporocistos, quando comparado com outras fases do verme, ao analisarmos resultados de SAGE (Williams *et al.,* 2007). Um exemplo de perfil de expressão gênica diferencial encontrado no banco de dados de SAGE OrganismDB está demonstrado abaixo (Figura 7).

Genes analisados neste trabalho, de acordo com nosso interesse específico em sua caracterização, foram: Glicoproteína K5, Zinc Finger 1 (SmZF1), Ring Box (SmRbx), Calmodulina 1, e as enzimas anti-oxidantes Glutationa Peroxidase (GPx), Tiorredoxina Peroxidase 1 (TPx-1) e Tiorredoxina Peroxidase 2 (TPx-2), Superóxido Dismutase (SOD), além de Glutationa S transferase 26 e 28 que também estão inseridas no grupo de proteínas que exibem expressão diferencial nas fases de miracídio e esporocistos.



Library	Raw Count	Percentage
Adult male - single sex infection	0 of 67195	0.00000%
Adult female - single sex infection	0 of 68195	0.00000%
Adult male - bisex infection	0 of 68430	0.00000%
Adult female - bisex infection	1 of 67891	0.00147%
Sub-adult liver stage	3 of 71326	0.00421%
Miracidia	17 of 68477	0.02483%
6d sporocysts (un-cond.)	85 of 68057	0.12490%
6d sporocysts (cond.)	83 of 60182	0.13791%
20d sporocysts (un-cond.)	58 of 30688	0.18900%
20d sporocysts (cond.)	93 of 52678	0.17654%
Cercariae	1 of 10321	0.00969%
4 hr schistosomula	0 of 69	0.00000%
24 hr schistosomula	0 of	0.00000%
5 day schistosomula	0 of 3791	0.00000%
10 day schistosomula	0 of 17843	0.00000%
Egg	6 of 13178	0.04553%

Figura 7: Exemplo de SAGE Tag com perfil de expressão gênica diferencial nos estágios larvais e intra-molusco encontrado no banco de dados OrganismDB. A tabela acima mostra o número de vezes e em quais bibliotecas a Tag4737 foi encontrada. O gráfico representa o perfil de expressão e, abaixo, a anotação da seqüência da etiqueta, indicando ser o transcrito do gene SPO-1 (gb:AF09181).

4.2 Síntese dos dsRNAs e análise fenotípica após tratamentos

Para iniciarmos este trabalho, cada dsRNA foi sintetizado, purificado e analisado em gel de agarose 1% não desnaturante. A Figura 8 mostra exemplos de alguns dsRNAs sintetizados. Notamos que a eficiência da síntese de dsRNA variou entre os diferentes transcritos alvo e isso pode ser devido à diferentes eficiências nas reações de PCR e na recuperação dos produtos da PCR dos genes alvo, previamente à produção do dsRNA. No entanto, para adição do dsRNA às culturas de esporocistos estes eram dosados e sempre adicionava-se uma quantidade em cada poço para obtenção da concentração final de 50 nM.

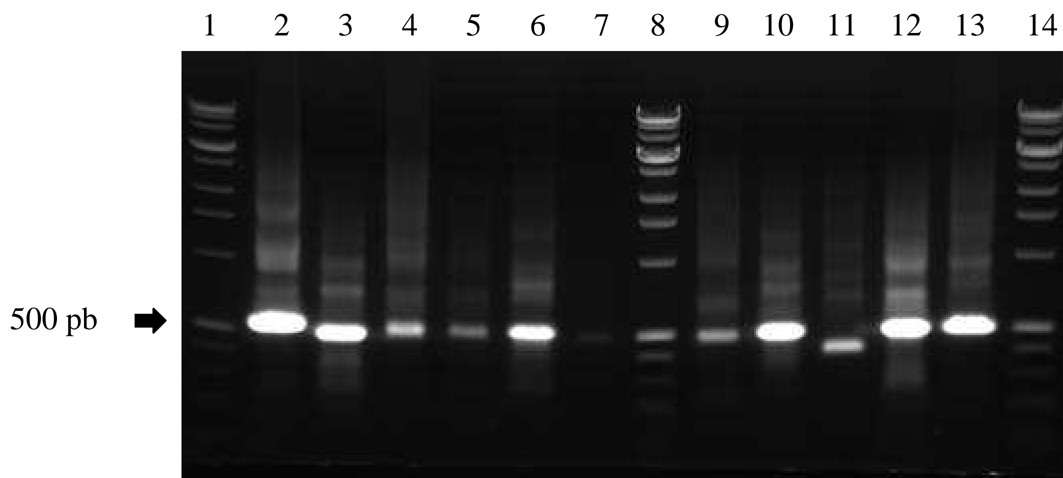


Figura 8: Representação de dsRNAs sintetizados e devidamente anelados em gel de agarose 1% não desnaturante. Canaletas: 1- Padrão de peso molecular 0,1-12 Kpb (Perfect DNA markers, Novagen); 2- dsRNA SmZF1; 3- dsRNA SOD; 4- dsRNA RHO1; 5- dsRNA RHO2; 6- dsRNA Lactato Desidrogenase; 7- dsRNA Smad2; 8- Padrão de peso molecular 0,1-12Kpb; 9- dsRNA Smad4; 10- dsRNA Miosina Cadeia Leve; 11- dsRNA SmRbx; 12- dsRNA Fator de Elongação 1α; 13- dsRNA Fibrilarina; 14- Padrão de peso molecular 0,1-12Kpb.

Parasitas tratados com os dsRNAs para os genes selecionados foram observados todos os dias por 14 dias, onde procuramos observar possíveis fenótipos que pudessem estar associados aos tratamentos com dsRNA. Na triagem por fenótipos, procuramos observar mudanças na forma, lise do tegumento e morte, perda ou aumento de movimento, falha/atraso na transformação dos parasitos e possivelmente, a ausência de indivíduos se transformando em esporocistos-mãe ou de progênie (esporocistos-filho). Medidas do comprimento dos esporocistos foram feitas aos sete dias de cultura para obtenção de dados referentes ao tamanho dos indivíduos. Esse dia foi escolhido devido aos resultados obtidos por Dinguirard e Yoshino (2006), na análise do fenótipo de esporocistos tratados com dsRNA para o gene *Scavenger Receptor Binding* (SRB).

Após a exposição dos esporocistos aos dsRNAs dos genes selecionados, o único fenótipo associado que pudemos distinguir entre os diferentes tratamentos foi o de alteração no comprimento dos parasitos. Exemplos de esporocistos após os tratamentos com dsRNA estão mostrados na Figura 9.

Ao sétimo dia, esporocistos foram amostrados em fotografias e cada parasita das imagens foram medidos, com exceção de organismos mortos. As medidas obtidas foram submetidas a tratamento estatístico usando o teste Mann-Whitney (ou Wilcoxon Sum of Ranks, significância $p<0,05$). Estes experimentos foram repetidos por, no mínimo, três vezes para cada tratamento.

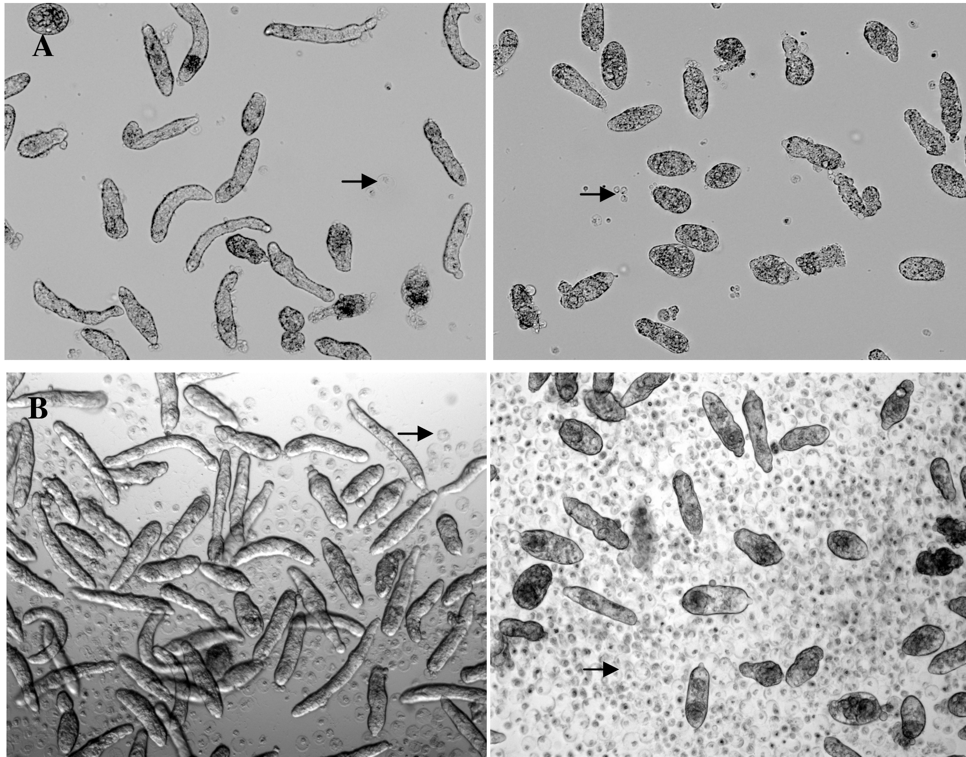


Figura 9: Imagens de esporocistos tratados com dsRNA por sete dias e exibindo fenótipo de redução de comprimento. Como controle negativo, parasitos foram incubados com dsRNA GFP (lado esquerdo). Na direita, estão representados parasitos tratados com dsRNA para GST26 e Smad4, A e B respectivamente. As placas ciliares liberadas pelos parasitas durante a transformação são mostradas com setas (imagens com aumento de 100x).

A mediana do tamanho dos esporocistos variou nos diversos grupos analisadas como pode ser visto nas Figuras 10 e 11. Portanto, cada experimento foi considerado separadamente e a mediana de cada amostra comparada com a mediana dos controles (esporocistos tratados com dsGFP e branco). Não houve diferença significativa entre os grupos de parasitas controles (dsRNA-GFP e branco). Os parasitos tratados com dsRNA para Smad4, Lactato Desidrogenase, Smad2, Cav2A, Fator de Elongação 1α, Smad1, SmRing Box, RHO2, Calcineurina B, SOD e GST26 apresentaram significante diminuição do tamanho em todos os experimentos realizados (Figuras 10 e 11).

No entanto, algumas amostras testadas como: Scmeg, HEXBP, SmZF1, Calpaína, Miosina cadeia leve, PKCβ, Calmodulina, SPO1, TPx1/2 e Canal de cálcio foram inconsistentes, apresentando comprimentos significativamente menores que esporocistos não tratados (branco) em três experimentos, assim como comprimentos significativamente menores que parasitos tratados com dsGFP em dois de três experimentos. Nestes casos, estas amostras foram consideradas inconsistentes e negativas para a presença de fenótipo, assim como os outros tratamentos que, apresentaram inconsistência, ou não apresentaram qualquer alteração de tamanho. Vale a pena ressaltar que o tratamento para silenciamento de Tiorredoxina peroxidase 1 e 2 apresentou-se letal para os esporocistos após oito dias de exposição.

Inesperadamente, observamos que 34% dos genes testados foram capazes de produzir um único fenótipo de redução de tamanho de esporocistos, a mesma alteração observada anteriormente no silenciamento do receptor de tegumento de esporocistos CD36-like scavenger (Dinguirard & Yoshino, 2006). Em *C. elegans* foi observado que apenas cerca de 10% dos genes apresentam algum fenótipo quando silenciados em escala genômica, sendo 2% dos fenótipos viáveis detectáveis relacionados com tamanho alterado, ou crescimento (Kamath *et al*., 2003). Recentes aplicações de RNAi em esquistossômulos, miracídios ou vermes adultos também produziram fenótipos de parasitas menores (Revisado por Kalinna & Brindley, 2007). Mais ainda, a eficiência de silenciamento e quais os genes eram silenciados em *C. elegans* foram, de certa forma, dependente dos métodos de RNAi empregados (O'Rourke & Bowerman, 2005), evidenciando assim, que a ausência de fenótipos e a inconsistência entre métodos de silenciamento é algo comum, até mesmo em *C. elegans,* organismo em que esta ferramenta foi desenvolvida e é largamente utilizada. Acreditamos que o fenótipo observado nos esporocistos possa ser resultante de algum desequilíbrio metabólico

desencadeado pela ausência da proteína codificada pelo gene silenciado, durante a transformação e desenvolvimento dos parasitas. Além disso, como na maioria das triagens de RNAi, apenas a observação dos fenótipos mais discrepantes se faz possível, devido à limitação do monitoramento dos sistemas em larga escala através dos equipamentos de imagem e da necessidade de elaboração de perguntas e experimentos altamente específicos capazes de permitir a percepção de diferenças e de comportamentos sutis. Este obstáculo nos mostra os limites de experimentos em larga escala e nos direciona, mais uma vez, para a necessidade de dedicação da técnica para caracterização gene-a-gene, até que a tecnologia nos provenha de métodos mais sensíveis e que possam ser utilizados em larga escala (Fuchs & Boutros, 2006).

Com relação à ausência na observação de fenótipos associados a muitos dos tratamentos com dsRNA, é provável que uma expressão mínima de uma determinada proteína seja suficiente para a execução de sua função no organismo. É necessário ter em mente que a técnica de RNAi não reproduz o "fenótipo nulo" que é obtido por mutações clássicas *knockout*, sendo uma possível explicação para tais observações (Kurreck, 2009). Mais ainda, não sabemos se as células/órgãos específicos, em que os transcritos alvo estariam sendo expressos e exercendo sua função estão sendo atingidos pelo mecanismo de RNAi. Além do mais, é possível que outras isoformas das proteínas, ou mesmo, outras proteínas estejam desempenhando o mesmo papel daquela codificada pelo gene silenciado (redundância funcional).

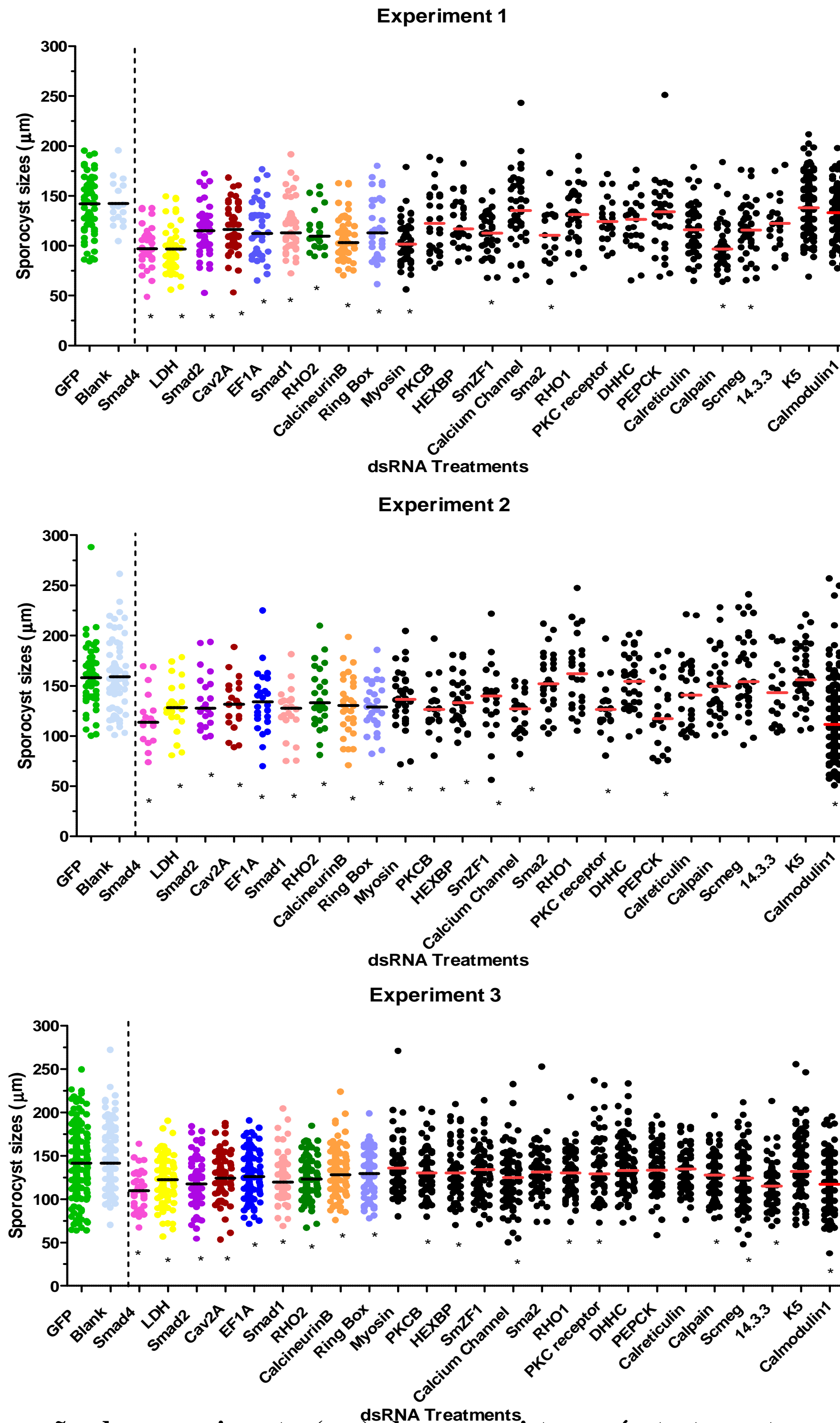


Figura 10: Comparação do comprimento (µm) de esporocistos após tratamento com dsRNAs em relação aos controles dsGFP e branco. Representação de três experimentos. Coloridas e com asteriscos (*) estão as amostras que apresentaram significativa diminuição do comprimento dos esporocistos em todos os experimentos, quando comparados com seus respectivos controles internos (separados pela barra preta pontilhada). As barras pretas e vermelhas horizontais representam a mediana dos comprimentos da população amostrada. Em preto e com asterisco (*) estão amostras que apresentaram diminuição de tamanho significativa em algum dos experimentos.

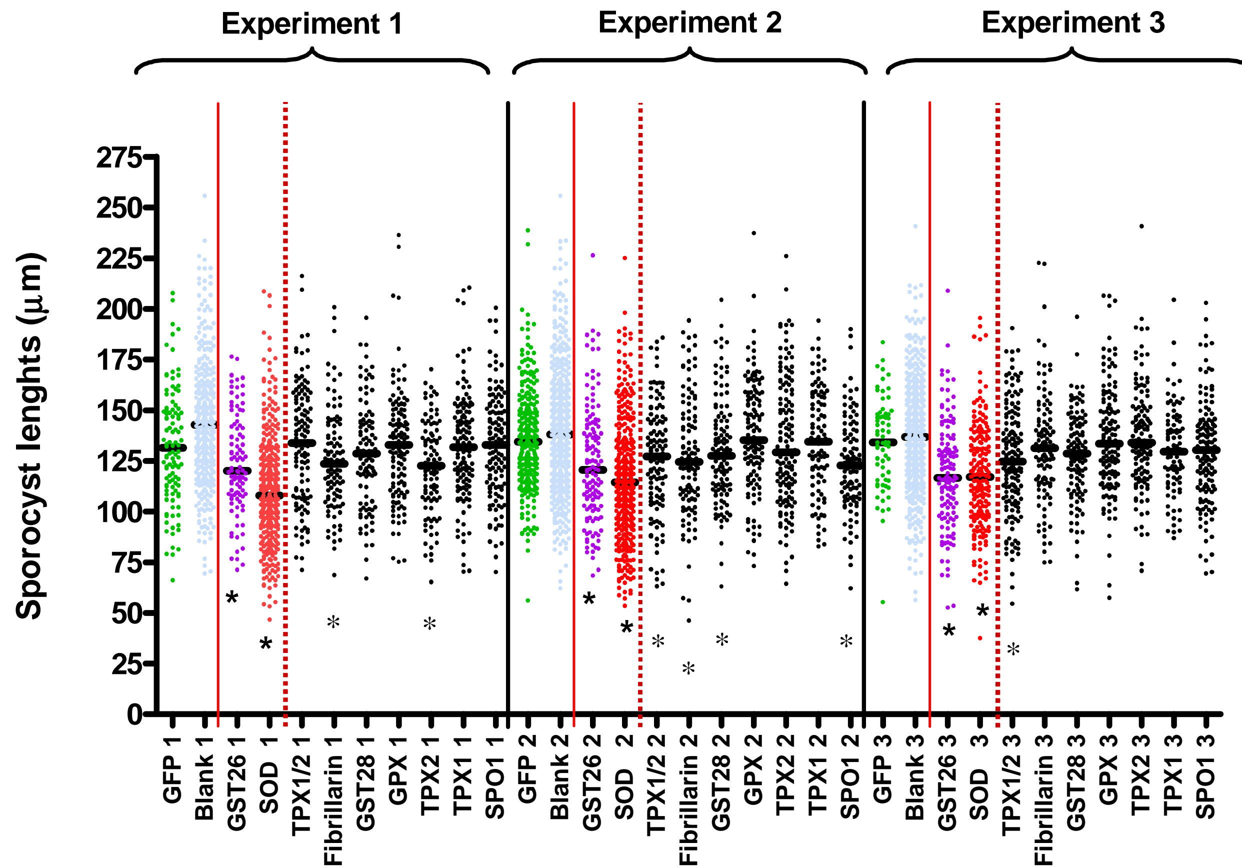


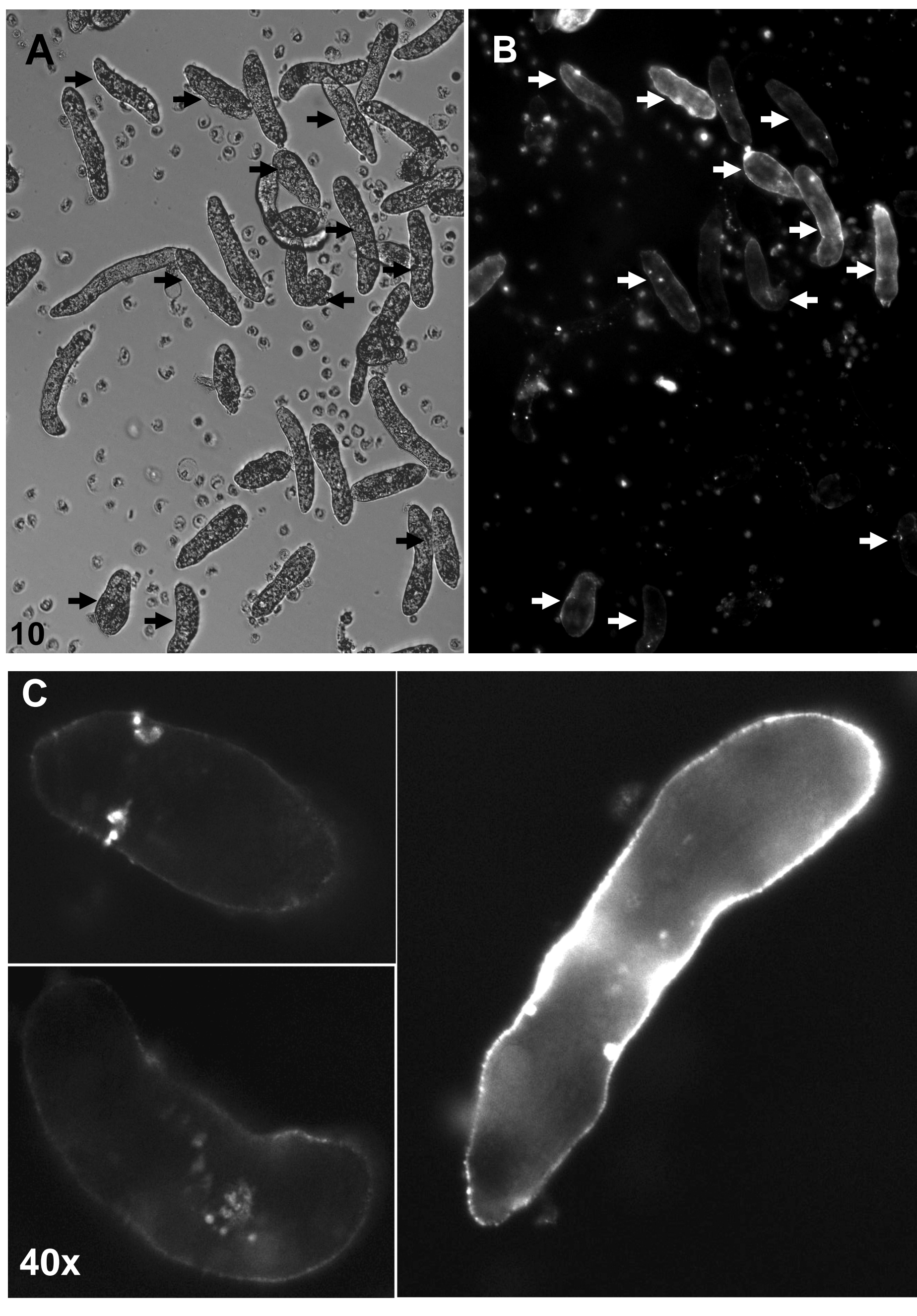
Figura 11: Comparação do comprimento (µm) de esporocistos após silenciamento de transcritos de enzimas anti-oxidantes em relação aos controles dsGFP e branco. Representadas em roxo e vermelho com asterisco (*) estão as amostras que apresentaram significativa diminuição em comprimento em todos os experimentos, quando comparados com seus respectivos controles internos (separados pela linha vermelha contínua). As barras pretas horizontais representam a mediana dos comprimentos dos indivíduos amostrados. Em preto e com asterisco (*) estão amostras que apresentaram diminuição de tamanho significativa em algum dos experimentos.

4.3 Absorção de dsRNA pelos esporocistos de *S. mansoni*

Para testarmos se os dsRNAs estavam sendo absorvidos pelos parasitos, dsRNAs de GFP (controle), Smad4 e Lactato Desidrogenase, foram marcados com Rodamina e adicionados à cultura dos parasitos. Após sete dias, estes foram fotografados e posteriormente medidos; como mostrado na Figura 12. Quando observados em microscópio invertido, os esporocistos não exibiram autofluorescência no comprimento de onda utilizado, sendo a fluorescência observada advinda da absorção dos dsRNAs marcados. A rodamina não interferiu na viabilidade dos parasitas e apenas os esporocistos viáveis foram analizados. Pudemos observar que todos os dsRNAs testados pareceram ser absorvidos pelos parasitos (Figura 12).

No entanto, dentro de um mesmo grupo de esporocistos houve uma grande variação de absorção, mostrando que alguns indivíduos podem ser refratários ao tratamento. Em alguns parasitas os dsRNAs parecem estar em grande quantidade na superfície (Figura 12C e D), já em outros a molécula parece estar em "órgãos" internos (muitas vezes nas células flama, como mostrado por setas na Figura 12), enquanto que alguns parasitas parecem não absorver o tratamento. Em resposta a todos os tratamentos com dsRNA marcados, os esporocistos exibiram ~70% de fluorescência, não existindo, portanto, diferença de absorção entre os diversos tratamentos testados.

Tentando melhorar a absorção do dsRNA pelos esporocistos, o reagente de transfecção Gene Silencer foi utilizado. Verificou-se que esse reagente de transfecção (e outros testados no laboratório) é tóxico para os esporocistos (dados não mostrados), fenômeno também observado em vermes adultos de *S. mansoni*. Recentemente foi descrito por Krautz-Peterson e colaboradores (2007) que a eletroporação reduz a viabilidade dos parasitas, demonstrando ser este um fator de estresse no processo, após o qual, não é possível afirmar a origem/causa e especificidade dos fenótipos observados. Há uma grande necessidade de melhoramento das técnicas de eletroporação e diminuição de toxicidade dos reagentes de transfecção.


A
B
10
C
40x

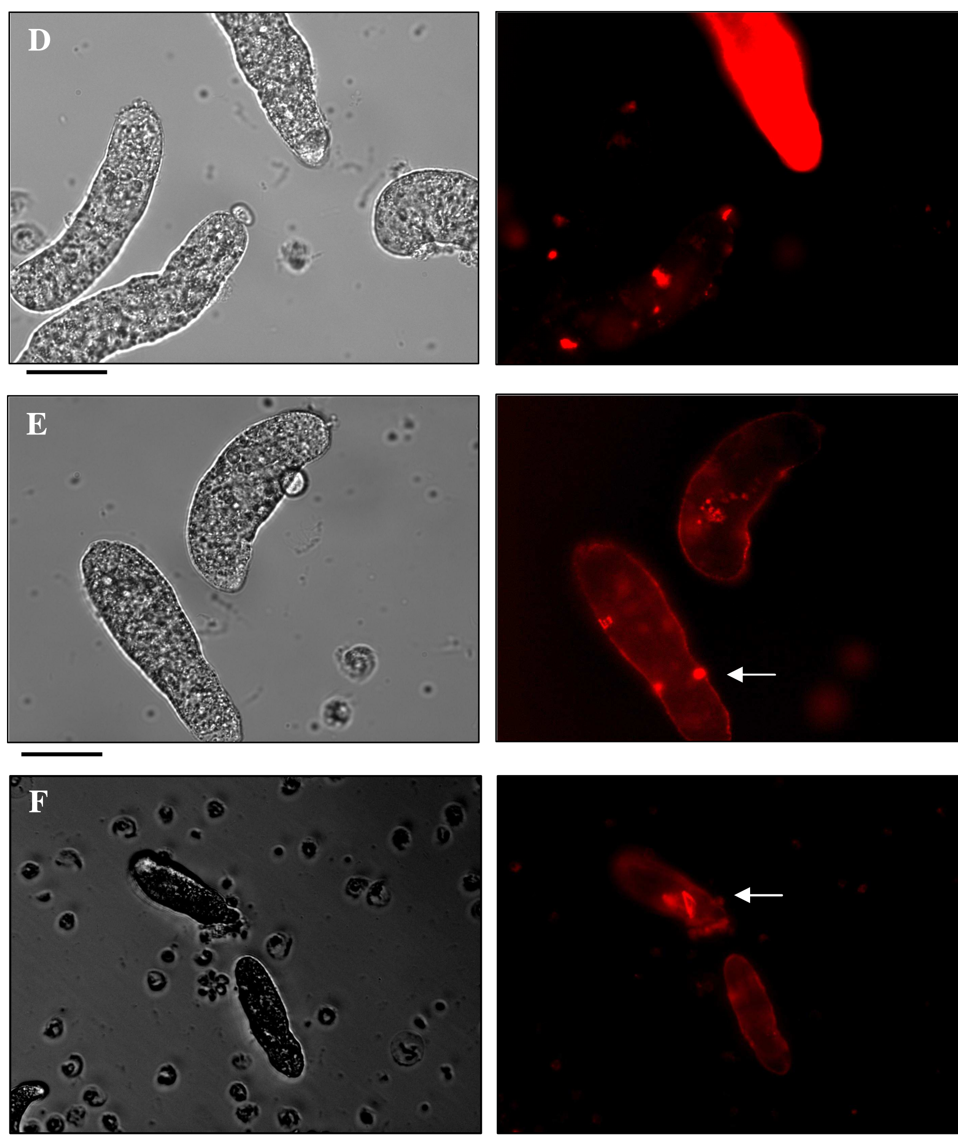


Figura 12: Imagens de esporocistos mostrando absorção de dsRNAs marcados com rodamina após 7 dias de tratamento. Imagem de campo claro de um grupo de parasitos tratados com dsRNA-GFP marcado (A) e sua imagem fluorescente correspondente (B) mostrando os diferentes níveis de absorção de dsRNA pelos parasitas submetidos a um mesmo tratamento. As setas mostram uma absorção média e alta dos dsRNA. Em maior aumento (40x) é possível observar diferentes níveis de absorção de dsRNA (C). Esporocistos expostos a Rod-dsRNA-Lactato Desidrogenase (D), Rod-dsRNA-Smad4 40x (E) e 20x (F), campo claro e imagem correspondente em fluorescência. As setas mostram possíveis células-flama, órgão excretor do parasita. A escala está representada como barras pretas (~50µm) no lado esquerdo das figuras.

Para avaliarmos se a marcação com rodamina influenciaria na absorção dos dsRNAs pelos parasitas, tratamos esporocistos com os mesmos dsRNA marcados e não marcados e medimos o seu comprimento como descrito anteriormente. Os resultados são mostrados na Figura 13.

Quando comparados com controles dsGFP e branco, todos os esporocistos tratados com dsRNAs para Lactato Desidrogenase e Smad4 apresentaram tamanho significativamente reduzido, independentemente de estarem marcados ou não com rodamina (Figura 13). No entanto, quando comparamos amostras tratadas com dsRNA para Lactato Desidrogenase e Smad4 marcadas e não marcadas, observamos que populações de parasitos expostos a dsRNAs marcados foram significativamente maiores que organismos incubados com seus respectivos dsRNAs não marcados. Não houve diferença significativa entre tratamentos com dsRNA GFP marcado e não marcado. Rodamina é amplamente utilizada para marcação de dsRNAs. É possível que este fluoróforo tenha afinidade pelo tegumento dos esporocistos e que dsRNA marcados com rodamina não penetrem nos organismos na mesma concentração que dsRNA não marcados, atenuando o fenótipo resultante destes silenciamentos e também interferindo no experimento de absorção. Portanto, é possível que a absorção de dsRNA pelos parasitas seja maior do que a observada previamente, quando se utilizou dsRNA marcado com rodamina.

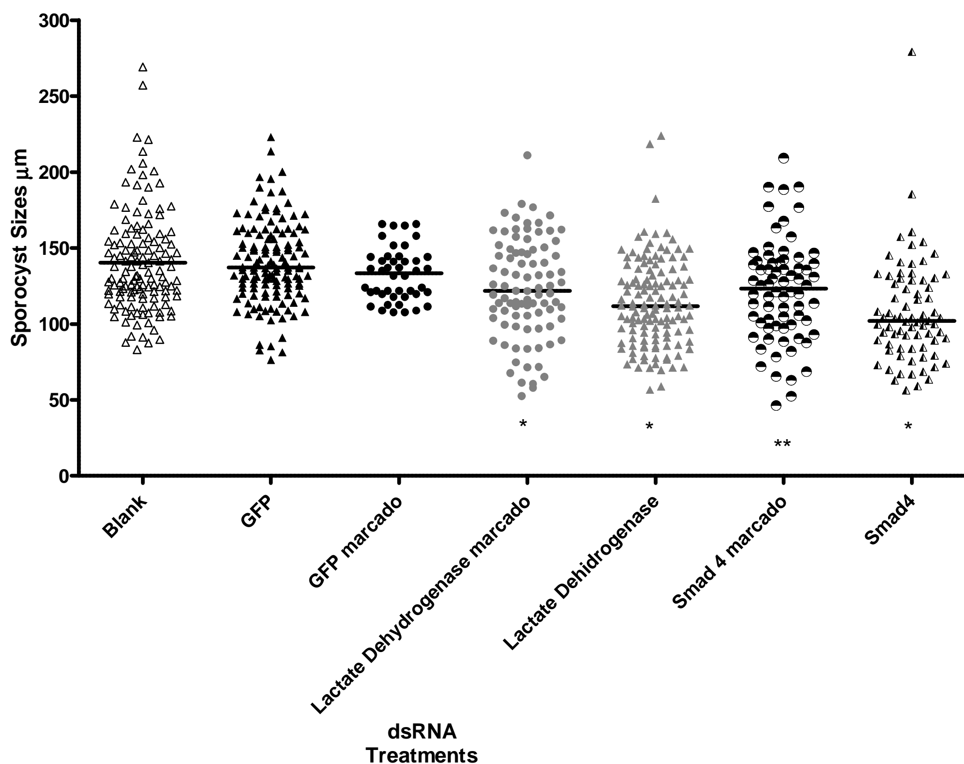


Figura 13: Comparação do comprimento de esporocistos tratados com dsRNA marcados e não marcados com rodamina. As barras pretas horizontais representam as medianas do comprimento do grupo de parasitas (µm). Mostrados com um asterisco (*), esporocistos que após tratamento apresentaram um decréscimo estatisticamente significativo em comprimento, quando comparados com tratamentos branco, dsGFP e Rod-dsGFP. Representada com dois asteriscos, o tratamento que se mostrou significativamente diferente quando comparado com controles não marcados, mas não quando comparada com dsGFP marcado (Wilcoxon-Sum of Ranks ou Mann-Whitney, significância $p<0,05$).

4.4 Estudo do nível de transcritos em parasitos tratados com dsRNA

Utilizamos PCR quantitativo (q-PCR) para avaliarmos o nível dos transcritos expressos pelos parasitos tratados com os dsRNA, comparando-os com o nível dos mesmos transcritos em esporocistos tratados com dsGFP, ou com parasitas tratados com dsRNA para outro gene, como um segundo controle para silenciamento inespecífico (*off-target*) (Figura 14). Primeiramente, avaliamos o nível de transcritos dos parasitos que, após tratamento, exibiram diminuição de tamanho significativo (Figura 14A). Posteriormente, selecionamos randomicamente parasitos que não exibiram fenótipo aparente, para a quantificação dos transcritos do gene alvo (Figura 14B).

Surpreendentemente, detectamos que em apenas sete dos onze tratamentos (Smad4, Lactato Desidrogenase, Cav 2A, Fator de Elongação 1α, CalcineurinaB, GST26, SOD) que exibiram redução de comprimento do parasita houve uma mudança significativa e consistente no nível de transcritos, quando comparados com esporocistos tratados com dsRNA GFP (Figura 14A). Podemos observar uma diminuição mais sutil, porém constante, nos níveis de transcritos de parasitas tratados com dsRNA-Smad4 e Lactato Desidrogenase (15%), após sete dias de exposição, enquanto que tratamentos para o EF1α e GST26 exibiram maiores níveis de silenciamento, 70% e 85%, respectivamente. Os tratamentos com Calcineurina B e Cav2A exibiram aproximadamente 65% e 42% de redução em seus níveis. Seis entre os treze tratamentos que não exibiram fenótipo aparente/consistente nos vermes tratados, apresentaram níveis reduzidos de transcritos, dentre estes tratamentos estão SmZF1 (30%), Calmodulina (55%) e Fibrilarina, GPx, GST28 e TPx1/2, com uma redução de ~75% .

Estes resultados sugerem que talvez não exista correlação direta entre a redução de comprimento e o silenciamento do transcrito, ou que, cada transcrito apresente um *turnover* diferente, sendo possível que no início da transformação dos miracídios em esporocistos o silenciamento promova uma redução do transcrito, mas ao sétimo dia, no momento em que acessamos o nível destes transcritos, a expressão gênica já tenha se normalizado. Inconsistência entre experimentos de RNA interferência em helmintos foi recentemente abordada por Geldholf, 2007, e tem sido freqüentemente observada até mesmo em parasitos nematódeos.

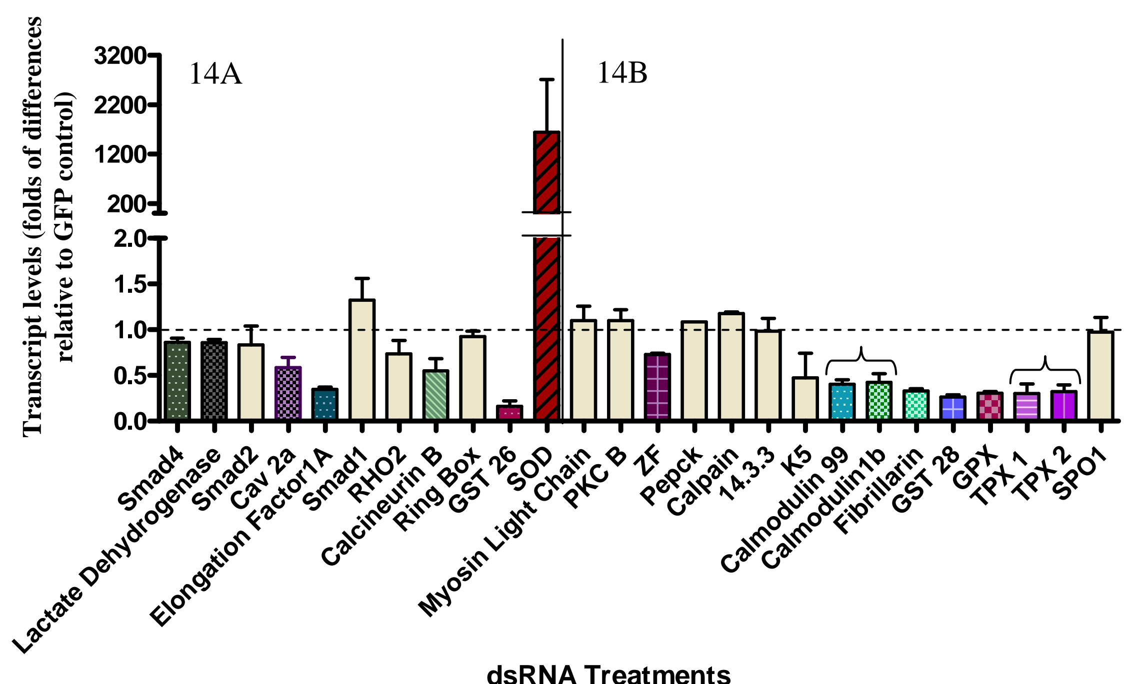


Figura 14: Níveis de transcritos dos genes alvo em parasitas tratados com dsRNA após sete dias. A: Tratamentos que apresentaram o "fenótipo" de redução de tamanho dos vermes. B: Tratamentos escolhidos randomicamente que não apresentaram diminuição significativa de comprimento dos esporocistos. As amostras coloridas exibiram uma diminuição de transcritos significativa em relação ao controle (dsRNA-Smad4/GFP, P=0.0056; -Lactato desidrogenase/GFP, P= 0.0358; -Cav2a/GFP, P= 0.0136; -EF1α/GFP, P= 0.0358; -Calcineurina B/GFP, P= 0.0189; -GST26/GFP, P= 0.0136; -SmZF1/GFP, P= 0.0189; -Fibrilarina/GFP, P= 0.0407; -GST28/GFP, P= 0.0284; -GPx/GFP, P= 0.0269 e -TPx1/GFP, P= 0.0358/-TPx2/GFP, P= 0.0358) ou aumento do transcrito alvo quando quando comparadas à esporocistos tratados com dsGFP (dsRNA-SOD/GFP, P= 0.0294). As barras em bege representam os tratamentos com dsRNA que não apresentaram alterações significativas no nível de transcritos (-Smad2/GFP, P= 0.0755; -Smad1/GFP, P= 0.8969; -RHO2/GFP, P= 0.0765; -SmRing box/GFP, P= 0.7642; -Miosina/GFP, P= 0.3725; -PKCB/GFP, P= 0.6579; -PEPCK/GFP, P= 0.3017; -Calpaína/GFP, P= 0.1642; -14.3.3/GFP, P= 0.6579; -K5/GFP, P= 0.3725 e -SPO1/GFP, P= 0.8969) (Wilcoxon Sum of Ranks, ΔΔCT, significância $p<0,05$, $N\geq3$). A linha pontilhada representa o nível normalizado de transcritos na população controle (dsGFP). As amostras unidas por chaves representam um único tratamento com dsRNA com seus níveis de silenciamento quantificados por pares de iniciadores desenhados para dois transcritos diferentes. As barras representam o erro padrão.

Interessantemente, ao invés de redução da expressão gênica, parasitos tratados com dsRNA para Superoxido Dismutase (SOD) apresentaram uma superexpressão gênica de 1690 vezes, comparado com esporocistos tratados com dsRNA GFP. Para nos certificarmos que não era artifício da técnica, amplificamos SOD por q-PCR em amostras com outro tratamento, estas apresentaram um nível normal de transcritos (resultados não mostrados).

Com relação ao gene Calmodulina, este foi escolhido não por estar diferencialmente expresso nas formas larvais e intra-molusco, mas porque está sendo caracterizado funcionalmente no laboratório do Dr. Yoshino. Dados de análise do rascunho do genoma do *S. mansoni* sugerem que existam duas cópias desse gene, que diferem ligeiramente entre si, produzindo duas isoformas proteicas com variações de aminoácidos na região carboxi-terminal. Por este motivo, foram desenhados dois iniciadores para avaliar os níveis dos transcritos de Calmodulina, que foram silenciados utilizando um único dsRNA para a região conservada entre as duas isoformas. Na Figura 14B, este gene está representado com os nomes de Calmodulina99 e Calmodulina1b. Observamos que os dois possíveis transcritos de Calmodulina parecem ser silenciados de maneira similar pelo mesmo dsRNA, com uma diminuição de aproximadamente 55%.

Com relação aos transcritos da Figura 14B, cujos tratamentos dos parasitas com dsRNA não geraram um fenótipo claro observado, mas que apresentaram alteração da expressão gênica medido por q-PCR, podemos sugerir uma série de hipóteses para esta observação. Dentre várias, destacamos: (1) a diminuição dos níveis do transcrito não seria suficientemente eficaz para levar a uma diminuição dos níveis protéicos no tempo observado (7dias), (a “traducibilidade” do transcrito é alta), (2) o fenótipo de silenciamento gênico produzido seria muito sutil, não sendo percebido pelos nossos métodos de avaliação, (3) a diminuição dos níveis do transcrito levaria a uma queda na síntese da proteína, mas a o produto protéico apresentaria uma meia-vida longa, o que reduziria os efeitos do silenciamento, (4) mesmo havendo queda, por vezes drásticas, dos níveis dos transcritos e das proteínas por eles codificadas, a presença de pequenas quantidades de proteínas seriam suficientes para manter sua função quase normal e, finalmente, (5) a função do gene silenciado poderia ser substituído por um outro gene funcionalmente análogo.

Para testarmos a hipótese de que, nas amostras que apresentaram redução do tamanho dos esporocistos, os transcritos dos genes que estavam sendo silenciados apresentavam *turnovers* diferentes e que era verificado um nível de transcritos normal após sete dias de

incubação com os dsRNAs, avaliamos a quantidade de mRNA após dois, quatro e sete dias de incubação para as amostras Lactato Desidrogenase, EF1α, Smad4, Calcineurina B, SOD, RHO2, Smad2, SmRing box e Miosina (Figura 15), esta última é pertencente ao grupo de tratamentos que não exibiu decréscimo de transcritos, tampouco um fenótipo consistente.

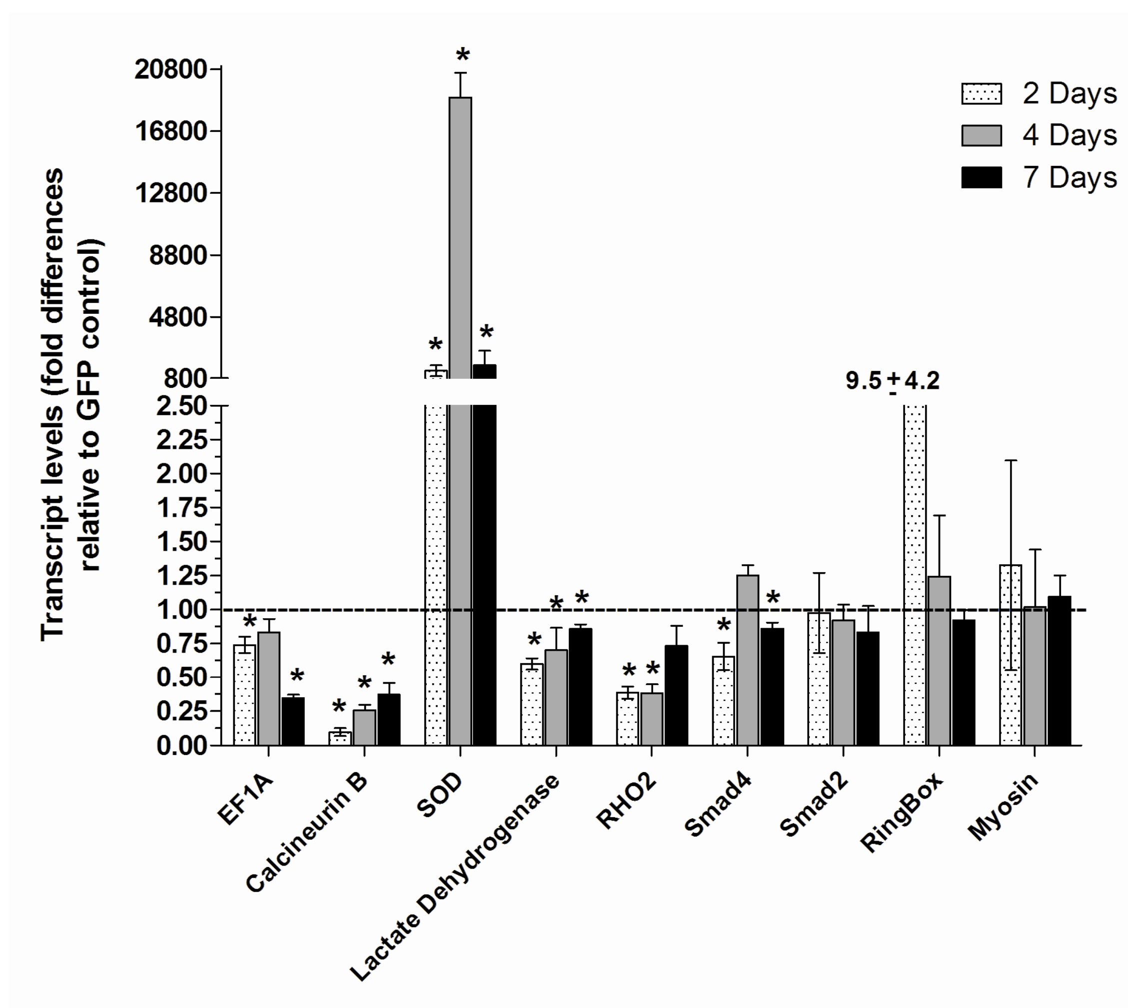


Figura 15: Níveis de transcritos dos genes alvo, relativos ao controle, em parasitas tratados com dsRNA após 2, 4 e 7 dias. Esporocistos foram tratados com os dsRNA específicos por 2 dias (barras pontilhadas), 4 dias (barra cinza) e 7 dias (barra preta). Os níveis de transcritos foram comparados com aqueles de parasitos tratados com dsRNA-GFP (controle). Os resultados foram analisados usando o metodo de ΔΔCT seguido do teste de Mann-Whitney (Wilcoxon sum of ranks); $P< 0.05$; $N=4$, decréscimo significativos nos níveis de transcritos são mostrados com asteriscos (*). A linha pontilhada representa o nível normalizado de transcritos na população controle (dsGFP).

Os resultados da Figura 15 mostraram vários perfis de silenciamento dos transcritos após diferentes tempos de exposição aos dsRNAs. Observa-se que os níveis dos transcritos de EF1α e Calcineurina B reduziram significativamente até ~70% no dia 7, consistente com os dados previamente observados. No entanto, após dois dias de exposição ao dsRNA, Calcineurina B apresentou um maior silenciamento chegando a ~90% de redução dos níveis do transcrito. Os níveis de mRNAs de Smad4 e Lactato Desidrogenase, os quais mostraram um pequeno, mas significativo decréscimo ao sétimo dia, também exibiram o maior silenciamento observado (~40%) após dois dias de exposição, indicando um efeito rápido do tratamento com dsRNA nos níveis destes transcritos. Observou-se que SOD estava super-expressa em todos os tempos testados, com um máximo de expressão ao quarto dia (aumento de ~17.000 vezes), retornando no sétimo dia aos mesmos níveis de transcritos observados após dois dias de exposição (~1.200x). Tratamento com dsRNA para RHO2, que previamente não havia mostrado qualquer efeito nos níveis de expressão deste transcrito no sétimo dia após tratamento, levou uma redução de 70% do transcrito no segundo e quarto dias após exposição. Tratamentos com dsRNA Smad2, SmRing box e Miosina não apresentaram diferença significante em nenhum tempo analisado.

Pudemos observar que há diferentes padrões de resposta ao tratamento com dsRNAs, parecendo este depender especificamente do gene alvo. Vimos que, enquanto alguns tratamentos pareciam não ter exibido efeito de silenciamento no nível dos transcritos (Smad2, Rho2, SmRing box), ou terem surtido apenas um leve efeito (Smad4, Lactato Desidrogenase) após sete dias de tratamento, três deles (Rho2, Smad4 e Lactato Desidrogenase) apresentaram uma significante diminuição com dois dias de tratamento, confirmando que o fenótipo observado pode ser devido a um silenciamento gênico no início da transformação dos esporocistos. Respostas rápidas ao tratamento com dsRNA são frequentemente observadas em diferentes organismos e tecidos, sendo que estas podem ter início e fim em poucas horas (Layzer *et al*., 2004, Bartlett & Davis, 2006).

Observamos que não houve diminuição dos transcritos específicos nos tratamentos com dsRNA de Smad2 e SmRing Box, apesar desses tratamentos terem levado a uma diminuição no tamanho dos parasitas, mas não sabemos a razão deste comportamento. Existe a possibilidade de ter havido *off-target*, ou seja, genes inespecíficos serem silenciados e, portanto, ter havido a ocorrência do fenótipo. *Off-target* é um efeito indesejável comum e é uma grande preocupação em experimentos de RNAi. Para evitarmos a ocorrência deste efeito podemos tomar várias precauções, tais como: (1) confirmar por pesquisa de similaridade

utilizando a ferramenta BLAST que os iniciadores desenhados para amplificar as sequências de cDNA sejam únicos e originem somente um amplicon, (2) sequenciar o amplicon obtido para a geração do dsRNA, (3) amplificar genes não relacionados como controles de silenciamento inespecífico por PCR quantitativo. Em nosso estudo, utilizamos longos dsRNAs de ~500pb, que após processamento pelo complexo DICER pode resultar em pequenos siRNAs com sequências diversificadas que poderiam ser uma potencial fonte de *off-target*. Tem sido mostrado que a eficiência do silenciamento em células de mamíferos é altamente dependente das sequências utilizadas nos experimentos (Reynolds *et al*., 2004) e que, em alguns casos, um pequeno grau de similaridade entre sequências do organismo e o siRNA utilizado podem causar este indesejável processo (Jackson *et al.,* 2003). Para complicar mais ainda, Fedorov e colaboradores (2006), mostraram que alguns siRNAs exibem efeitos tóxicos, não específicos, que afetam processos transcricionais, sem afetar os níveis dos transcritos alvo do silenciamento.

O resultado observado em relação aos níveis inalterados de transcritos de Miosina em parasitos tratados com dsRNA em todos os tempos examinados, corroboram com a ausência de um fenótipo visualizável. Esta observação nos leva a sugerir que talvez a sequência utilizada para a síntese do dsRNA não seria a sequência ideal para o silenciamento. Adicionalmente, é possível que a maquinaria de processamento do dsRNA (DICER) esteja clivando regiões do transcrito que produzam siRNAs inefetivos para o silenciamento gênico. Já foi demonstrado que a efetividade do silenciamento é altamente dependente da sequência utilizada para a síntese dos RNAs de fita dupla (Reynolds *et al.,* 2004).

Talvez, o resultado mais intrigante deste estudo seja o consistente alto nível de expressão dos transcritos de SOD após o tratamento com dsRNA de SOD. Uma possível explicação, mas altamente especulativa, é que após o processamento da sequência de dsRNA pelos esporocistos, alguma parte esteja de alguma forma estimulando um mecanismo similar ao processo de ativação por RNA (RNAa) (Li *et al*., 2006, Pushparaj *et al*., 2008). Se isto se confirmar, esta será uma nova faceta do mecanismo de interferência por RNA. Pretendemos avaliar com atenção especial este interessante resultado. Sabe-se que a enzima SOD possui função de anti-oxidante neste parasita (Guillou *et al*., 2007; Kuntz *et al*., 2007; Bender *et al*., 2007). No entanto, sua essencial função no parasita ainda não foi demonstrada.

4.5 Avaliação dos níveis protéicos de GST26, EF1α e Tiorredoxina 1 e 2 em esporocistos tratados com dsRNA para estes genes

Usamos a técnica de *Western blot* para detectar se os níveis protéicos de GST26, EF1α e Tiorredoxina 1 e 2 nos parasitos tratados com dsRNA apresentavam uma correlação com o decréscimo dos transcritos observados por q-PCR (Figuras 16, 17 e 18). Utilizando extrato protéico total de parasitos tratados com dsRNA para GST26, EF1α, Tiorredoxina Peroxidase 1/2 e dsRNA-GFP (controle), juntamente com anticorpos primários específicos para Glutationa-S-Transferase 26, EF1α e Tiorredoxina Peroxidase 1 e 2 pudemos confirmar, também no nível protéico, o silenciamento destes genes.

Os esporocistos silenciados para GST26, EF1α e Tiorredoxina Peroxidase 1/2 após 7 dias de tratamento, apresentam uma redução específica dos transcritos alvos e, conseqüentemente, do nível de proteína correspondente (~90%, 80% e 50%, respectivamente) quantificados por densitometria (Figura 16,17 e 18), sendo esta mais uma importante confirmação do silenciamento dos referidos genes.

Fatores de elongação são essenciais para o processo de tradução e tem como papel carrear aminoacil tRNAs aos ribossomos durante a elongação da síntese proteica (Negrutskii & El'skaya, 1998). Dada esta função do EF1α, pensaríamos que seu silenciamento no nível dos transcritos e proteico (80% de redução da proteína após o tratamento com o dsRNA específico) levaria a uma diminuição da taxa da tradução como um todo na célula e no organismo silenciado. As razões por não termos visto mudanças nos níveis da proteína GST26 (controle normalizador de proteínas) no experimento de *Western blot* de silenciamento de EF1α poderia ser devido, talvez, ao lento *turnover* de GST26 ou que esta proteína esteja sendo sintetizada em células e tecidos não afetados pelo silenciamento de EF1α.

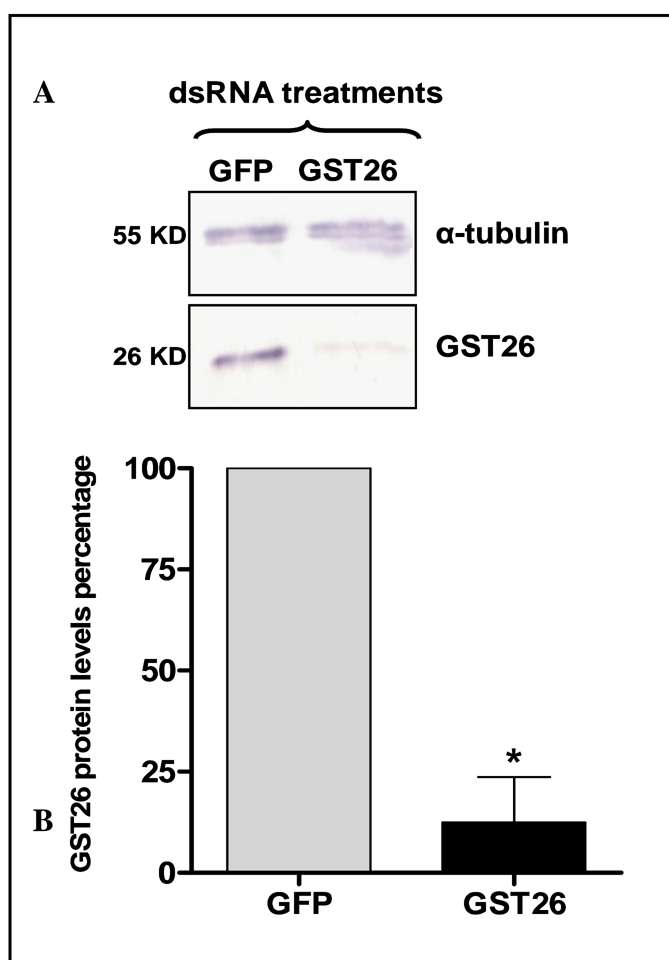


Figura 16: *Western blot* mostrando o nível da proteína Glutationa-S-Transferase 26 em extratos proteicos de parasitos tratados por 7 dias com dsRNA-GFP e dsRNA-GST26 após 7 dias. A) *Western blot* mostrando nível de proteína GST26 (26KDa) e α-Tubulina (55KDa) (controle interno). Foram aplicados no gel ~8µg de proteína total de parasitos tratados com dsGFP e de esporocistos tratados com dsGST26, respectivamente. B) Nível normalizado (obtido por densitometria ótica) da proteína GST26, média de três experimentos de *Western blot*, em parasitos silenciados para GST26 (preto) comparados com amostras tratadas com dsGFP (cinza) (*)P<0,01.

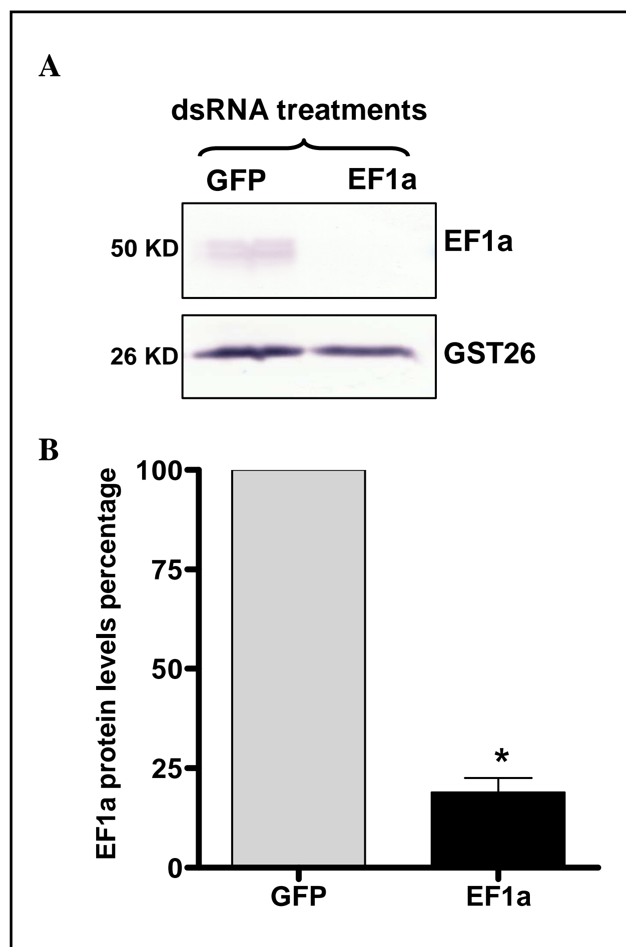


Figura 17: *Western blot* mostrando o nível da proteína Fator de Elongação 1 α em extratos proteicos de parasitos tratados por 7 dias com dsRNA-GFP e dsRNA-EF1α. A) *Western blot* mostrando nível de proteína do Fator de Elongação 1α (50KDa) e GST26 (26KDa) (controle interno). Foram aplicados no gel ~10μg de proteína total de parasitos tratados com dsRNA-GFP e dsRNA-Fator de Elongação 1 α, respectivamente. B) Nível normalizado (obtido por densitometria ótica) da proteína Fator de Elongação 1α, média de três experimentos de *Western blot*, em parasitos silenciados para Fator de Elongação 1α (preto) comparados com amostras tratadas com dsRNA-GFP (cinza). (*)P<0,01.

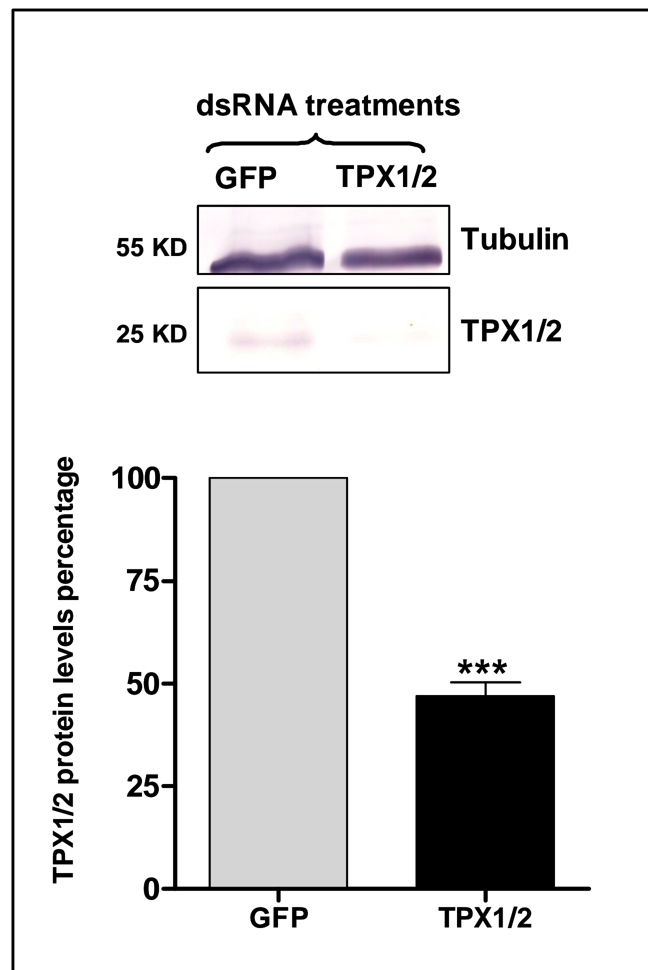


Figura 18: ***Western blot*** **mostrando os níveis das proteínas Tiorredoxina Peroxidase 1 e 2 em extratos proteicos de parasitos tratados por 7 dias com dsRNA-GFP e dsRNA-TPx1/2.** A) *Western blot* mostrando nível das proteínas Tiorredoxina Peroxidase 1 e 2 (25KDa) e alfa-Tubulina (55KDa) (controle interno). Foram aplicados no gel ~10µg proteína total de parasitos tratados com dsRNA-GFP e dsRNA-TPx1/2, respectivamente. B) Nível normalizado (obtido por densitometria ótica) das proteínas Tiorredoxina Peroxidase 1 e 2, média de três experimentos de *Western blot*, em parasitos silenciados para Tiorredoxina Peroxidase 1/2 (preto) comparados com amostras tratadas com dsRNA-GFP (cinza). (***)$P<0.001$

4.6 Detecção *in situ* de GST26, EF1α e TPx1 e 2 em parasitos silenciados

Detectamos o nível protéico por imunofluorescência de GST26, EF1α e TPx 1/2 *in situ* em esporocistos controle e silenciados. Verificamos que há uma diminuição das proteínas GST26 e EF1α em parasitas silenciados para estes genes. Parasitas tratados mostraram muito pouca imunoreatividade (fluorescência), quando comparados com esporocistos controle que mostraram uma alta reatividade com os anticorpos anti-GST26 e anti-EF1α evidentes nas células e tecidos, refletindo os baixos níveis proteícos após o silenciamento destes genes. Estes resultados são consistentes com as reatividades préviamente observadas por Western Blot, ficando assim, evidenciada a correlação entre diminuição dos transcritos e proteica (Figuras 19 e 20). No entanto, a imunolocalização de anti-TPx1 e 2 apresentou uma pequena diminuição na intensidade da imunoreatividade entre os grupos de parasitas tratados com dsRNA para GFP e TPx 1 e 2 (Figura 21), exceto por um maior decrécimo de coloração (imunoreatividade) observado na superfície dos esporocistos silenciados. Estes resultados são consistentes com a menor diminuição na expressão proteica (~50% redução), observada anteriormente por Western blot, em relação ao decrécimo observado para os tratamentos com GST26 e EF1α.

Infelizmente a falta de anticorpos compatíveis com as proteínas de *S. mansoni*, que fizeram parte deste estudo, nos impossibilitou de examinar o nível proteico de outros tratamentos com dsRNA.

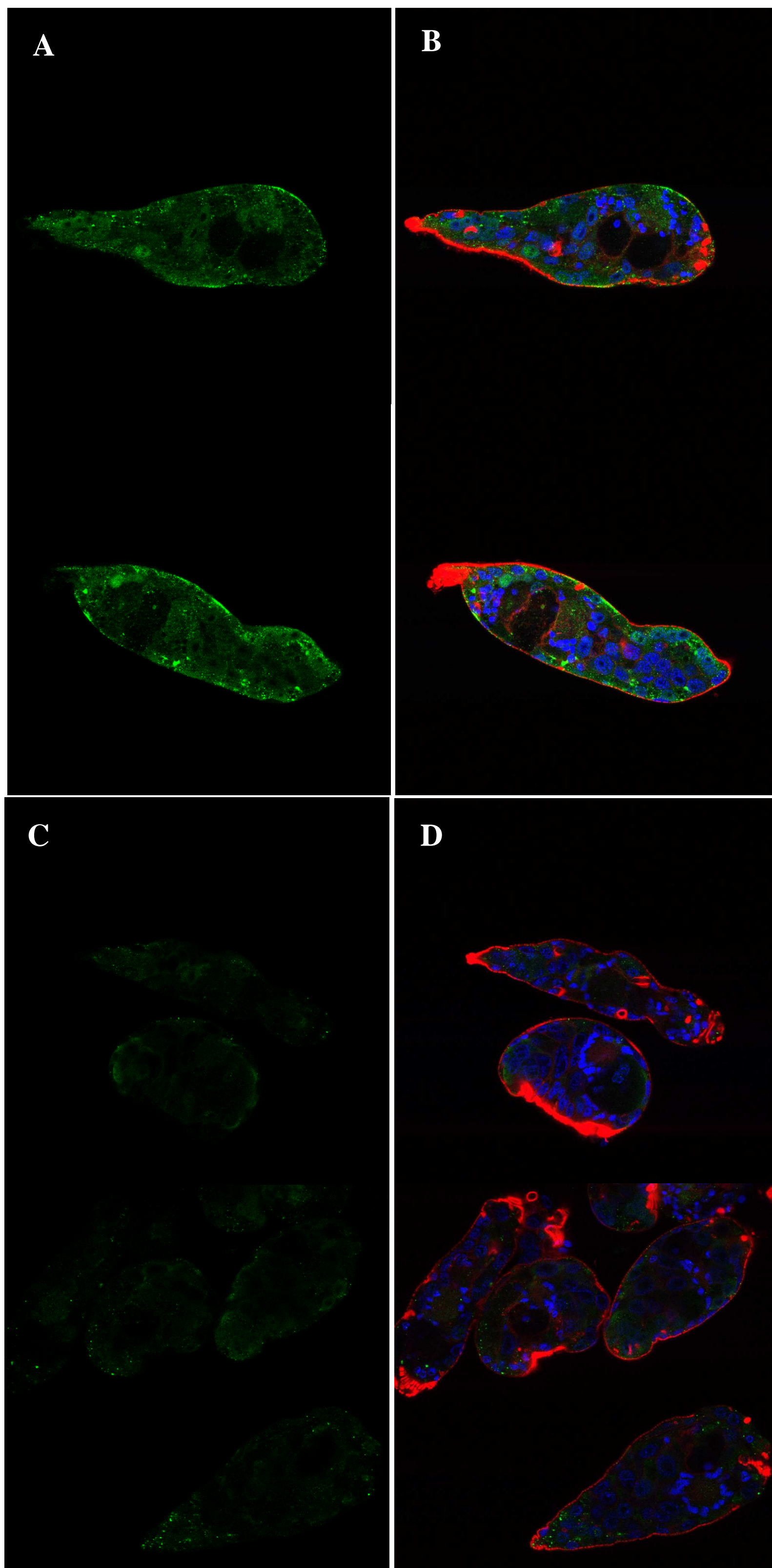


Figura 19: Imunofluorescência dos esporocistos após silenciamento do transcrito de GST26. Imagens mostrando (A) Proteína GST26 (alexa488, fluorescência verde) em esporocistos tratados com dsRNA-GFP por 7 dias (controle); (B) imagem fluorescente correspondente, mostrando a proteína GST26 (fluorescência verde), DNA (Hoechst, azul) e filamentos de actina (Faloidina, vermelho). (C) Imagens de esporocistos tratrados com dsRNA-GST26, (D) Diminuição de proteína GST26 (verde), DNA (Hoechst, azul) e filamentos de actina (Faloidina, vermelho) nos mesmos parasitos.

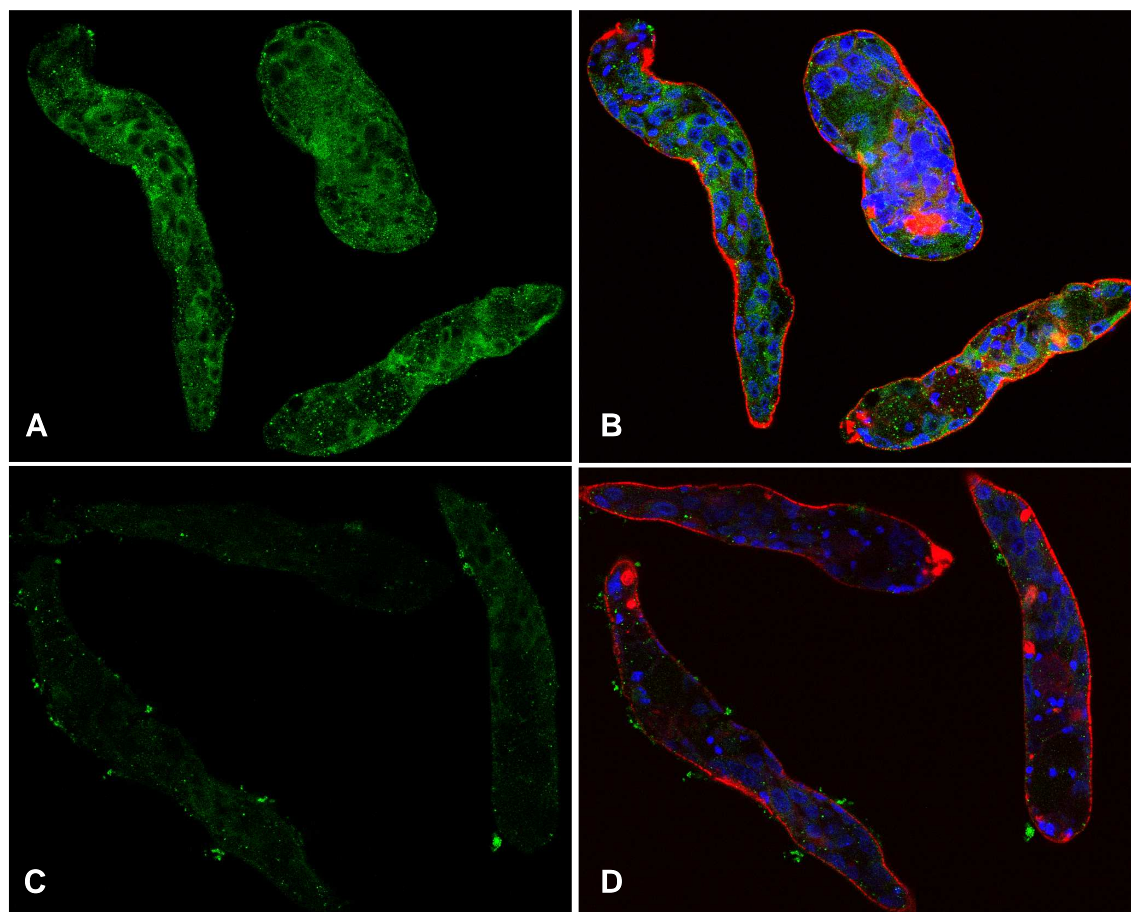


Figura 20: Imunofluorescência dos esporocistos após silenciamento do transcrito para EF1α. Imagens mostrando (A) Proteína EF1α (alexa488, fluorescência verde) em esporocistos tratados com dsRNA-GFP apos 7 dias de tratamento (controle); (B) imagem fluorescente correspondente, mostrando a proteína EF1α (fluorescência verde), DNA (Hoechst, azul) e filamentos de actina (Faloidina, vermelho). (C) Imagens de esporocistos tratados com dsRNA-EF1α, mostrando a diminuição de (fluorescência verde), (D) imagem fluorescente correspondente, mostrando a proteína EF1α (fluorescência verde), DNA (Hoechst, azul) e filamentos de actina (Faloidina, vermelho) nos mesmos parasitos.

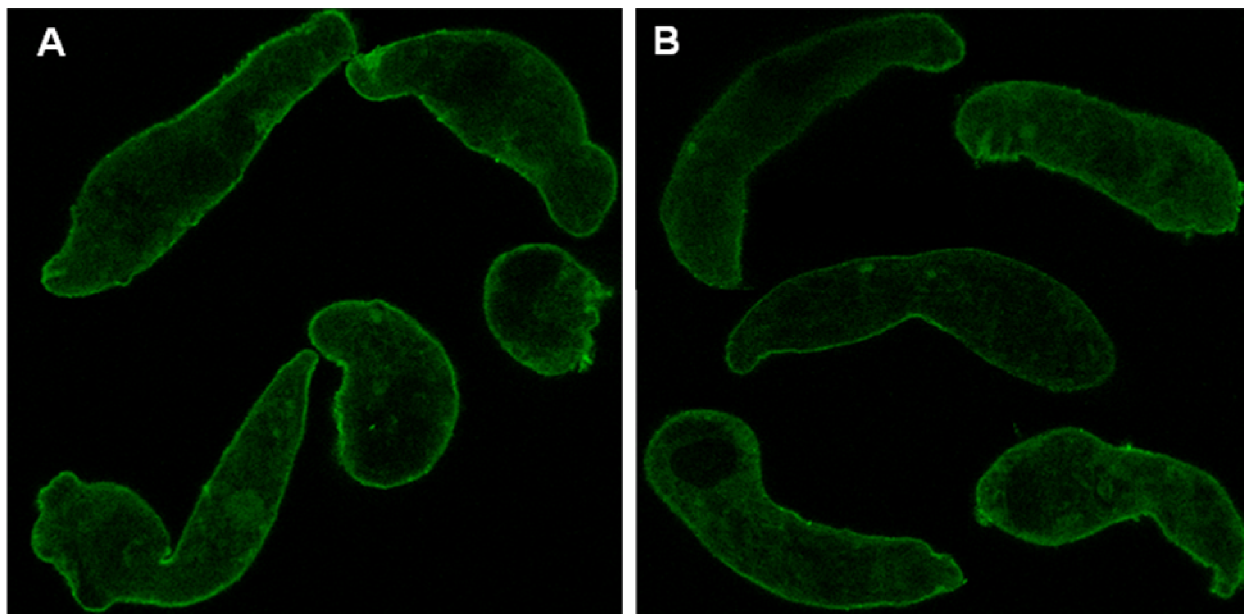


Figura 21: Imunofluorescência dos esporocistos após silenciamento dos transcritos das Tiorredoxinas peroxidases 1 e 2. Imagens mostrando (A) Proteínas TPx 1 e 2 (alexa488, fluorescência verde) em esporocistos tratados com dsRNA-GFP apos 7 dias de tratamento (controle); (B) imagem fluorescente de esporocistos tratados com dsRNA TPx1 e 2, mostrando uma pequena diminuição das proteínas TPx 1 e 2 (fluorescência verde).

4.7 Mortalidade dos esporocistos após tratamento com dsRNA

Após observarmos que alguns silenciamentos foram muito bem sucedidos em nossa triagem, resolvemos aprofundar no estudo funcional das enzimas anti-oxidantes, supostamente envolvidas na função de proteção dos esporocistos contra a ação de espécies reativas de oxigênio. Discutiremos adiante o papel central dessas enzimas na interação do parasita *S. mansoni* e seu hospedeiro intermediário.

Após oito dias de incubação com dsRNA, para o silenciamento de transcritos de enzimas com capacidade oxi-redutora, notamos que alguns grupos apresentavam um maior número de parasitas mortos nas placas de cultivo. Portanto, para analisarmos a existência de um fenótipo letal nestas amostras, adicionamos iodeto de propídio e contamos o número de organismos mortos após o tratamento, em cinco experimentos (Figura 22).

Foi verificado que apenas parasitos silenciados para Tiorredoxina peroxidase 1 e 2 apresentaram um porcentagem maior (17%) de organismos mortos ($P<0.004$), indicando que o silenciamento das duas Tiorredoxina peroxidases tornam o organismo mais vulnerável e sensível a fatores externos presentes no meio, possivelmente radicais livres (Figura 22). Portanto, a viabilidade dos esporocistos, em longo prazo, parece depender de níveis normais das enzimas Tiorredoxina peroxidase 1 e 2, indicando que estas enzimas são importantes para sobrevivência do parasita em qualquer de seus hospedeiros, pois, foi previamente observado a letalidade do silenciamento de TPx1 em esquistossômulos (Sayed *et al.*, 2006). Portanto, estas observações indicam que estas enzimas podem ser importantes candidatas para o desenvolvimento de drogas anti-esquistossomóticas. Interessantemente, a mesma sensibilidade não foi observada quando TPx1 e 2 foram silenciadas em esquistossômulos de *S. japonicum* (Kumagai *et al.*, 2009).

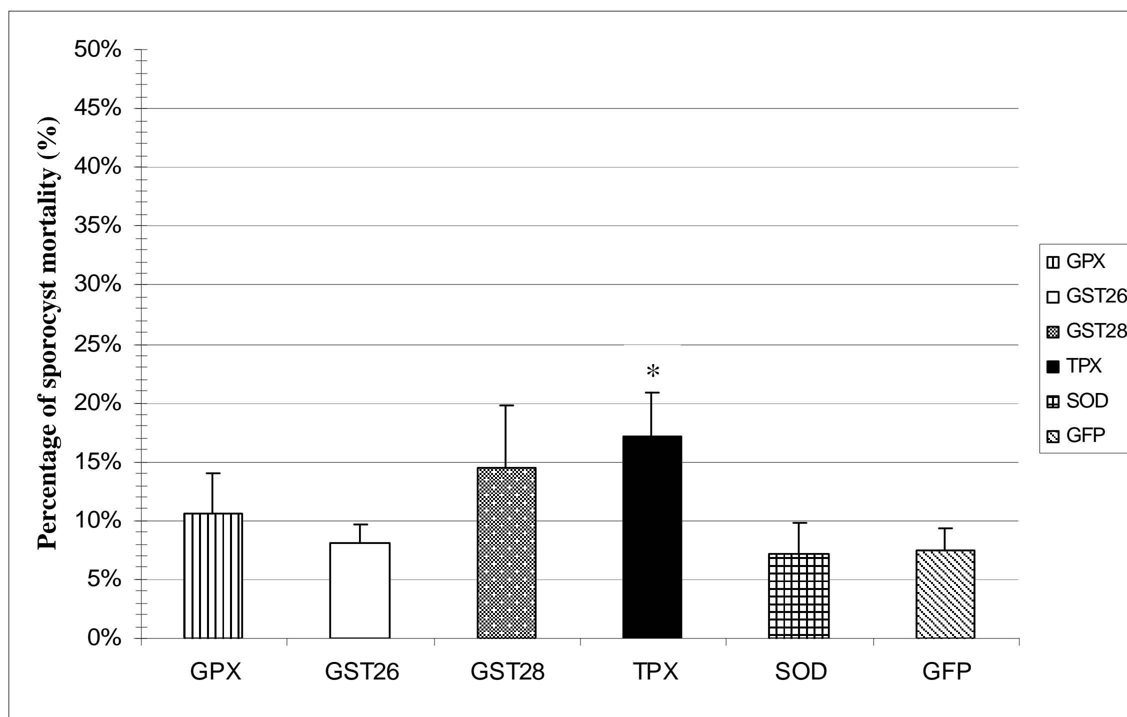


Figura 22: Mortalidade de esporocistos silenciados com dsRNA para enzimas relacionadas ao estresse oxidativo, após 8 dias de tratamento. Com asterisco (*) está representada a amostra estatisticamente significante. Significância analisada por Student's T test ($p<0.004$, n=5).

4.8 Experimento de estresse oxidativo

No intuito de avaliarmos funcionalmente o efeito do silenciamento gênico nas enzimas relacionadas ao estresse oxidativo, expusemos esporocistos tratados por oito dias com dsRNA de GFP (controle), SOD, GST26, GST28, TPx1/2 e GPx ao meio contendo peróxido de hidrogênio. A concentração de uso de 50µM de H_2O_2 foi determinada após a realização de uma curva de mortalidade utilizando diversas concentrações de peróxido de hidrogênio em esporocistos controle. O experimento de estresse oxidativo foi repetido seis vezes e todos os tratamentos possuíam seu respectivo controle negativo, ou seja, parasitas tratados com os dsRNAs sem a adição de H_2O_2. A viabilidade dos esporocistos foi avaliada após 4h, 24h e 48h de tratamento com H_2O_2 e a percentagem de mortalidade calculada. Resultados obtidos estão exibidos na Figura 23:

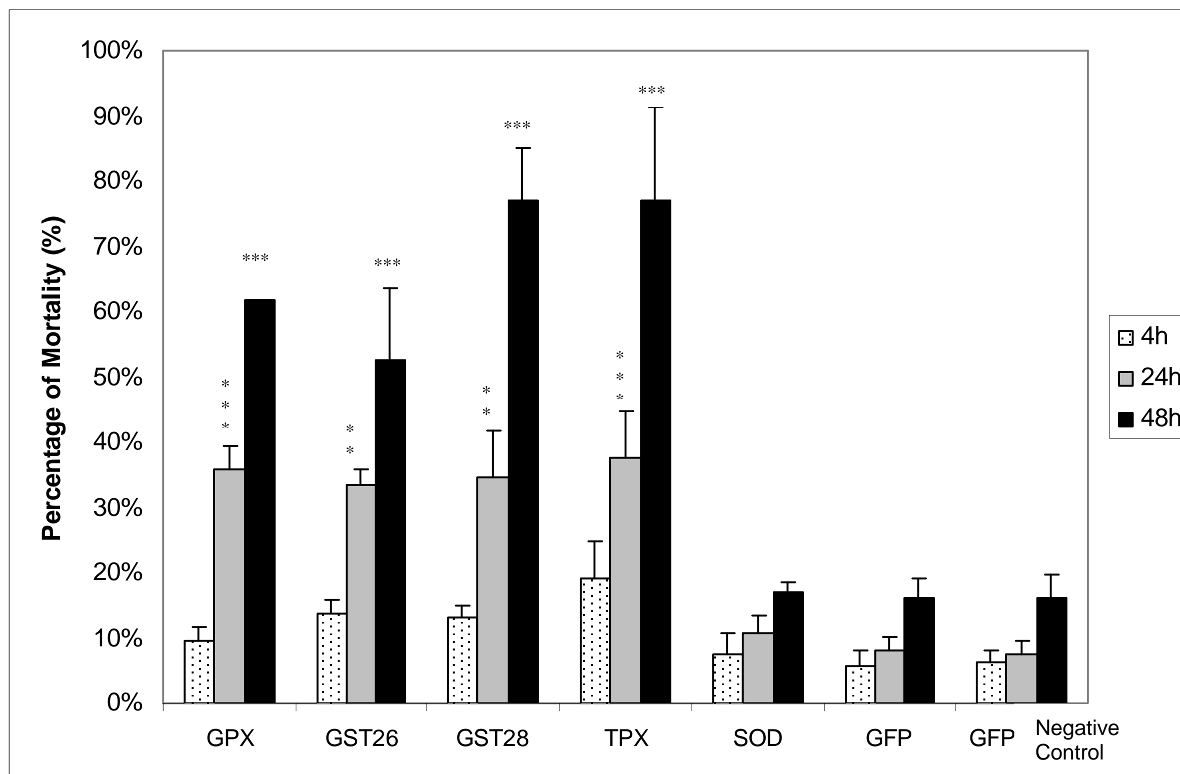


Figura 23: Mortalidade de esporocistos silenciados para enzimas antioxidantes, após exposição a estresse oxidativo. Parasitos após 8 dias de tratamento com dsRNA para GPx, GST26, GST28, TPx1/2, SOD e GFP, foram expostos a 50 µM de peróxido de hidrogênio e sua viabilidade avaliada após 4h, 24h e 48h. À direita, parasitas controle sem adição de H2O2 (GFP Controle negativo). Significância analisada por Two-way ANOVA e os resultados significativos foram tratados pelo teste de Bonferroni, comparando-se cada tempo do tratamento com seus respectivos tempos em esporocistos tratados com GFP (controle) (significância (**) p<0,01 (***) p<0,001, N=6). As barras representam o erro padrão entre experimentos.

Parasitos tratados com dsRNA para silenciamento das enzimas anti-oxidantes (SOD, GST26, GST28, TPx1/2 e GPx) se mostraram significativamente ($p<0,01$) mais suscetíveis ao estresse oxidativo, após 24h e 48h de exposição a H_2O_2 quando comparados à parasitos controle (GFP e GFP controle negativo), com exceção de SOD que apresentou comportamento semelhante aos esporocistos controle durante todos os intervalos de tempo observados. Parasitos silenciados para GST26, GST28, TPx1/2 e GPx apresentaram uma média de aumento de mortalidade de ~35% após 24h ($P <0.0001$) e, 60 a 80% após 48h de exposição ao estresse oxidativo ($P < 0.0001$, N=6), comparado com 8% e 18% de mortalidade dos controles, respectivamente. A resistência ao estresse oxidativo de esporocistos tratados com dsRNA para Superoxido Dismutase corrobora com os achados de que esta enzima poderia estar sendo super-expressa após tratamento com dsRNA, como observado por q-PCR.

Um fato muito importante, tanto na validação dos experimentos com RNAi como no estudo das enzimas anti-oxidantes, foi a possível correlação entre o decrécimo no nível de transcritos e diminuição proteica de GST26 e TPx1/2 e o aumento da mortalidade dos parasitas silenciados após 24 e 48 h de exposição à H_2O_2. Além disso, apesar da impossibilidade de averiguação dos níveis proteicos de GST28 e GPx, estas enzimas também demonstraram uma correlação funcional comparável com os níveis de transcritos observados e comparável com os resultados observados para as enzimas GST26 e TPx1/2. Esses resultados indicam uma função direta destas enzimas do parasita na ação contra o estresse oxidativo.

Nossas descobertas estão de acordo com o intenso trabalho que vem sendo desenvolvido sobre os mecanismos redox em vermes adultos do *S. mansoni* e, possivelmente, sobre a existência de uma única enzima que combina as atividades das enzimas tiorredoxina redutase e glutationa redutase (TGR) presentes em mamíferos (Alger & Williams, 2002, Kuntz *et al*., 2007). Esta enzima de *S. mansoni* é responsável por manter a tiorredoxina e a glutationa nos seus estados ativos reduzidos, permitindo assim, a ativação de três tiorredoxinas peroxidases (TPx1-3) e da glutationa peroxidase (GPx), as quais serão então capazes de reduzir H_2O_2 e outras espécies reativas de oxigênio (Sayed & Williams, 2004). Portanto, há indicações de que esporocistos, assim como vermes adultos, dependem de um sistema de anti-oxidantes endógeno robusto para se estabelecer em seu hospedeiro através da superação dos ataques de espécies reativas de oxigênio.

Para certificarmos de que peróxido de hidrogênio era o fator oxidante no meio e que este seria o causador da morte dos parasitos, expusemos esporocistos silenciados à mesma concentração subletal de peróxido de hidrogênio (50µM) na presença e ausência de catalase bovina 0,025% e como controle, apenas catalase foi adicionada ao meio de cultura (Figura 24).

De forma geral, a porcentagem de mortalidade dos parasitas tratados com dsRNA para GPx, GST26, GST28 e TPx1/2 quando expostos à H_2O_2+catalase, ou à apenas catalase foram bastante similares ($P>0,05$; não sendo estatisticamente significantes) (Figura 24). Estes resultados estão em contraste com os efeitos observados após exposição à H_2O_2 pura, no qual a mortalidade dos esporocistos silenciados para GPx, GST26, GST28 e TPx foram significativamente maiores, com uma média de mortalidade de 48%, comparado à ~15% de mortalidade no grupo controle. A taxa de mortalidade exibida por esporocistos tratados apenas com a enzima catalase, não foi significativamente diferente daquela de parasitos expostos a H_2O_2 adicionado de catalase. No entanto, a percentagem de esporocistos mortos quando expostos a peróxido de hidrogênio é significativamente elevada para organismos silenciados para GPx, GST26, GST28 e TPx 1 e 2. Assim, a catalase foi capaz de neutralizar os efeitos do peróxido de hidrogênio, mostrando que H_2O_2 é o fator oxidante no meio e a causa da morte dos esporocistos silenciados para as enzimas envolvidas em detoxificação dos parasitos.

Foi demonstrado por Vermeire e Yoshino (2007) que as enzimas Tiorredoxinas são secretadas durante a transformação de miracídios em esporocistos e estas devem funcionar no mecanismo de defesa do parasita, protegendo-o de H_2O_2 produzida exógenamente. Mais uma vez, pudemos observar que esporocistos silenciados para TPx 1 e 2 apresentaram uma maior vulnerabilidade às agressões do meio e, consequentemente, 76% de mortalidade ($p<0.01$), confirmando a importância destas enzimas na proteção do parasita frente a exposição ao peróxido de hidrogênio.

Como observado, o tratamento de esporocistos com dsRNA para SOD, mais uma vez, não apresentou nenhuma diferença na taxa de mortalidade entre os diferentes tratamentos (H_2O_2+catalase, apenas catalase e H_2O_2) e nem quando comparado às taxas de mortalidade observadas em parasitas controle, pois esse gene se encontrava super-expresso após tratamento com dsRNA.

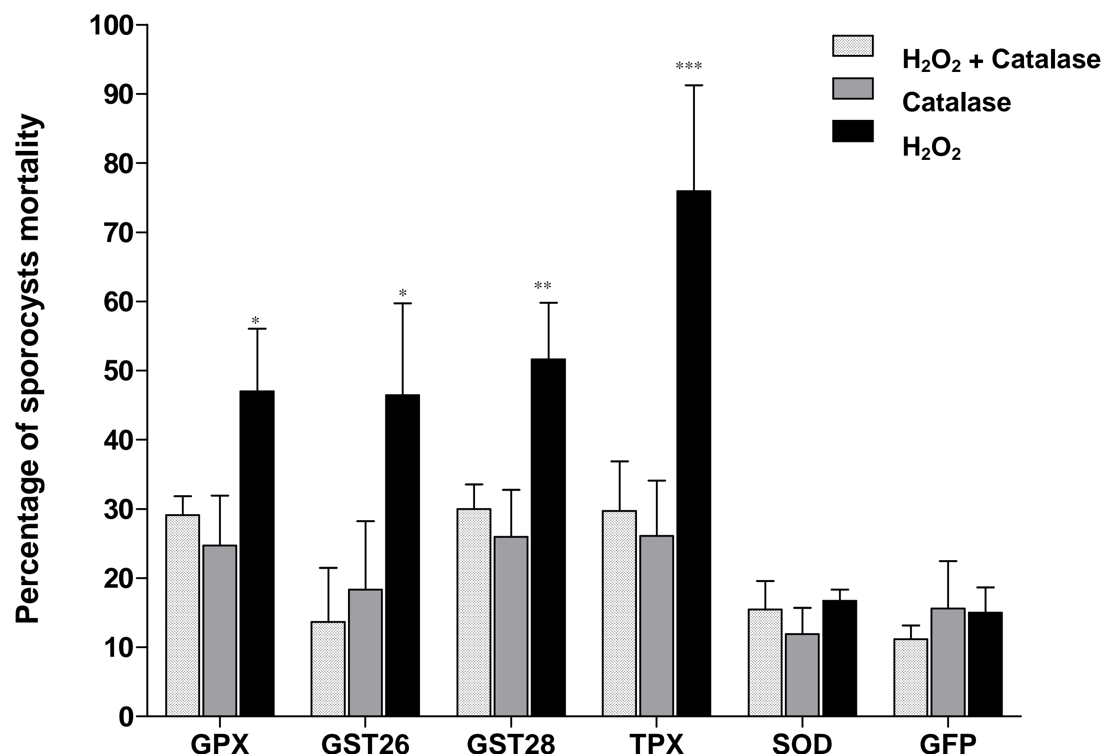


Figura 24: Mortalidade de esporocistos silenciados para enzimas antioxidantes, expostos a peróxido de hidrogênio e a peróxido de hidrogênio acrescido de catalase. Parasitos após 8 dias de tratamento com dsRNA para GPx, GST26, GST28, TPx1 e 2, SOD e GFP, foram expostos a 50 µM de peróxido de hidrogênio, na presença ou ausência de catalase bovina (0,025%) e sua viabilidade avaliada após 48h de exposição. Significância analisada por Two-way Anova e os resultados significativos foram tratados pelo teste de Bonferroni, comparando-se cada tempo do tratamento com seus respectivos tempos em esporocistos tratados com GFP (controle) (significância (*) $p<0,05$, (**) $p<0,01$, (***) $p<0,001$, N=6). As barras representam o erro padrão entre experimentos.

4.9 Ação dos hemócitos do hospedeiro na morte do parasita

É sabido que na interação parasita-hospedeiro o sistema de defesa do hospedeiro tenta combater a infecção de diversas maneiras, uma delas seria por produção de superoxido (O_2^-) pelo complexo NADPH oxidase. A liberação de espécies de oxigênio reativo (ROS) por este complexo enzimático gera H_2O_2, radical hidroxil ($^{\cdot}OH$), ácido hipoclórico (HOCl) e oxigênio singlete ($^1O_2$) na maioria das células fagocíticas (Bayne *et al*; 2001). No entanto, foi mostrado por Hahn e colaboradores (2001) que, apesar destes compostos serem produzidos por *B. glabrata,* são as espécies oxidantes peróxido de hidrogênio e óxido nítrico os compostos diretamente envolvidos na morte de esporocistos de *S. mansoni* mediada por hemócitos (células reativas de defesa) de *Biomphalaria* resistente à infecção.

Finalmente, para melhor entendermos o papel das enzimas responsáveis pela neutralização de espécies reativas de oxigênio no parasita, frente ao sistema de defesa do hospedeiro, expusemos hemócitos isolados de *B. glabrata* da cepa NMRI (susceptível a infecção) a esporocistos silenciados para as enzimas GPx, GST26, GST28, TPx1 e 2 e SOD (co-cultura). Analisamos a mortalidade dos parasitos frente às células do hospedeiro após 24h de co-cultivo (teste de toxicidade mediada por célula, como descrito por Hahn *et al*., 2001). Os resultados obtidos são mostrados na Figura 25.

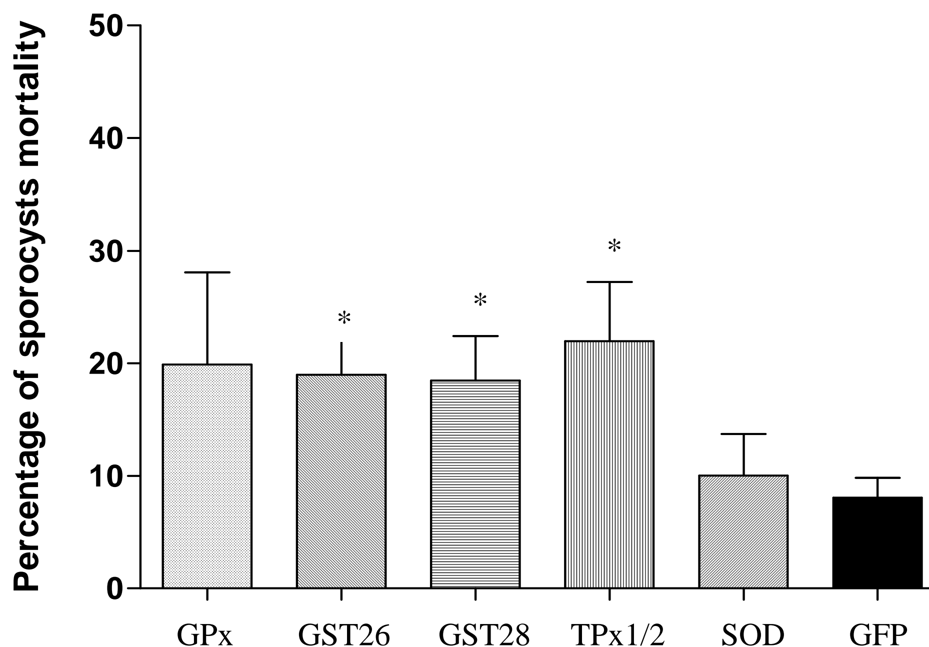


Figura 25: Mortalidade de esporocistos silenciados para enzimas antioxidantes após interação com hemócitos do hospedeiro *B. glabrata* (NMRI). As barras com asterisco (*) correspondem às amostras que apresentaram um aumento significativo de mortalidade quando comparadas com controle (GFP), após 24h de exposição. Significância analisada por Student's Ttest ($p<0.01$, n=4).

Após 24 horas de incubação *in vitro,* parasitos silenciados para GST26 ($p<0.01$, N=4), GST28 e TPx1 e 2 ($p<0.04$; N=4) mostraram um aumento significativo de vulnerabilidade a hemócitos de *B. glabrata* da cepa suscetível NMRI (~20%), quando comparados ao controle (8%) (Figura 25). Esporocistos silenciados para Glutationa Peroxidase mostraram mortalidade aumentada quando expostos as células do hospedeiro, mas, no entanto, os valores não foram estatisticamente significativos ($p>0.05$). Mais uma vez, esporocistos tratados com Superoxido Dismutase não mostraram relevante alteração quando comparados ao grupo controle, possivelmente, devido à superexpressão desta enzima nos parasitos tratados.

Hemócitos de caramujos da cepa susceptível NMRI são capazes de encapsular os esporocistos, mas são incapazes de matá-los, provavelmente devido à menor produção de peróxido de hidrogênio pelas células (Bayne *et al*., 1980; Hahn *et al*., 2001a, Bender *et al*., 2005). Neste experimento, pretendíamos testar a hipótese de que esporocistos silenciados possuiriam uma capacidade antioxidante reduzida, aumentando sua vulnerabilidade aos níveis subletais de espécies reativas de oxigênio normalmente produzidas por hemócitos de caramujos da cepa suceptível. Assim, demonstramos uma significante função protetora das enzimas TPxs e GSTs na interação dos esporocistos com as células de defesa reativas do hospedeiro intermediário, reforçando a importância destas enzimas na proteção do parasita contra o sistema de defesa do hospedeiro. Através do observado aumento de mortalidade dos esporocistos silenciados pode ser inferido que tratamentos com dsRNA para GST28 e GPx também induziram uma diminuição específica dos produtos proteicos destes alvos.

Foi proposto por Bayne e colaboradores (2001) que enzimas envolvidas em vias de redução e oxidação celular são componetes essenciais na regulação das interações moleculares entre *S. mansoni* e hospedeiro intermediário *B. glabrata* (Bayne *et al*., 2001; Bayne. 2009). Hoje se tem conhecimento de que células de defesa (hemócitos) de certas cepas do caramujo *B. glabrata* produzem uma quantidade substancial de espécies de oxigênio reativo e nitrogênio (Hahn *et al*., 2001). Isso acontece como consequência da estimulação dos hemócitos por ativadores de espécies reativas de oxigênio ou nitrogênio (ROS/RNS) quando de encontro com esporocistos de *S. mansoni* (Hertel *et al*., 2005; Hahn *et al*., 2001), e que, estes últimos são altamente sensíveis a estes compostos, especialmente H_2O_2. A enzima Cu/Zn-superoxido dismutase (SOD) parece ter uma função central nestes hemócitos de caramujos resistentes, agindo na atividade de defesa através da conversão de superóxido à H_2O_2 citotóxica. Estudos demostraram expressão aumentada dos transcritos de SOD e uma

maior atividade desta enzima em hemócitos de algumas cepas de *B. glabrata* resistentes à infecção, quando comparadas à cepas suceptíveis (Goodall *et al*., 2004) e isto foi correlacionado com maior produção de H_2O_2 (Bender *et al*., 2005). Dentro deste contexto foi sugerido que diferenças na expressão de SOD por diferentes cepas de caramujo possa estar correlacionado aos fenótipos de resistência e susceptibilidade (Bayne, 2009).

Enquanto os hemócitos de *B. glabrata* produzem peróxido de hidrogênio como molécula efetora anti-parasítica, foi mostrado que larvas de *S. mansoni* possuem atividades anti-oxidantes, basicamente através de enzimas específicas (Williams *et al*., 2007; Taft *et al*., 2009). Devido a aparente falta da enzima catalase, principal enzima responsável pelo metabolismo de H_2O_2 na maioria dos organismos (Mkoji *et al*., 1988a, 1988b), este parasita necessita de diferentes formas de neutralização de H_2O_2. Como claramente demonstrado na fase adulta de *S. mansoni*, a neutralização deste composto acontece por sistemas tiol-dependentes que envolvem a enzima tiorredoxina glutationa redutase como pivô das reações anti-oxidantes (Alger & Williams, 2002). Similarmente, os estágios de miracídios e esporocistos primários também possuem elementos do sistema redox, incluindo SOD, GPx, GST, TPx1 e TPx2 e estas apresentam uma expressão gênica extremamente aumentada em resposta à exposição às espécies reativas do molusco hospedeiro (Zelck & Janowsky, 2004; Vermeire & Yoshino, 2007). Em adição, algumas destas enzimas (Cu/Zn SOD, GSTs e TPxs) foram identificadas entre proteínas secretadas durante a transformação *in vitro* de miracídios em esporocistos, indicando que enzimas anti-oxidantes liberadas durante o desenvolvimento larval podem ter uma função protetora do parasita frente ao seu hospedeiro (Guillou *et al*., 2007; Wu *et al*., 2009).

Nossos resultados sugerem que a produção de espécies reativas de oxigênio por hemócitos de *B. glabrata* da cepa suceptível são capazes de se impor frente à parasitas deficientes em enzimas antioxidantes. Anteriormente, foi proposto por Zelck e Janowsky (2003), que moluscos susceptíveis à infecção geram uma pequena quantidade de ROS, na qual por sua vez, induzem a produção de anti-oxidantes em esquistossomas que neutralizam este ataque efetivamente. Neste estudo, demonstramos que com a existência de deficiência em níveis normais de uma ou mais enzimas antioxidantes (GST26, GST28, TPxs ou GPx), a sobrevivência de esporocistos é significantemente reduzida quando confrontados por H_2O_2 ou desafiados por hemócitos, suportando a idéia da importância de um sistema anti-oxidante eficiente no estabelecimento de infecções viáveis.

A enzima superoxido dismutase foi uma grande exceção ao que foi até agora discutido sobre o aumento da susceptibilidade dos parasitas silenciados ao estresse oxidativo. Durante as exposições à água oxigenada ou à encapsulação por hemócitos os parasitas submetidos ao tratamento mediado à dsRNA para SOD não demonstraram qualquer alteração de resposta. Este efeito diferencial corrobora com o fato que este gene esteja superexpresso após o tratamento com RNAi, o que por sua vez, induziria a produção da enzima SOD. Até o momento, os mecanismos de sinalização envolvidos nesta resposta não são conhecidos. É possível que tenha havido um rebote compensatório, após um rápido silenciamento desta enzima, mas isto é extremamente especulativo e talvez pouco provável, já que com dois dias os níveis de transcrito da enzima já estavam aumentados. Outra especulação seria a estabilização do transcrito através de ligação de possíveis produtos da degradação do dsRNA, ou dos próprios siRNAs, a algumas regiões instáveis do mRNA de SOD, tornando-o mais estável.

5. Conclusão

Os resultados apresentados neste trabalho mostram nitidamente a enorme variabilidade de intensidades de silenciamento que podem ser obtidas, considerando os tempos medidos e as concentrações de dsRNA utilizadas, quando se utiliza RNAi como ferramenta para manipulacão gênica em *S. mansoni.* Existe uma carência de técnicas refinadas e sensíveis para a detecção de variações sutis de fenótipos e há necessidade de perguntas específicas para cada gene silenciado. Além disso, há muito que se melhorar nos métodos atuais de absorção de dsRNA para o uso desta ferramenta neste organismo. Todas estas restrições ilustram a dificuldade do uso da técnica de RNAi em larga escala em esquistossomas.

Este estudo é o primeiro a utilizar múltiplos genes para acessar a eficácia de tratamentos com dsRNAs na caracterização das mudanças fenotípicas e transcricionais em esporocistos de *S. mansoni* cultivados *in vitro*. Foi mostrado que genes diferentes exibem diferentes cinéticas de silenciamento e, portanto, é necessário averiguar a efetividade do silenciamento em diferentes tempos após o tratamento. Ademais, como mostrado, genes individuais diferem significantemente em suas susceptibilidades ao tratamento e nem sempre existe uma correlação clara entre o fenótipo e os níveis do transcrito silenciado.

Neste estudo associamos claramente funções essenciais às enzimas anti-oxidantes GST26, GST28, GPx, TPx1/2 para a sobrevivência do parasita *S. mansoni* na presença de estresse oxidativo exógeno e frente a células de defesa do hospedeiro intermediário *B. glabrata*. A caracterização funcional destas enzimas foi possível após bem-sucedidos silenciamentos no nível de transcrito e proteico destas. Neste cenário, as Tiorredoxinas peroxidases parecem ser uma das mais importantes enzimas no combate ao estresse oxidativo.

Este trabalho ilustra também o nosso pouco entendimento sobre os mecanismos de regulação gênica em eucariotos e, especialmente, neste complexo parasita. Inusitadamente, utilizando a técnica de silenciamento gênico, observamos um exacerbado e consistente aumento específico do transcrito para a enzima SOD. Esse resultado demonstra o nosso desconhecimento a respeito do universo dos pequenos RNAs e de como eles estão envolvidos no controle da degradação de transcritos codificadores de proteínas.

6- Perspectivas

- Estudos futuros envolvendo otimização da utilização de reagentes de transfecção e eletroporação, ajudarão a melhorar a consistência dos resultados de experimentos utilizando RNAi.

- Caracterizar funcionalmente outros genes que exibiram silenciamento após o tratamento com dsRNA.

- Aprofundar no estudo dos mecanismos que parecem gerar a superexpressão do gene da superoxido dismutase.

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ANEXOS

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Plos Neglected Tropical Diseases

esse trabalho foi capa da revista

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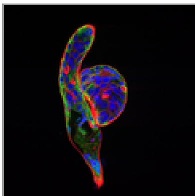

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About This Image

RNAi-altered gene expression in larval schistosomes. Confocal fluorescence micrograph of two in vitro-derived primary sporocysts of *Schistosoma mansoni* showing the somatic distribution of immunoreactive elongation factor 1a (EF1a) protein (green). Larvae are counterstained for actin (red) and DNA (blue) using Alexa-phalloidin and Hoechst dye, respectively. EF1a was one of 32 genes selected for larval phenotypic profiling by RNA interference (see article by de Moraes Mourão, et al. doi:10.1371/journal.pntd.0000502). *Image Credit: M. de Moraes Mourão, N. Dinguirard. Image was captured using a Nikon Eclipse TE2000 epifluorescent microscope equipped with a Bio-Rad Radiance 2100 MP Rainbow confocal imaging system (Keck Laboratory for Biological Imaging, University of Wisconsin).*

Editorial

A Constitutional Amendment for Deworming
Peter J. Hotez

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PLoS NEGLECTED TROPICAL DISEASES

Phenotypic Screen of Early-Developing Larvae of the Blood Fluke, *Schistosoma mansoni*, using RNA Interference

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Abstract

RNA interference (RNAi) represents the only method currently available for manipulating gene-specific expression in *Schistosoma* spp., although application of this technology as a functional genomic profiling tool has yet to be explored. In the present study 32 genes, including antioxidants, transcription factors, cell signaling molecules and metabolic enzymes, were selected to determine if gene knockdown by RNAi was associated with morphologically definable phenotypic changes in early intramolluscan larval development. Transcript selection was based on their high expression in *in vitro* cultured *S. mansoni* primary sporocysts and/or their potential involvement in developmental processes. Miracidia were allowed to transform to sporocysts in the presence of synthesized double-stranded RNAs (dsRNAs) and cultivated for 7 days, during which time developing larvae were closely observed for phenotypic changes including failure/delay in transformation, loss of motility, altered growth and death. Of the phenotypes evaluated, only one was consistently detected; namely a reduction in sporocyst size based on length measurements. The size-reducing phenotype was observed in 11 of the 33 (33%) dsRNA treatment groups, and of these 11 phenotype-associated genes (superoxide dismutase, Smad1, RHO2, Smad2, Cav2A, ring box, GST26, calcineurin B, Smad4, lactate dehydrogenase and EF1α), only 6 demonstrated a significant and consistent knockdown of specific transcript expression. Unexpectedly one phenotype-linked gene, superoxide dismutase (SOD), was highly induced (~1600-fold) upon dsRNA exposure. Variation in dsRNA-mediated silencing effects also was evident in the group of sporocysts that lacked any definable phenotype. Out of 22 nonphenotype-expressing dsRNA treatments (myosin, PKCB, HEXBP, calcium channel, Sma2, RHO1, PKC receptor, DHHC, PepcK, calreticulin, calpain, Smeg, 14.3.3, K5, SPO1, SmZF1, fibrillarin, GST28, GPx, TPx1, TPx2 and TPx2/TPx1), 12 were assessed for the transcript levels. Of those, 6 genes exhibited consistent reductions in steady-state transcript levels, while expression level for the rest remained unchanged. Results demonstrate that the efficacy of dsRNA-treatment in producing consistent phenotypic changes and/or altered gene expression levels in *S. mansoni* sporocysts is highly dependent on the selected gene (or the specific dsRNA sequence used) and the timing of evaluation after treatment. Although RNAi holds great promise as a functional genomics tool for larval schistosomes, our finding of potential off-target or nonspecific effects of some dsRNA treatments and variable efficiencies in specific gene knockdown indicate a critical need for gene-specific testing and optimization as an essential part of experimental design, execution and data interpretation.

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Introduction

Digenetic trematodes (parasitic flatworms) of the genus *Schistosoma* infect more than 200 million people in over 70 developing countries [1], with an additional 770 million people worldwide at risk of becoming infected [2]. As causative agents of chronic, often severe morbidity and responsibility for an estimated 280,000 death per year in Africa alone [3], schistosomiasis ranks as one of the most important of neglected tropical diseases [4].

Although significant research effort and funding have been dedicated to the treatment and control of schistosomiasis, including sanitary measures, suppression of the snail intermediate host, and chemotherapeutic interventions, there has been little change in the overall disease prevalence [5]. Progress in vaccine development has been very slow, and although several antigens, some of which are currently under clinical trial, have shown limited promise in rodent and primate challenge experiments, prospects are not good for an effective, highly protective vaccine in the foreseeable future [6,7]. Clearly there continues to be a pressing need for new strategies to break the cycle of schistosome transmission to the human population [8–10].

In view of the limited options available for controlling schistosomiasis in both the human host and snail vector, it is important that research focus on obtaining information that can be translated into new tools for parasite control. To that aim, genomic, transcriptomic and proteomic approaches offer strong

Author Summary

RNA interference (RNAi) represents the only method currently available for manipulating gene-specific expression in human blood flukes, *Schistosoma* spp., although its application as a functional genomics tool in early intramolluscan larval stages has been limited to single gene analyses. Accelerating gene discovery efforts over the past 10 years have resulted in extensive, ever-increasing databases of genomic, transcriptomic and EST sequences. Unfortunately, our understanding of the function of the vast majority of these genes has not kept pace with their discovery, and this represents a significant barrier and the next real challenge for investigators of schistosomes, and other parasitic helminths. In the present study, we selected an array of 32 genes expressed in *S. mansoni* sporocysts to evaluate their susceptibility to double-stranded (ds)RNA treatment and to begin characterizing morphological phenotypes associated with a potential RNAi effect. Results demonstrate that gene knockdown and/or resulting phenotypes are highly transcript-dependent (specific dsRNA sequence used) and vary with time post-dsRNA exposure. Because of this potential variability in both transcript and phenotype expression in response to dsRNA treatment, our findings illustrate that, although a RNAi-type approach holds great promise as a functional reverse-genetics tool for larval schistosomes, its application requires caution in the design and execution of experiments and interpretation of results.

possibilities to discover new potential targets for vaccines, develop new drug candidates, and provide a better understanding of basic molecular mechanisms underlying host-parasite interactions. The *S. mansoni* Genome Project and data generated by various gene discovery efforts using expressed sequence tags (ESTs) and serial analysis of gene expression (SAGE), have resulted in a massive amount of gene sequence and expression information [11–19]. However, without reliable reverse or forward genetics methods, this vast amount of data cannot be placed into any functional context that can then be used to determine the value or importance of specific genes as targets for disease control.

Unlike the parasitic nematodes, which have benefited from reverse genetic methods developed in the model free-living worm *Caenorhabditis elegans* [20,21], no analogous model system is available for schistosomes. This has further delayed the application of new genomic technologies to problems related to disease control and drugs development [22]. However, despite this lack of a *C. elegans*-type model for parasitic flatworms, important advances have been made in trematode transgenesis with the introduction and transient expression of various reporter constructs in schistosomes [22,23] and fasciolids [24], although these approaches did not permit the functional assessment of specifically introduced genes. With the first demonstrations of gene expression knockdown by RNA interference (RNAi) in the mammalian [25] and snail [26] stages of *S. mansoni*, this reverse-genetics approach has now been applied to a limited number of genes expressed in primary (= mother) sporocysts [27], schistosomula [28,29] and adults [30]. In a recent review of RNAi in parasitic helminths, however, Geldhof and collaborators [31] admonish researchers for, at times, providing insufficient data that more firmly connects RNAi-mediated gene expression changes with specific phenotypes, and/or reporting only on genes that are susceptible to double-stranded (ds)RNA-mediated knockdown. Therefore, in order to gain a broader profile of RNAi efficacy in schistosomes, in the current study we performed an *in vitro* phenotypic screening of 32 genes known to be expressed in primary sporocysts of *S. mansoni.* These genes covered a variety of functional categories including antioxidants, transcription factors, cell signaling molecules and metabolic enzymes. Out of the 32 genes (comprising 33 dsRNA treatments) targeted for silencing, one-third (11 genes) exhibited a dsRNA treatment-associated phenotype that consisted of a reduction in sporocyst size (i.e., larval length). Interestingly of the 11 phenotype-yielding genes, only 7 demonstrated a significant and consistent alteration in transcript expression after the 7-day treatment period, although time-course experiments suggest that transient gene knockdown during earlier times of exposure may, in part, account for the observed phenotype.

Materials and Methods

Schistosoma mansoni in vitro culture and dsRNA treatments

All experiments were performed using the NMRI strain of *S. mansoni*. Eggs were obtained from 7–8 weeks infected mouse livers. After hatching in an artificial "pond water" [32] containing antibiotics (50 µg/mL streptomycin and 60 µg/mL penicillin), miracidia were immobilized on ice for 15 min, washed twice in cold pond water by centrifugation (1 min, 700×g) and gently resuspended in Chernin's balanced saline solution (CBSS) [33], supplemented with glucose and trehalose (1 g/L each), streptomycin (50 µg/mL) and penicillin (60 µg/mL) [34]. Larvae were then counted and distributed into either 48- or 96-well polystyrene tissue culture plates (Costar, Corning Incorporated, NY) at concentrations of ~6000 and 500 miracidia/well, respectively, depending on the experiments being performed. The general procedure used in all RNAi experiments involve treatment of miracidia starting at day 0 in culture with specific dsRNAs or control media containing irrelevant dsRNAs or medium alone for 7 days followed by assessment of an RNAi-type effect [26]. Details of dsRNA preparation and experimental designs are presented below. All research protocols involving mice used in the course of this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin-Madison under assurance no. A3368-01.

Targeted genes

In the present study, a total of 32 genes were selected for quantitative and qualitative assessment. Twenty-three of these genes were chosen based on their abundant expression in *in vitro* cultured *S. mansoni* miracidia and/or primary sporocysts, using the SAGE database OrganismDB [18] http://gmod.mbl.edu/perl/site/s_mansoniest]: calcineurin B (AJ276885.1), lactate dehydrogenase (LDH; U87629.1.), Smad4 (AY371484.1.), Smad2 (AF232025.1), Smad1 (AF215933.1), 14.3.3 (U24281.1), epidermal growth factor receptor (Scmeg; M86399.1), phosphoenolpyruvate carboxykinase (PepCK; AF120929.1), calpain (M74233.1), hexamer-binding protein (HEXBP; putative, organismDB:-Tag623), fibrillarin (putative, OrganismDB: Tag 428), elongation factor 1α (EF1α; Y08487.1), Rho 1 GTPase (Rho1; AY158212.1), Rho 2 GTPase (Rho2; AY158214.1), calcium ATPase 2, (Sma2; AF074400.1), SPO1 (AF109181), protein kinase Cß (PKCß; AY337620.1), protein kinase C receptor (PKC receptor; AF422164.1), zinc finger DHHC domain (DHHC; putative, OrganismDB: Tag 1180), myosin-light chain (AF071011.1), calreticulin (L24159.1), high voltage-activated calcium channel subunit α (Cav2A; AF361883.1) and high voltage-activated calcium channel ß-subunit 2 (calcium channel; AY277532.1).

The remaining 9 genes were chosen for their predicted putative functions in the parasite (antioxidants, transcription factors) and

ongoing characterization by our group: glycoprotein K5 (AY903301.1), zinc finger 1 (SmZF1; AF316827.1.), ring box (SmRbx; DQ466078.1.), glutathione peroxidase (GPx; M86510.1), thioredoxin peroxidase 1 (TPx-1; AF121199.1), thioredoxin peroxidase 2 (TPx-2; AF157561.1), superoxide dismutase (SOD; M27529.1), 26 kDa glutathione-S-transferase (GST26; M73624.1), and 28 kDa glutathione-S-transferase (GST28; S71584.1). TPx-1 and TPx-2 were used in combination to simultaneously silence both thioredoxin peroxidases. All of the above sequences are available from GenBank (http://www.ncbi.nlm.nih.gov/Genbank) or OrganismDB as indicated above.

Double-stranded (ds) RNA synthesis

T7 promoter-tagged specific PCR primers were designed to amplify ~500 base pair (bp) products for each of the targeted genes (Dataset S1; Table 1). A 500-bp green fluorescent protein (GFP) gene segment also was synthesized from the vector pAcGFP (Clontech, Mountain View, CA) to serve as a nonspecific dsRNA treatment-control. Following amplification, PCR products were separated on 1% agarose gels and purified using QIAquick gel extraction kit (Qiagen, Valencia, CA), following the manufacturer's protocol. Each PCR product was sequenced and their sequences verified using the Basic Local Alignment Search Tool (BLASTn, National Center for Biotechnology Information, NCBI). Double-stranded RNAs were synthesized from isolated sporocyst cDNA using T7 RiboMAX Express RNAi Kit (Promega, Madison, WI), according to procedures outlined by the manufacturer. Briefly, dsRNAs synthesis reactions were allowed to incubate for 16 hr at 37°C prior to DNAse treatment. DsRNA products were then phenol/chloroform-extracted and purified by precipitation with isopropanol. The purified products were resuspended in diethylpyrocarbonate (DEPC)-treated water, quantified by measurement at OD_{260} and their integrity verified by 1% agarose gel electrophoresis. Samples were stored at −20°C until further use.

Phenotypic screening and dsRNA uptake experiments

Effects of dsRNA treatment on *in vitro* cultured *S. mansoni* larvae were performed in 96-well culture plates (Costar) in which approximately 500 miracidia were added to wells containing 50 nM of specific or control green fluorescent protein (GFP) dsRNA diluted in 200 µL CBSS or medium lacking any dsRNA (no dsRNA control). Cultures were maintained at 26°C for 4 days, after which time an additional 10 nM of dsRNA was added to each well due to possible RNA degradation in culture [35], followed by incubation for 3 more days. Over the 7 days culture period, sporocysts were monitored for the following phenotypes: failure/delay in transformation, loss of motility, tegumental lysis and granulation (lethality) and changes in larval growth. Parasite viability and morphological changes were monitored daily using a Nikon Eclipse TE 300 inverted epifluorescent microscope (Nikon Instrument Inc., Melville, NY). In addition digital images of live treated and control parasites were captured using a CoolPix EZ digital camera (Nikon Instruments Inc.) throughout the 7-day incubation period, allowing more detailed observations of larval morphology and to quantify sporocyst growth (length measurements) in treated vs. control larvae at day 7. Length measurements from captured images were obtained and analyzed by Metamorph software version 7.0 (Meta Imaging series, Molecular Devices, Sunnyvale, CA). Sporocysts exhibiting tegumental lysis or loss of surface/somatic integrity were excluded from measurements. Larval growth datasets for each experimental replicate were statistically analyzed using the Mann-Whitney *U*-test (Wilcoxon-Sum of Ranks test) with significance set at $P \leq 0.05$. All treatments were performed in duplicate wells, and the experiment was independently replicated a minimum of 3 times on miracidia isolated from different batches of infected mouse livers. In addition, to verify dsRNA uptake by sporocysts we labeled Smad4, lactate dehydrogenase and GFP (specificity control) dsRNAs with rhodamine using the Label IT kit (CX-Rhodamine Labeling Kit; Mirus, Madison, WI), according to manufacturer's recommendations. Miracidia were *in vitro*-transformed to sporocysts in CBSS containing 50 nM labeled dsRNAs and subjected to epifluorescence photomicrography after 7 days of incubation.

Effect of dsRNA treatment on larval gene expression

In order to demonstrate an association between phenotype and transcript expression real-time quantitative PCR (q-PCR) was used to determine steady-state transcript levels in specific dsRNA-treated sporocysts. In these experiments ~6000 miracidia were distributed into a 48-well plate (Costar) and treated with 50 nM dsRNA diluted in CBSS (500 µL/well). Cultures were maintained at 26°C for 2, 4 or 7 days prior RNA extraction and isolation. Cultures maintained for 7 days were supplemented with 10 nM dsRNA at day 4. Sporocysts were extensively washed with CBSS in order to eliminate unabsorbed dsRNAs and shed ciliary epidermal plates, followed by extraction in Trizol reagent (Invitrogen, Carlsbad, CA) to isolate both total RNA and protein fractions from cultured larvae. The protein pellet was dissolved in the protein solubilization buffer (3 M Urea, 2% CHAPS, 40 mM Tris) for use in Western blot analyses (see below), while the isolated RNA fraction was resuspended in DEPC-treated water and subjected to DNAse treatment using the DNA-Free kit (Ambion, Austin, TX) to eliminate any contaminating genomic DNA. RNA samples were quantified and their purity assessed on a Nanodrop Spectometer ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE).

Real-time quantitative PCR (q-PCR) analysis

Quantitative PCR analysis was used to compare steady-state transcript levels between specific dsRNA-treated sporocysts and control treatments (GFP-dsRNA). To accomplish this 0.5 to 1 µg total RNA, derived from at least three different extractions, was used to synthesize cDNA using Superscript III cDNA Synthesis kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The q-PCR reaction mixtures consisting of 2.5 µL of cDNA, 12.5 µL of Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA), 10 µL of 600 or 900 nM primers (determined after primer concentration optimization), were added to 96-Well Optical Reaction Plates (ABI PRISM, Applied Biosystems) for amplification and quantification in a AB7500 Real Time PCR System (Applied Biosystems). In order to avoid the possibility of false amplification of the originally applied dsRNA, specific pairs of primers were designed outside of the region used to synthesize the original interfering dsRNA products (Dataset S1; Table 2). In addition to the targeted gene-specific primers used to assess potential knockdown, primers for *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and α-tubulin were used as endogenous normalization controls in all samples tested. Other controls for verifying the specificity of RNA treatment effects included (1) larval treatment with irrelevant GFP dsRNA and (2) treatment with a nontarget *S. mansoni* dsRNA. Finally, each q-PCR run was performed with 2 internal controls assessing both potential genomic DNA contaminations (no reverse transcriptase added) and purity of the reagents used (no cDNA added). For each specific set of primers, all individual treatments (including specificity controls) were run in three technical replicates. Each experiment was repeated 3–5 times ($N = 3–5$) as independent biological replicates and the ΔΔCt method [36], using GAPDH

and α-tubulin as endogenous loading controls to normalize the quantification of all cDNA targets was used to quantitatively compare treatment and control steady-state transcript levels. Due to the nonparametric distribution of data, statistical analysis of ΔΔCt values was performed using the Mann-Whitney *U*-test with significance set at $P \leq 0.05$.

Effect of specific dsRNA treatment at the protein level

Using a sporocyst-reactive rabbit anti-elongation factor 1α (anti-EF1α) antibody (Cell Signaling Technology, Danvers, MA), Western blot analysis and immunocytochemical localization experiments were performed to monitor EF1α protein levels in EF1α dsRNA-treated sporocysts. For Western blots miracidia, transformed in the presence of 50 nM EF1α or control GFP dsRNA and cultivated *in vitro* for 7 days, were extracted in Trizol reagent (Invitrogen) as previously described, separated by standard 12.5% SDS-PAGE methods [37], and electroblotted to nitrocellulose membranes (Biorad Lab, Richmond, CA) using a semi-dry protein transfer apparatus (Hoefer TE 70, Amersham Biosciences). After transfer, membranes were blocked overnight in TBS (2.42 g Tris base and 8 g NaCl/L, pH 7.6) containing 5% bovine serum albumin (BSA), followed by incubation in a mixture of anti-EF1α (1: 1000) and rabbit anti-SmGST26 (loading control, 1:1000 dilution; Cell Signaling Technology), for 16 hr at 4°C. Membranes were washed 3 times in TBS-Tween (0.1%) and incubated for 1 hr in TBS 5% BSA containing either goat anti-rabbit IgG-alkaline phosphatase-tagged (AP) or AP-labeled goat anti-rabbit IgG ($1{:}10^4$ and 1:5000, respectively). Colorimetric immunoreactivity was detected with the chromogen substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium (NBT), diluted in AP buffer (0.1 M Tris, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5). To quantify the relative levels of anti-EF1α in specific dsRNA- and control GFP dsRNA-treated sporocyst extracts, target and control immunoreactivities were measured using an Ultraviolet Trans-illuminator BioImaging Systems (UVP, Inc., Upland, CA) with the co-processed anti-GST26 band serving as a normalizing signal (loading control). Quantitative comparisons of protein expression for EF1α in control and target dsRNA-treated sporocysts were analyzed by LabWorks Image Acquisition and Analysis Software version 4.6.

For immunocytochemical studies, 7-day old dsRNA-EF1α or -GFP-treated sporocysts were washed in CBSS (allowing removal of ciliated epidermal plates), transferred to siliconized-microcentrifuge tubes in 500 µL PT buffer (2% paraformaldehyde, 1% Triton-X100/sPBS), and incubated overnight at 4°C with constant rotation. Fixed-parasites were washed 5 times in sPBS by centrifugation at 1600 rpm (2 min), resuspended in 500 µL of blocking buffer (5% normal goat serum/0.02% azide/sPBS) for 16 hr, under constant agitation before addition of rabbit-anti-EF1α primary antibodies (1:200 dilution in blocking buffer) and incubation overnight at 4°C. Parasites were washed for 10 min in sPBS, resuspended in 500 µL of AlexaFluor 488-conjugated goat anti-rabbit IgG (4 µg/mL blocking buffer) and incubated for 16 hr at 4°C with agitation. Following antibody treatments, sporocysts were washed 5 times in sPBS by centrifugation (1600 rpm, 2 min), resuspended in 40 µL of sPBS and mounted on coverslips. Specimens were examined and photographed using a Nikon Eclipse TE2000 (Nikon Instrument Inc.) inverted microscope equipped with a Bio-Rad Radiance 2100 MP Rainbow Confocal/Multiphoton Imaging System (W.M. Keck for Biological Imaging, Instrumentation, UW-Medical School).

Results

In our initial phenotype analysis of 24 different dsRNA treatments, no differences between specific dsRNA-treated larvae and controls (GFP dsRNA-treated and untreated sporocysts) were

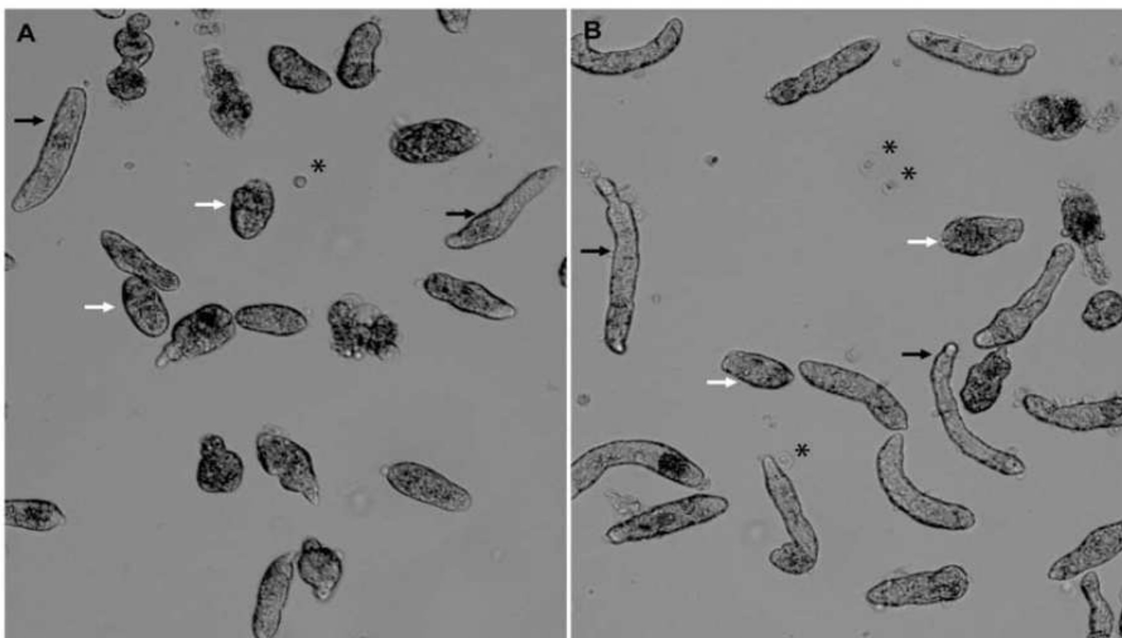

Figure 1. *In vitro* cultured *S. mansoni* larvae 7 days post-dsRNA treatments. Brightfield photomicrographs of *in vitro* cultured *Schistosoma mansoni* sporocysts after 7 days of treatments with a specific GST26-dsRNA (A) compared to the control GFP-dsRNA (B), illustrating the effects of exposure to phenotype-inducing GST26-dsRNA on sporocyst lengths. Arrows indicate examples of shortened (white arrows) and normal elongate (black arrows) sporocysts measured in both treatments. Asterisks indicate rounded ciliated epidermal plates that were shed from the miracidial surface after transformation.
doi:10.1371/journal.pntd.0000502.g001

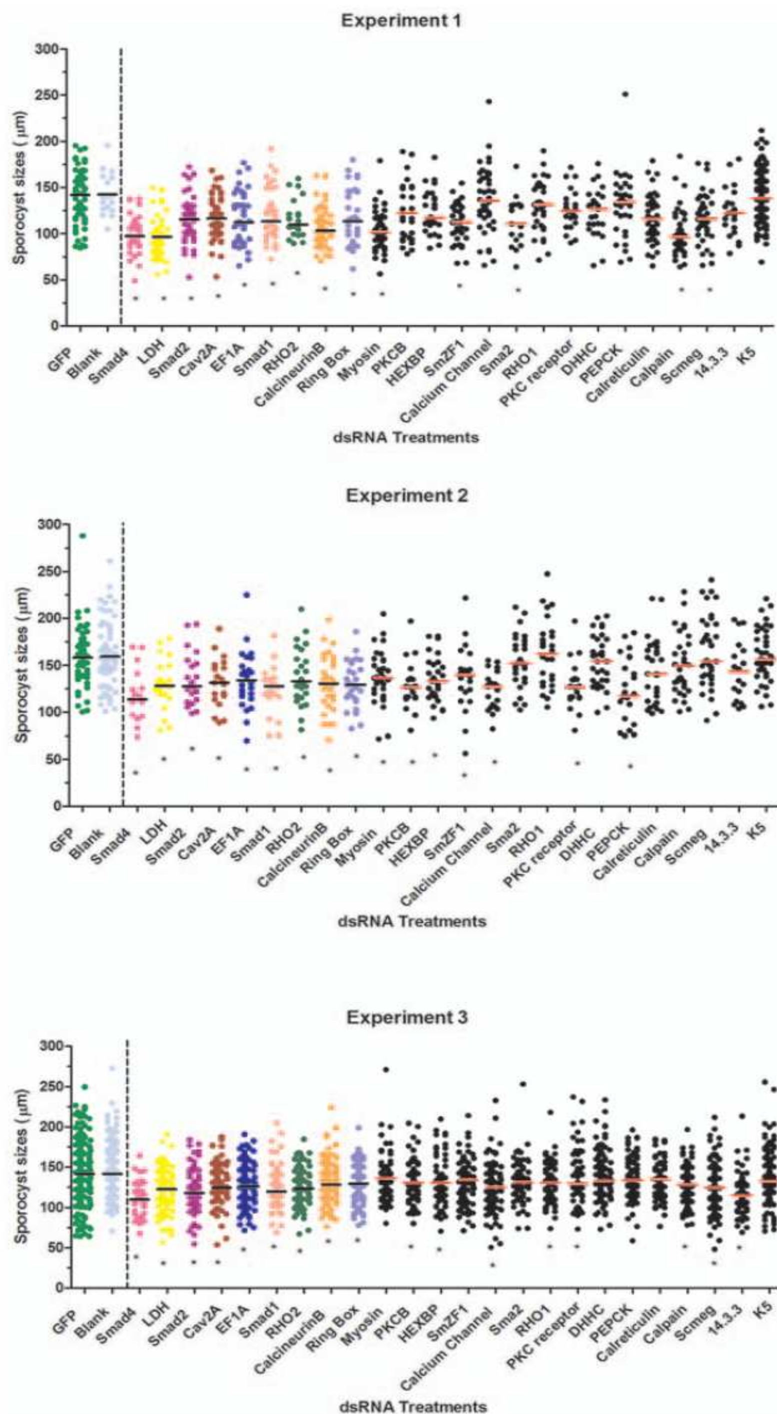

Figure 2. *S. mansoni* sporocyst length measurements post-dsRNA treatments. Graphic representation of sporocyst length measurements (μm) after 7 days of dsRNA treatments, from 3 independent experiments (A–C). Sporocyst length measurements are represented by scatter plots with the calculated median values indicated by the horizontal bars within each dsRNA treatment. The median values for specific dsRNA treatments were compared to both GFP-dsRNA (green plots) and blank (no dsRNA; blue plots) treatment controls. For each of the 3 experiments, the pair of controls is shown in between solid and dashed vertical black lines, immediately followed by the gene-specific dsRNAs groups. The dsRNA treatments exhibiting significant differences from GFP and blank controls in all 3 replicate experiments are represented as colored plots, marked with an asterisk (Smad4, lactate dehydrogenase, Smad2, Cav2A, EF1α, Smad1, RHO2, calcineurin B, and ring box), while those yielding inconsistent phenotypic differences when compared to the controls (myosin, PKCB, HEXBP, SmZF1, calcium channel, Sma2, RHO1, PKC receptor, DHHC, Pepck, calreticulin, calpain, Smeg, 14.3.3, and K5) are indicated by black dot scatter plots. Of this latter group, asterisks denote those individual replicates that were significantly different from controls. All treatments were statistical analyzed using Mann-Whitney *U*-test within each experiment, $^{*}P \leq 0.05$.
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noted in miracidial transformation rates, larval motility or mortality during the *in vitro* cultivation period. The only notable phenotype observed in treated 7-day cultured sporocysts was an apparent greater number of small-sized or shortened sporocysts possibly involving a growth-related defect(s) (Fig. 1A, 1B). However, because sporocysts in a given culture population typically represented a range of sizes, live sporocyst images were captured, from which larval lengths were measured and digitally-analyzed using Metamorph software. Within each biological replicate, such measurements were taken for the dsRNA treatment groups and statistically compared to both the GFP dsRNA-treated and no treatment (blank) groups. For a given dsRNA to be identified as having a putative dsRNA-mediated effect, the median larval length had to significantly differ ($P \leq 0.05$) from both the GFP dsRNA and the blank controls in each of the biological replicates. Using these criteria, we observed significant decreases in parasite lengths in 9 of 24 dsRNAs in the first screening trial: Smad4, lactate dehydrogenase (LDH), Smad2, Cav2A, elongation factor 1α (EF1α), Smad1, RHO2, calcineurin B, and ring box (Fig. 2). Similar results were found in a second experimental series, which included nine additional dsRNA treatments. In this case, using the same criteria for significance, 2 of the 9 dsRNAs treatments (GST26 and SOD) exhibited a consistent size-related phenotype effect when compared to controls (Fig. 3). As before, greater frequency of shortened larvae was the only observable dsRNA-associated phenotype.

To illustrate the variability in parasite response to the different dsRNA at the population level, and to underscore the importance of biological replication in phenotypic analysis of dsRNA treatment effects, 9 of the 33 dsRNA treatments (Scmeg, HEXPB, Sm zinc finger1 (SmZF1), -calpain, -myosin light chain, PKCß, SPO1, TPx1/2 and calcium channel) exhibited significant length decreases in 2 of 3 experiments suggesting a possible, but inconsistent, connection with the observed phenotype. Sporocyst treatment by the remaining dsRNAs (14-3-3 protein, Sma2, RHO1, PKC receptor, DHHC, PEPCK, calreticulin, glycoprotein K5, fibrillarin, GST28, GPx, TPx1, and TPx2) had no measurable effect on larval phenotype (Figs. 2 and 3).

In order to document potential difference in dsRNA uptake within larval populations and between treatments, miracidia were exposed to rhodamine (Rh)-labeled dsRNA-GFP, -Smad4 or -LDH. After 7 days of exposure, dsRNA-uptake in sporocysts was assessed by fluorescent microscopy. As shown in Figure 4, larvae within a single population exhibited wide variation in their abilities to take up labeled dsRNA, regardless of transcript species (Figs. 4A and B). Indeed, a one-way ANOVA comparing the 3 dsRNA-treated groups for the prevalence of tegumental or internal staining was non-significant ($F = 1.159$; $P = 0.2555$) indicating no differences in staining distribution between groups or locations. The most prominent sites of Rh-dsRNA localization in positively-stained sporocysts (67% of larvae) were in the tegument (~28%), excretory pores/flame cells and in unidentified parenchymal-like cells (~39%)

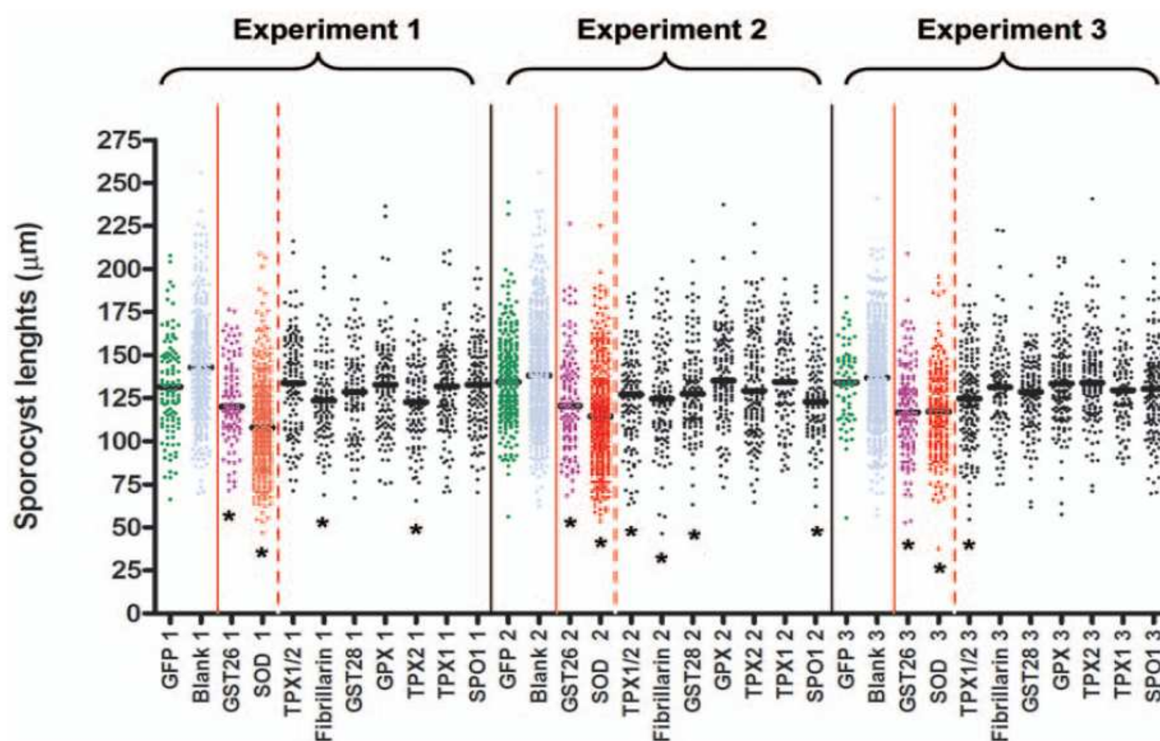

Figure 3. Additional *S. mansoni* sporocyst length measurements 7 days post-dsRNA treatments. Graphic representation of sporocyst lengths (µm) after 7 days post-dsRNA treatments, generated from 3 independent experiments covering an additional group of sporocyst-expressed genes. Larva lengths are represented by a dot scatter plots with the median length for each sporocyst treatment group shown as a short horizontal bar. Calculated medians for each target dsRNA group were compared to both GFP-dsRNA (green dots) and no-dsRNA (blank, blue dots) control median values. For each experiment, controls are the first 2 scatter plots shown between the solid red and black vertical lines, followed by the 2 dsRNA treatments that showed significant phenotypic differences in all 3 experiments (marked with *; GST26 and SOD) when compared to both controls. Black scatter plots represent dsRNA-treated sporocysts whose median length measurements exhibited inconsistent differences when compared to both GFP and blank controls. Of this latter group (TPx1/2, fibrillarin, GST28, GPx, TPx2, TPx1, and SPO1), asterisks denote those individual replicates that were significantly different from controls. Each experiment was analyzed using Mann-Whitney *U*-test, $^*P \leq 0.05$.
doi:10.1371/journal.pntd.0000502.g003

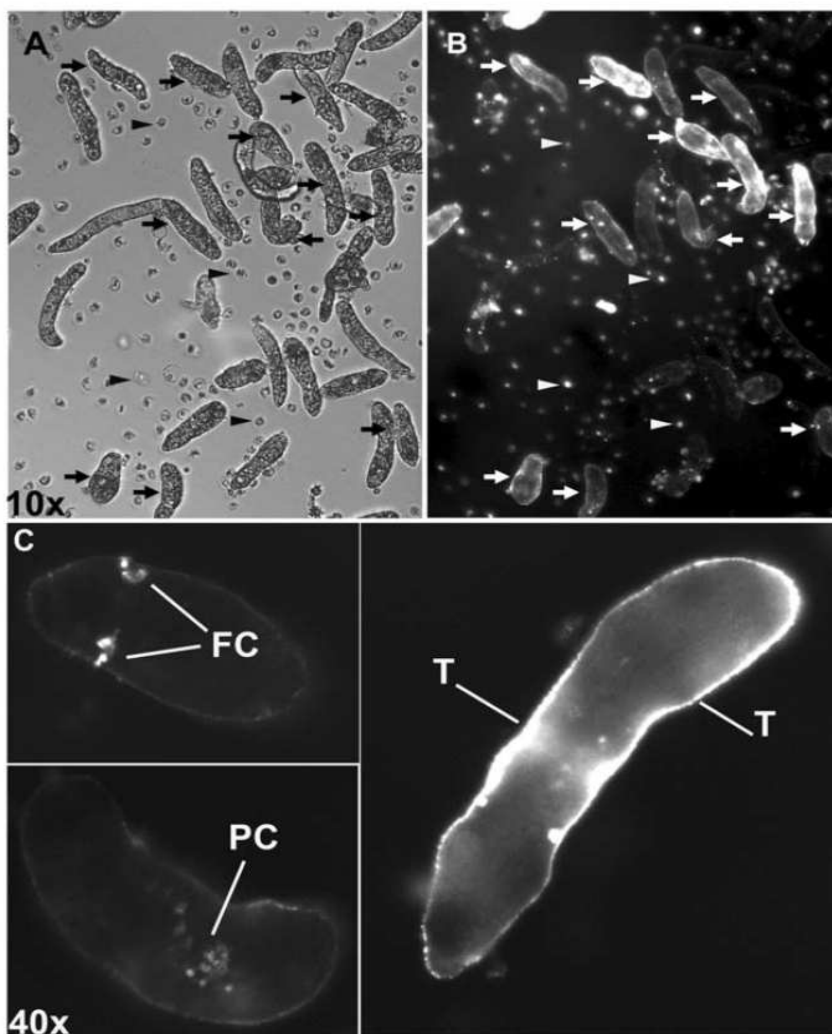

Figure 4. Localization of rhodamine-labeled-dsRNA in *S. mansoni* larvae 7 days post-exposure. Brightfield (A) and fluorescent (B) photomicrographs showing *S. mansoni* sporocysts and localization of rhodamine-dsRNA taken up after 7 days of labeled dsRNA exposure, respectively (100×). Arrowheads indicate rounded epidermal plates that were shed from miracidia during transformation to sporocysts. (B) Fluorescent images show the different levels of dsRNA penetrance within the same treatment and the same population. (C) The higher magnification (400×) illustrating the heterogeneity of dsRNA uptake within individual sporocysts in a given population including excretory ducts/flame cells (FC), cells within the parenchyma (PC), and tegument (T).
doi:10.1371/journal.pntd.0000502.g004

(Fig. 4C). Negative controls consisting of larvae treated with unlabeled-dsRNA did not display any fluorescent signal (data not shown). Sporocysts within a given population exhibited heterogeneous (+/−) Rh-staining indicating specific dsRNA uptake by larvae, and not a nonspecific uptake via Rh-binding.

Because the phenotypic screen revealed both phenotype-associated and nonphenotype-associated dsRNA species, we selected a subset of 24 genes to assess the effect of dsRNA treatments on steady-state transcript levels using real-time quantitative PCR (q-PCR). Comparisons of normalized-levels of dsRNA-targeted messenger RNAs to their corresponding control treatment (GFP dsRNA-treated group) resulted in 12 transcripts that exhibited significantly reduced expression levels (Fig. 5). Unexpectedly, SOD transcripts consistently increased, rather than decreased, to very high levels of expression (>1600-fold) upon specific dsRNA treatment.

A comparison was made between dsRNA species that produced a detectable phenotype and those generating a significant transcript knockdown (or induced expression) in an attempt to directly correlate phenotype and gene expression. Notably, only 7 of the 11 target dsRNA-treatments that produced a "shortened" larval phenotype presented a significant alteration in transcript levels (Smad4, lactate dehydrogenase, Cav2A, EF1α, calcineurin B, GST26 and SOD) when compared to dsRNA-GFP treated sporocysts (Fig. 5). Smad4 and LDH dsRNA treatments showed a small, but consistently significant 15% decrease, while Cav2A, calcineurin B, EF1α and GST26 exhibited knockdowns of 42%, 65%, 70% and 85%, respectively. SOD, whose transcript levels were dramatically increased in dsRNA-treated sporocysts, also was phenotype-associated. In addition, as noted in Figure 5, 6 dsRNA treatment groups that did not exhibit significant or consistent changes in larval length expressed significantly lower transcript levels than controls ranging from an approximately 30% (SmZF1) to 75% (fibrillarin, GST28, GPx, TPx1, and TPx2) after 7 days of exposure. No changes in transcript levels were observed for phenotype-associated Smad1, Smad2, RHO2 and ring box dsRNA

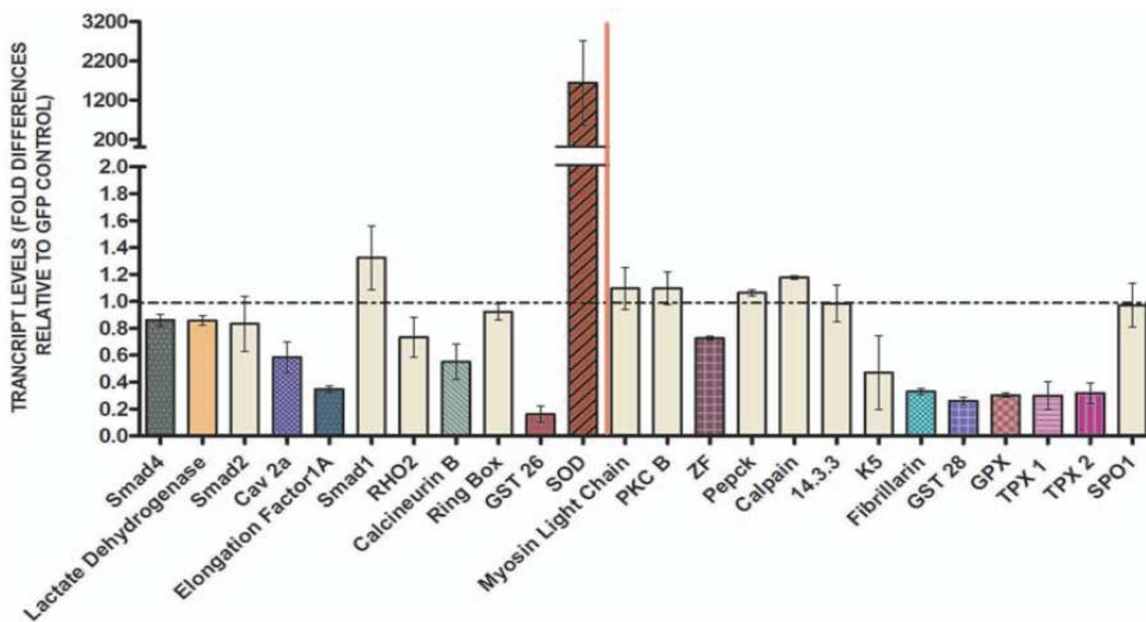

Figure 5. Transcript levels of dsRNA-treated sporocysts 7 days after dsRNA exposure. Bar graph depicting the relative steady-state transcript levels of dsRNAs-treated sporocysts after 7 days of exposure compared to the GFP-dsRNA control. For each dsRNA tested, data are represented as mean fold-differences (+/−S.E.) relative to the GFP control (1.00). Colored bars represent sporocyst mRNA levels showing consistent and statistically significant decrease (dsRNA-Smad4/GFP, $P = 0.0056$; -lactate dehydrogenase/GFP, $P = 0.0358$; -Cav2A/GFP, $P = 0.0136$; -EF1α/GFP, $P = 0.0358$; -calcineurin B/GFP, $P = 0.0189$; -GST26/GFP, $P = 0.0136$; -SmZF1/GFP, $P = 0.0189$; -fibrillarin/GFP, $P = 0.0407$; -GST28/GFP, $P = 0.0284$; -GPx/GFP, $P = 0.0269$ and -TPx1/GFP, $P = 0.0358$/-TPx2/GFP, $P = 0.0358$) or increase (dsRNA-SOD/GFP, $P = 0.0294$) in target transcript levels when compared to the GFP-dsRNA control treatment. Tan-colored bars represent transcript levels for dsRNA-treated sporocysts that showed no differences when compared to GFP-dsRNA treated controls (-Smad2/GFP, $P = 0.0755$; -Smad1/GFP, $P = 0.8969$; -RHO2/GFP, $P = 0.0765$; -ring box/GFP, $P = 0.7642$; -myosin/GFP, $P = 0.3725$; -PKCB/GFP, $P = 0.6579$; -PEPCK/GFP, $P = 0.3017$; -calpain/GFP, $P = 0.1642$; -14.3.3/GFP, $P = 0.6579$; -K5/GFP, $P = 0.3725$ and -SPO1/GFP, $P = 0.8969$). In addition, bars located on the left of the solid red vertical line represent treated-sporocysts previously shown to express the shortened phenotype (dsRNA-Smad4, -lactate dehydrogenase, -Smad2, -Cav2A, -EF1α, -Smad1, -RHO2, -calcineurin B, -ring box, -GST26 and -SOD). Transcript levels were determined by q-PCR and data analyzed using the ΔΔCt method [36] followed by statistical analysis using the Mann-Whitney *U*-test. Significance levels were set at $P \leq 0.05$. Data were generated from 3–5 independent experiments.
doi:10.1371/journal.pntd.0000502.g005

treatments and phenotype-non-associated myosin, PKCB, Pepck, calpain, 14.3.3 protein, glycoprotein K5, and SPO1 dsRNAs.

Since we typically used day 7 as our temporal end-point for assessing RNAi phenotypic effect, we also investigated the possibility that transcripts may have been knocked down prior to day 7. Using a subsampling of dsRNA species that represented a range of transcript knockdown levels, *S. mansoni* miracidia were treated with dsRNA-EF1α, -calcineurin B, -SOD, -LDH, -RHO2, -Smad2 -Smad4, myosin light chain and -ring box, and sporocyst transcript levels analyzed after 2 and 4 days postexposure. Compared to our previous 7-day treatment effects, results yielded various patterns of transcript silencing (Fig. 6). For example, although EF1α and calcineurin B transcripts were significantly reduced by ~70% by day 7, calcineurin B knockdown was actually greatest (~90%) at 2 days postexposure to dsRNA. Smad4 and LDH mRNAs, which previously showed a small, but significant, decrease at day 7 exhibited highest knockdown (~40%) on day 2 indicating an early effect of dsRNA treatment. SOD was found to be over-expressed at all time points, with an initial increase of ~1200% at day 2, followed by a maximum ~17,000-fold expression at day 4, before again returning to day-2 levels after 7 days of exposure. SOD transcript levels, however, were unaffected by heterologous exposure of larvae to several non-SOD-related dsRNAs (data not shown). RHO2 dsRNA, previously displaying no effect on homologous transcript expression in sporocysts at 7 days, showed significant transcript knockdown at 2 and 4 days post-exposure before recovering to control levels at the 7-day time point. In contrast, Smad2, myosin light chain and ring box dsRNA treatments demonstrated no consistent effect on their respective transcript levels regardless of the sampling interval.

Finally, because we had available an antibody that was specifically reactive to the *S. mansoni* EF1α protein, we assessed the effect of EF1α dsRNA treatment on EF1α protein levels using Western blot and immunofluorescence imaging. Western blot analysis clearly showed that EF1α dsRNA-treated 7-day sporocyst extracts were significantly reduced in EF1α protein (50 kDa band) compared to the GFP dsRNA-treated control group (Fig. 7). The presence of a 25 kDa GST26 band (used as an antibody specificity and loading control) in both the EF1α and GFP dsRNA-treated samples suggested both a specific EF1α transcript silencing and associated protein knockout (Fig. 7). This result was quantitatively confirmed by densitometry showing that, following normalization of transcripts to the loading control, EF1α protein was highly reduced by >80% in the EF1α dsRNA-treated sample compared to the GFP dsRNA control. Confocal immunolocalization of EF1α in intact dsRNA-treated sporocysts was consistent with the Western blot analysis: EF1α dsRNA-treated larvae displayed little immunoreactivity, while abundant anti-EF1α-reactivity was evident within cells and parenchymal tissues of GFP dsRNA-treated sporocysts (Fig. 8).

Discussion

RNA interference (RNAi) has been widely used in a variety of organisms as a reverse-genetic approach to generate functional gene

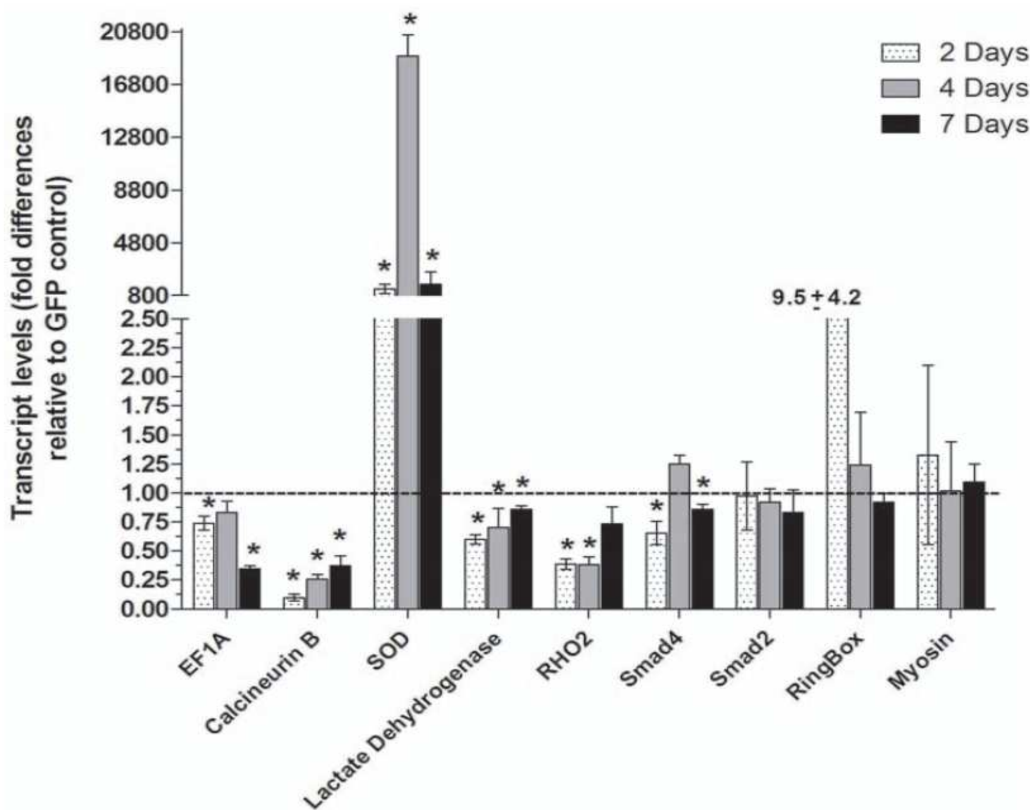

Figure 6. Transcript levels in *S. mansoni* sporocysts at different times post-dsRNA treatment. Time-course of steady-state transcript levels was assessed in sporocysts treated with dsRNAs under culture conditions. Sporocysts were treated with dsRNA-EF1α, -calcineurin B, -SOD, -lactate dehydrogenase, -RHO2, Smad2, -Smad4, -myosin and -ring box for 2 days (stippled bars) or 4 days (gray bars), and compared to 7 day dsRNA treatment effects (black bars). Transcript levels were assessed by q-PCR at each time and compared to its matched GFP-dsRNA control. For each dsRNA tested, data are represented as mean fold-difference (+/−S.E.) relative to the GFP control (1.00). However, statistical analyses were based on raw q-PCR values using the $\Delta\Delta$Ct method followed by statistical analysis using the Mann-Whitney *U*-test, N = 4, $^*P\leq 0.05$. Two-day comparisons (stippled bars): dsRNA-EF1a/GFP, $P = 0.028$; -calcineurin B/GFP, $P = 0.021$; -SOD/GFP, $P = 0.015$; -lactate dehydrogenase/GFP, $P = 0.041$; - RHO2/GFP, $P = 0.021$; –Smad4/GFP, $P = 0.028$; -ring box/GFP, $P = 0.3$; -Smad2/GFP, $P = 1.0$; and -myosin/GFP, $P = 0.059$. Four-day comparisons (gray bars): dsRNA-EF1a/GFP, $P = 0.0319$; -calcineurin B/GFP, $P = 0.03$; -SOD/GFP, $P = 0.028$; -lactate dehydrogenase/GFP, $P = 0.029$; - RHO2/GFP, $P = 0.021$; –Smad4/GFP, $P = 0.0286$; -ring box/GFP, $P = 0.884$; -Smad2/GFP, $P = 0.98$; and -myosin/GFP, $P = 0.9$. Data for 7 day dsRNA treatments (black bars) were taken from identically performed experiments (data shown previously in Fig. 5), and are reproduced in Fig. 6 for graphic comparisons only. Statistics for this group of genes are provided in the Fig. 5 legend.
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knockdowns with associated phenotypic changes [38–40]. In combination with complete and well-annotated genome databases, tools developed for RNAi now permit systematic, whole-genome screening leading to putative functional assignments for unknown genes, or direct functional confirmation of genes identified by sequence homology (orthologues) [41]. Some RNAi libraries are already available and have taken advantage of this functional genomics approach including for *D. melanogaster* [39] and *C. elegans* [42,43]. However, many newly-defined functions for any given gene tend to be organism-specific and may not always be identical to, or even homologous with, a similar gene's function in other species. Therefore, one of the current challenges we face is to integrate this organism-specific RNAi-derived functional information into the existing, ever-growing genomic databases of diverse organisms [44]. In addition, as noted by Geldhof [31], application of RNAi approaches to parasitic helminths have at times lacked convincing evidence of an RNAi effect or have not provided information on the full spectrum or diversity of target transcript susceptibilities to dsRNA treatments.

The RNAi screening approach described in the current study, to our knowledge, is the first to profile morphological phenotypes associated with exposure of larval schistosome blood flukes to dsRNAs representing a diversity of expressed genes. From our sampling of dsRNAs for 32 different *S. mansoni* genes known to be expressed in primary sporocysts, only 34% (11 transcripts) produced a consistent, highly reproducible phenotype; namely a reduced larval length (shortening), morphologically resembling a type of growth inhibition. Interestingly, this was the same phenotype that was observed in an earlier study involving dsRNA-mediated knockdown of a CD36-like scavenger receptor at the tegumental surface of *S. mansoni* sporocysts [27]. Thus, the genes associated with this phenotype are quite varied, including signaling molecules, Ca-interactive proteins, redox enzymes and a membrane receptors/ion channels. Although it would be premature to speculate on specific gene-phenotype linkages, it may not be particularly surprising that such a general phenotype as larval size might be regulated by many different genes expressed in variety of cell or tissue-types. In whole genome RNAi studies of *C. elegans*, an overall ~2% of detectable viable phenotypes were growth- or size-related [41] and recent RNAi applications on schistosomules, miracidia, or adults also produced a similar

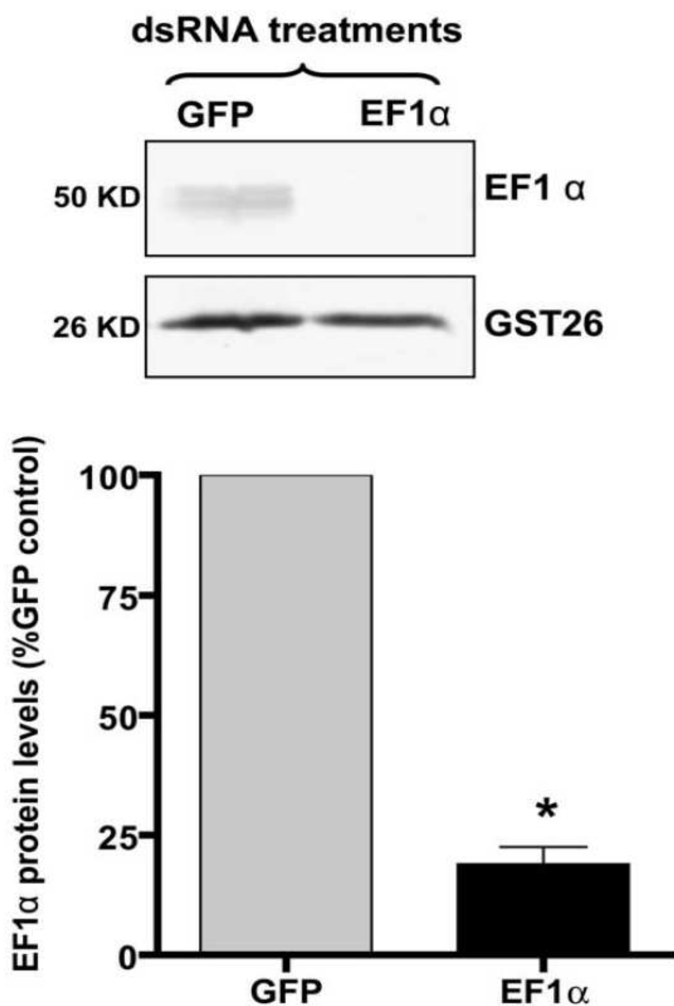

Figure 7. Quantification of differential EF1α protein levels in *S. mansoni* sporocysts post-dsRNA exposure. Western blots of SDS-PAGE separated total proteins extracted from sporocysts treated for 7 days with elongation factor1α (EF1α) or GFP (control) dsRNA. A rabbit anti-EF1α was used to detect a 50 kDa *S. mansoni* EF1α, while a rabbit anti-SmGST26 antibody served as both a protein specificity and loading control. Note the presence of EF1α protein in dsRNA-GFP treated-sporocysts, but reduced reactivity in EF1α-dsRNA-silenced parasites. Significant knockdown of EF1α protein in EF1α dsRNA-treated parasites was confirmed by optical densitometry comparing protein band intensities of test and control dsRNA treatment groups after anti-GST26 normalization of each band. The bar graph shows an 80% reduction (+/−S.E.) in dsRNA-induced EF1α protein level in EF1α dsRNA-treated sporocysts relative to GFP controls. Statistical analyses were performed using Students *t*-test. $^{*}P \leq 0.05$; N = 3.
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consistent "shorter" phenotypes [45]. Our finding that only a proportion of sporocyst exhibited the shortened phenotype might be explained, at least in part, by results of the rhodamine-labeled dsRNA uptake experiment demonstrating that ~67% of dsRNA-treated larvae within a population (in all treatments) exhibited signs of labeling, and of those, cellular localization of Rh-dsRNA within sporocysts varied considerably (tegument, flame cells, parenchymal tissues). Thus the degree and site of dsRNA penetrance may be among several critical determinants influencing the observed phenotype. Moreover, this differential dsRNA uptake also could explain the variation in the levels of transcript knockdown observed in q-PCR analyses.

Attempts to correlate phenotype and knockdown of target gene expression also yielded variable results in that 7 of the 11 genes associated with the shortened phenotype were significantly altered in their expression after the treatment period. One explanation as to why all phenotype-expressing transcripts were not reduced is the possibility that some genes possess different kinetic profiles (i.e., may have exhibited knockdown prior to day 7). Of 5 genes whose transcript levels were unaffected (Smad2, Rho2, ring box) or marginally affected (Smad4, LDH) by dsRNA at 7-days of treatment, 3 transcripts (Rho2, Smad4 and LDH) showed an early significant knockdown at day 2 suggesting a temporal reduction in transcript levels that could be phenotype-associated. Why transcript expression of the other 2 phenotype-associated genes (Smad2 and ring box) was not affected by specific dsRNA treatments remains unclear, although RNAi off-targeting, i.e., a mis-targeting of specific dsRNA to other unidentified mRNAs [46–49] could be involved. Off-target effect of introduced dsRNAs seems to be a common occurrence in helminth RNAi experiments, and presents a challenge in controlling such effects, as recently reviewed by Geldhof and colleagues [31]. Yet, it has been shown in mammal cells that knockdown efficiency is highly dependent on the specific dsRNA sequence of a particular gene [50], and that in some cases, a small degree of similarity may invoke off-target gene silencing [51]. In our current study, we exposed larvae to gene-specific long (500 bp) dsRNA, which, upon dicer cleavage, results in short unpredictable RNA sequences that represent potential sources of off-target gene silencing. To complicate matters further, some siRNA also have been shown to exhibit nonspecific toxic effects that may directly affect transcriptional processes without altering specific transcript levels [52].

Although it was not the goal of this study to provide in-depth analyses of each the gene investigated herein, followup experiments involving *S. mansoni* elongation factor 1α (EF1α) illustrates the importance of providing several lines of evidence of an RNAi effect. In this case, larval treatment with EF1α dsRNA resulted in a demonstrable phenotype, specific transcript knockdown, and approximately 80% inhibition of EF1α protein expression as measured by both Western blot and immunocytochemical assays. Elongation factors are known to be essential in the translational process by functioning to catalyze the aminoacyl-tRNA delivery to ribosomes during protein elongation [53]. Given this putative function of EF1α, and its widespread knockdown at the transcript and protein levels, its involvement either directly or indirectly in generating the shortened larval phenotype is supported by the data presented here. Reasons why we did not see any changes in GST26 protein levels in the EF1α Western blot assay maybe due to a slow protein turnover rate for GST26 or the possibility that GST26 is synthesized in cells/tissues that were unaffected by EF1α dsRNA knockdown.

Variation in dsRNA treatment effects also was evident in the group of sporocysts that lacked any definable phenotype. Of the 13 nonphenotype-expressing larval groups, half (6) exhibited consistent, significant reductions in transcript levels as measured by q-PCR, while transcript levels in the other half (7) were unaffected. This type of result is not unexpected as this has been demonstrated previously in RNAi screens of model organisms such as *C. elegans* [54], as well as parasitic nematodes [31,55]. For those transcripts whose expression was unaffected by specific dsRNA treatment, there would not be an *a priori* expectation of phenotypic change. There are several ways to potentially explain a lack of differential phenotype in larvae presenting with dsRNA-induced transcript knockdown : (1) the gene targeted for dsRNA knockdown is functionally unrelated to the observed phenotype, (2) since typically an RNAi-like effect does not lead to a complete gene (and presumably protein) knockout, sufficient protein synthesis/activity remains to continue support of the normal "phenotype",

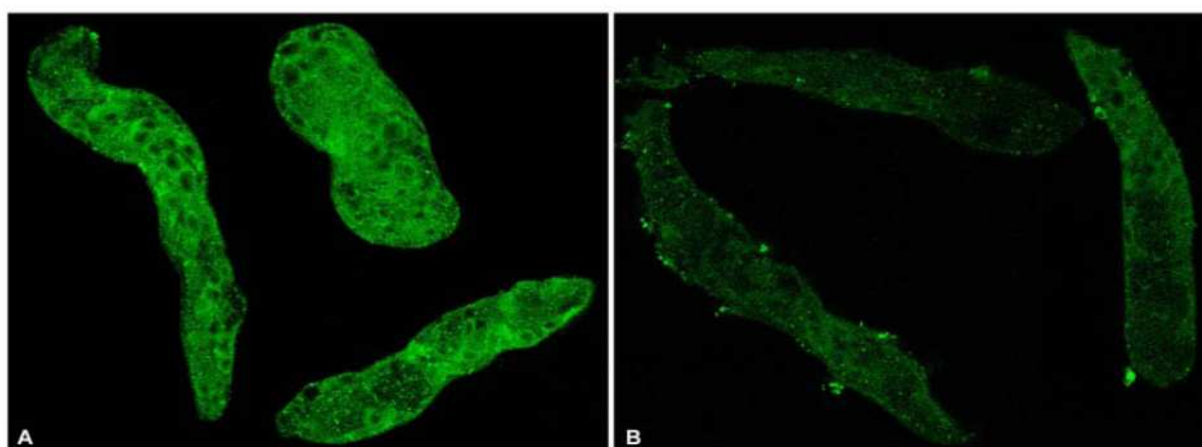

Figure 8. Observations of differential expression of EF1α protein levels in *S. mansoni* sporocysts after dsRNA treatments. Immunfluorescence photomicrographs of GFP dsRNA (control; A) and EF1α dsRNA (B) treated sporocysts showing EF1α protein-knockdown post RNAi treatments. Larvae were cultured with dsRNAs for 7 days and fixed prior to treatment with anti-EF1α antibody and Alexa 488-conjugated secondary antibody. Strong immunoreactivity (green fluorescence) is distributed among various cells and tissues within interior of control sporocysts (A), compared to only weak reactivity in EF1α dsRNA-treated sporocysts (B). Confocal images; 400×. N = 2.
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(3) other related proteins and/or isoforms may be supplementing or replacing the protein (and its function) initially targeted for dsRNA-mediated silencing, and (4) the protein product of the targeted transcript may have a lengthy half-life (i.e., slow turnover rate), hence delaying potential gene knockdown effects at the protein level. These results further underscore the wide variation in susceptibilities of individual *S. mansoni* genes to RNAi procedures, and the fact that dsRNA knockdown may not be associated with any demonstrable phenotype.

One of the more intriguing results of our study was the consistent, high level upregulated expression of SOD in sporocysts upon treatment with SOD dsRNA. Even more impressive than the 1600-fold transcript expression following the standard 7-day incubation period was the ~17,000-fold expression 2 days earlier (day 4). At present we do not have an explanation as to how larval exposure to SOD dsRNA may be triggering such high expression levels. One possibility is that the yet unknown sequence(s) in processed SOD dsRNA may be stimulating reactions similar to RNA activation (RNAa) [56,57]. If this is indeed the case, this would be a novel facet of RNAi in parasitic helminths. Although the function of SOD as a protective anti-oxidant has been suggested [58–60], its essential role in parasite development has not been established. If the overexpression response seen in this study is linked to SOD depletion or SOD sequence activation, this would imply a critical role in sporocyst survival, and perhaps in larval development as evidenced by its association with the sporocyst size phenotype. The role of endogenous SOD, and other anti-oxidants, in sporocysts confronted with oxidative stress is the subject of ongoing investigations in our laboratory.

To date, RNAi is the only reverse genetic tool available in schistosomes [22], and although it has been successfully applied as a functional genomics tool in both mammalian [25,28,61] and snail [26,27] stages of infection, a lack of consistency in the RNAi-induced knockdown and resulting phenotypes indicates a pressing need to more fully investigate RNAi to gain a better understanding of this complex mechanism in *S. mansoni*, and other parasitic flatworm species [31]. The results presented here provide an overview of the variability that may be encountered as transcript-specific dsRNA sequences are applied as a tool for targeted gene manipulation and morphological phenotyping in larval schistosomes. It is anticipated that further improvements in dsRNA delivery methods likely would be beneficial in attaining more consistent transcript/protein knockdowns and resulting phenotypes, as will further detailed analyses of specific siRNA for individual genes. Future studies involving optimization of transfection reagent- and electroporation-based gene delivery approaches are currently being planned. In assessing RNAi effects, in addition to low and/or inconsistent dsRNA penetrance, we also are hindered by the numbers of parasites that can be processed for dsRNA treatments at a given time, and, as reported in this study, a very limited phenotype repertoire, due to a lack of more sensitive detection tools. These restrictions illustrate some of the limitations facing large-scale RNAi experiments, and demonstrate the necessity of small-scale or gene-by-gene characterizations, until development of more sensitive, higher-throughput methodologies [44].

In summary, this study is the first to provide a multi-gene assessment of the efficacy of dsRNA treatments in characterizing phenotypic and transcriptional changes brought about by introduction of gene-specific dsRNAs into cultured *S. mansoni* larvae. Prolonged exposure to dsRNA, when selectively applied to target genes expressed in early larval stages, can generate significant transcript knockdown, thus facilitating the investigation of potential gene-associated function. However, as shown in the present study, individual genes may differ significantly in their abilities to render RNAi-like effects, and this is likely due, at least partially, to efficacy of their intracellular processing. Although RNAi approaches continue to be potentially valuable tools for functional genomics in parasitic helminths, caution should be taken in the design, set-up and execution of RNAi experiments. As a followup to this study, we are now focusing on the group of enzymes involved in reduction-oxidation (redox) reactions, especially those with antioxidant activity, and that have exhibited consistent transcriptional knockdown by RNAi. These functional studies were made possible by the data provided in this initial multi-gene profiling of dsRNA effects.

Supporting Information

Dataset S1 Target genes and primers. Table 1: List of genes targeted in the RNAi screening, includes specific forward and

reverse primers used to amplify the 500 bp templates for dsRNA synthesis. Also, protein functions were included in the context of *S. mansoni* when possible. Gb: GenBank. Table 2: List of primers used to quantify specific transcripts during real-time Q-PCR analysis. Found at: doi:10.1371/journal.pntd.0000502.s001 (0.12 MB XLS)

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Author Contributions

Conceived and designed the experiments: MdMM GRF TPY. Performed the experiments: MdMM ND. Analyzed the data: MdMM ND TPY. Contributed reagents/materials/analysis tools: TPY. Wrote the paper: MdMM ND TPY. Reviewed and revised the manuscript: GRF. Secured CAPES fellowship support for MdMM to conduct the reported research: GRF.

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PLoS NEGLECTED TROPICAL DISEASES

Role of the Endogenous Antioxidant System in the Protection of *Schistosoma mansoni* Primary Sporocysts against Exogenous Oxidative Stress

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Abstract

Antioxidants produced by the parasite *Schistosoma mansoni* are believed to be involved in the maintenance of cellular redox balance, thus contributing to larval survival in their intermediate snail host, *Biomphalaria glabrata*. Here, we focused on specific antioxidant enzymes, including glutathione-S-transferases 26 and 28 (GST26 and 28), glutathione peroxidase (GPx), peroxiredoxin 1 and 2 (Prx1 and 2) and Cu/Zn superoxide dismutase (SOD), known to be involved in cellular redox reactions, in an attempt to evaluate their endogenous antioxidant function in the early-developing primary sporocyst stage of *S. mansoni*. Previously we demonstrated a specific and consistent RNA interference (RNAi)-mediated knockdown of GST26 and 28, Prx1 and 2, and GPx transcripts, and an unexpected elevation of SOD transcripts in sporocysts treated with gene-specific double-stranded (ds)RNA. In the present followup study, *in vitro* transforming sporocysts were exposed to dsRNAs for GST26 and 28, combined Prx1/2, GPx, SOD or green-fluorescent protein (GFP, control) for 7 days in culture, followed by assessment of the effects of specific dsRNA treatments on protein levels using semi-quantitative Western blot analysis (GST26, Prx1/2 only), and larval susceptibility to exogenous oxidative stress in *in vitro* killing assays. Significant decreases (80% and 50%) in immunoreactive GST26 and Prx1/2, respectively, were observed in sporocysts treated with specific dsRNA, compared to control larvae treated with GFP dsRNA. Sporocysts cultured with dsRNAs for GST26, GST28, Prx1/2 and GPx, but not SOD dsRNA, were significantly increased in their susceptibility to H_2O_2 oxidative stress (60–80% mortalities at 48 hr) compared to GFP dsRNA controls (~18% mortality). H_2O_2-mediated killing was abrogated by bovine catalase, further supporting a protective role for endogenous sporocyst antioxidants. Finally, *in vitro* killing of *S. mansoni* sporocysts by hemocytes of susceptible NMRI *B. glabrata* snails was increased in larvae treated with Prx1/2, GST26 and GST28 dsRNA, compared to those treated with GFP or SOD dsRNAs. Results of these experiments strongly support the hypothesis that endogenous expression and regulation of larval antioxidant enzymes serve a direct role in protection against external oxidative stress, including immune-mediated cytotoxic reactions. Moreover, these findings illustrate the efficacy of a RNAi-type approach in investigating gene function in larval schistosomes.

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Introduction

Miracidial penetration and entry into the molluscan intermediate host represent a critical transition period in which the previously free-living larval stage is now confronted with a potentially hostile environment as it attempts to establish a viable infection [1,2]. Miracidia of the human blood fluke *Schistosoma mansoni* shed their ciliated epidermal plates soon after entry into the host snail *Biomphalaria* spp., transforming to primary or mother sporocysts. It is during this time of transition and early sporocyst development that larvae are especially vulnerable to oxidative stress generated from products of oxidized plasma hemoglobin [3], or reactive oxygen or nitrogen species (ROS and RNS, respectively) resulting from hemocyte-mediated immune responses [4–7]. In such a potentially damaging environment, it is vital that parasites possess the capability of maintaining a redox equilibrium in order to counteract the effects of ROS/RNS generated both internally (products of endogenous metabolic oxidative reactions) and externally (environmental insults) [1,8].

Recent studies have shown that *S. mansoni* larvae possess numerous enzymes involved in ROS metabolism and detoxification of oxidative products [9–14], and, like their adult stage counterparts [15–18], appear to complement each other to maintain the redox balance in the parasite. Included among these enzymes are the following: (i) glutathione-S-transferases 26 and 28 (GST26 and GST28) that function to neutralize potential membrane damage by the linked catalysis of glutathione (GSH) reduction with detoxification reactions involving thiol-conjugation to xenobiotics [19], (ii) peroxiredoxin (Prx1 and Prx2) that are involved in maintaining redox balance, by reducing hydrogen

Author Summary

Species of the human blood fluke *Schistosoma* are estimated to infect approximately 200 million people worldwide, resulting in loss of health, vitality and productivity mainly among the world's poorest inhabitants. Since snail intermediate hosts represent an essential part of the flukes' life cycle, an understanding of the strategies used by the intramolluscan schistosome larvae to survive within this host may provide novel approaches for disrupting larval development and thus transmission to humans. Anti-oxidant enzymes produced by the parasite *Schistosoma mansoni* are believed to play a critical role in the maintenance of cellular redox balance, contributing to larval survival in their snail host, *Biomphalaria glabrata*. In this study, we have incorporated a RNA interference approach attempting to knock down specific anti-oxidant enzymes, including gluthatione-S-transferases 26 and 28 (GST26 and 28), gluthatione peroxidase (GPx), peroxiredoxins 1 and 2 (Prx1/2) and superoxide dismutase (SOD), and to evaluate their endogenous anti-oxidant function in the sporocyst stage of *S. mansoni*. Results clearly demonstrated a significantly higher susceptibility of antioxidant double-stranded (ds)RNA-treated larvae to in vitro H_2O_2 treatment or hemocytic encapsulation compared to GFP dsRNA controls. Taken together, our findings support the hypothesis that endogenous expression and regulation of larval antioxidant enzymes serve a direct role in protection against external oxidative stress, including immune-mediated cytotoxic reactions.

peroxide (H_2O_2) using a thioredoxin as an electron donor [20], (iii) superoxide dismutases (SOD), metalloenzymes responsible for catalyzing the dismutation of the superoxide radical to hydrogen peroxide as a defense mechanism against oxygen toxicity [21], and (iv) glutathione peroxidase (GPx), an H_2O_2-metabolizing enzyme that protects membranes from damage by phospholipid peroxidation [20,22]. It is noteworthy that unlike most organisms, catalase, an enzyme responsible for H_2O_2 metabolism, is absent in *S. mansoni* [18,23,24], but is functionally replaced by Prx and GPx [16]. Interestingly, for schistosome GPx, whose H_2O_2-reactivity is typically very low in adult worms [8], exposure to the mammalian host environment induces enzyme activity and appears to be positively correlated to the parasite's resistance to oxidative stress [22]. In contrast to GPx, high levels of Prx activity are found in adult *S. mansoni* worms, and these enzymes are believed to be key components in maintaining redox balance, as well as are major contributors to antioxidant activity [16].

Previous findings have demonstrated that *in vitro* cultured *S. mansoni* sporocysts are highly sensitive to H_2O_2 toxicity [5], and that sublethal exposure of sporocysts *in vitro* to ROS, in particular H_2O_2, elicits an upregulation of genes encoding various antioxidant proteins [7,11]. These data support the hypothesis that the primary sporocyst is capable of interfering with, or deactivating ROS-mediated damage, through activity of an endogenous antioxidant system [1]. However, to date, a functional role of specific antioxidant enzymes within intact larvae in providing protection against external ROS insults has not been demonstrated. Recently Mourão *et al.* [25] demonstrated consistent transcript knockdown for various antioxidant/redox-active detoxicant mRNA species in *S. mansoni* sporocysts using RNA interference as originally described [26]. These included transcripts for GST26 and 28, Prx1 and 2, and GPx. As a followup to these findings, the present study was conducted to determine the functional consequences of these induced antioxidant gene changes, especially their relevance to *S. mansoni* sporocyst interactions with the intermediate snail host *B. glabrata*.

Materials and Methods

Ethics statement

Research procedures involving mice used in the course of this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin-Madison under assurance no. A3368-01.

In vitro cultivation of larval *Schistosoma mansoni*

The NMRI strain of *S. mansoni* was used for all experiments. *S. mansoni* eggs were isolated from livers obtained from mice harboring 7-week old infections, and miracidia hatched in an artificial "pond water" supplemented with antibiotics (50 µg/mL streptomycin and 60 µg/mL penicillin) [27]. Larvae were washed twice in ice-cold, sterile pond water by centrifugation, before being resuspended in Chernin's Balanced Saline Solution (CBSS; [28]), containing glucose and trehalose (1 g/L each) streptomycin and penicillin (50 µg/mL and 60 µg/mL, respectively). Miracidia were then counted and distributed into 48- or 96-well polystyrene tissue culture plates (Costar, Corning Incorporated, NY), at concentrations of ~500, 1000 or 8000 miracidia/well for oxidative stress experiments, immunocytochemistry or Western blot analyses, respectively. Finally, double-stranded RNAs were synthesized from isolated sporocyst cDNA using T7 RiboMAX Express RNAi Kit (Promega, Madison, WI), according to manufacturer protocol. Briefly, dsRNAs synthesis reactions were allowed to incubate for 16 hr at 37°C prior to DNAse treatment. DsRNA products were then extracted by phenol/chloroform and purified by precipitation with isopropanol. DsRNAs (50 nM final concentration) for specific antioxidant genes or green-fluorescent protein (GFP; specificity control dsRNA) were added to cultures containing 100 µL of CBSS for the oxidative stress assays and immunocytochemistry and 400 µL for the Western blot experiments. Because of the sequence and functional similarities of Prx1 and 2, dsRNAs for these transcripts were combined as a single treatment, designated hereafter as Prx1/2. Larvae were incubated for 7 days as previously detailed [25], after which time the functional consequences of dsRNA treatments were determined in functional assays described below. It should be noted that in a previous series of RNAi experiments conducted in parallel with the present study [25], a consistent, significant knockdown of steady-state transcript levels for each of the antioxidant genes currently under study was well documented. The only exception was the Cu/Zn superoxide dismutase (SOD) gene, in which larval exposure to SOD dsRNA resulted in a consistent increase, not knockdown, of SOD transcripts.

Western blot analysis

To assess the effects of antioxidant dsRNA on the expression of specific proteins in sporocysts, we analyzed protein extracts of dsRNA-exposed sporocysts by Western immunoblot analysis [29] incorporating specific antibodies to two antioxidant species; namely SmGST26 (Cell Signaling Technology, Danvers, MA) and SmPrx1/2 (gift from Dr. D. Williams). Briefly, protein samples (~8 µg) and Precision Plus Dual Color Marker (Bio-Rad, Bio-Rad Laboratories, Inc., Hercules, CA) were separated on 12.5% SDS-PAGE gels and transferred by semi-dry electroblotting (Amersham Biosciences) to nitrocellulose membranes (Bio-Rad). After blocking overnight in TBS (2.42 g Tris base, 8 g NaCl, pH 7.6) containing 5% bovine serum albumin (BSA), membranes were incubated in specific antibodies or a mouse anti-α tubulin

antibody (serving as loading control, 1:1000 dilution; Upstate Biotechnology Inc., Lake Placid, NY) for 16 hr at 4°C with gentle rocking. Membranes were then washed for 30 min in TBS-Tween (0.1%), and incubated for 1 hr in TBS-BSA (5%) containing either alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG or AP-rabbit anti-mouse IgG at dilutions of $1{:}10^4$ and 1:5000, respectively (Promega, Madison, WI). The colorimetric immunoreactivity was detected with the chromogen 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium (NBT) diluted in AP buffer (0.1 M Tris, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5).

To quantify the observed immunoreactivity of each target protein in sporocysts treated with specific dsRNA and control GFP dsRNA, the intensities of reactive target bands were measured using Ultraviolet Transilluminator BioImaging Systems (UVP, Inc., Upland, CA) and normalized to the αtubulin band with LabWorks Image Acquisition and Analysis Software (version 4.6) in order to quantitatively evaluate the effects of antioxidant dsRNA treatment on specific protein levels. Three independent experimental replicates were performed and analyzed by Student's *t*-test, with significance set at $P \leq 0.05$.

Immunocytochemistry

In order to compare *in situ* GST26 and Prx protein levels in antioxidant dsRNA-treated parasites, we prepared whole, intact sporocysts for immunofluorescent observations. All washing steps, in eppendorf tubes, were performed by centrifugation at 1600 rpm for 2 min and repeated 5 times, or as otherwise mentioned. Following transformation and *in vitro* cultivation (24 hr), sporocysts were washed 3 times in CBSS, to remove detached ciliated plates, prior transfer to siliconized-tubes containing 2% paraformaldehyde and 1% Triton-X100/sPBS. Larvae were fixed overnight at 4°C under gentle agitation, then washed in snail phosphate-buffered saline (sPBS; [30]) and resuspended in blocking buffer (5% normal goat serum + 0.02% sodium azide in sPBS) for 16 hr at 4°C. Rabbit-anti-GST26 or mouse anti-Prx1/2 primary antibodies, diluted at 1:2000, and 1:200, respectively, were then added to the larvae in fresh blocking buffer for 16 hr at 4°C under gentle agitation. This was followed by 5 washes, 10 min each, in sPBS, and resuspension in blocking buffer containing 4 µg/mL Alexa-Fluor 488-conjugated anti-rabbit/mouse antibody, 7 units/mL phalloidin-Alexa 546 and 10 µg/mL Hoechst 33258 dye (Invitrogen). Tubes containing samples were incubated for 16 hr at 4°C under constant rotation, followed by washing in sPBS, resuspension in 40 µl of sPBS and mounting on coverslips. A Nikon Eclipse TE2000 (Nikon Instrument Inc., Melville, NY) inverted epifluorescence microscope equipped with a Bio-Rad Radiance 2100 MP Rainbow Confocal/Multiphoton Imaging System (W.M. Keck Laboratory for Biological Imaging, Instrumentation, UW-Medical School) was used for specimen imaging and evaluation.

Oxidative stress experiments

Peroxide toxicity. A series of *in vitro* experiments were designed to test the potential functional consequences of antioxidant protein knockdown in sporocysts treated with a sublethal concentration of H_2O_2. In preliminary tests, to determine our working sublethal peroxide concentration we exposed 7-day cultured GFP dsRNA-treated sporocysts to increasing amounts of H_2O_2 (Sigma-Aldrich, St Louis, MO) in a 96-well plate containing 0 µM (buffer only control), 5 µM, 10 µM, 25 µM, 50 µM, 100 µM, and 5 mM H_2O_2 in CBSS. Propidium iodide (PI; Invitrogen, Carlsbad, CA), used as a vital dye (5 µg PI/mL CBSS), was added at the time of viability scoring and the percentage of dead sporocysts, determined by intense staining with PI (PI-positive or PI+) was calculated at 24- and 48-hr post-treatment according to the following formula:

$$\%\ \text{dead sporocysts} = \left[\text{number of dead larvae (PI+)}/\text{total number of larvae}\right] \times 100$$

Larval mortality in ≤50 µM H_2O_2 was comparable to untreated sporocysts (CBSS alone) at both time intervals (data not shown), and therefore 50 µM was chosen as our sublethal H_2O_2 concentration.

Effect of antioxidant gene knockdown on sublethal H_2O_2-mediated sporocyst killing. To evaluate the functional relevance of the antioxidant enzymes GPx, GST26, GST28, Prx1/2 and SOD on parasite survival under oxidative stress conditions, 7-day dsRNA-treated sporocysts were exposed to 50 µM H_2O_2 for 4, 24 and 48 hr prior to evaluating larval viability. Freshly-hatched miracidia were axenically isolated and soaked in CBSS containing 50 nM of GPx, GST26, GST28, Prx1/2, SOD or control GFP dsRNAs and cultured for 7 days in 24-well plate as previously described [25]. Each treatment group was then divided into 3 wells of a 96-well plate: 2 of the wells were exposed to 50 µM of H_2O_2, and the third well was used as a no-treatment control (no H_2O_2). Cultures were incubated at 26°C and dead parasites (PI+) were counted at 4 h, 24 h and 48 h using an epifluorescent inverted microscope Nikon Eclipse TE 300 (Nikon Instruments Inc.). Data were represented as mean percentage dead sporocysts: $\left[(\#\text{PI+ larvae})/(\text{total}\ \#\ \text{larvae})\right] \times 100$, at each time point for each treatment group. Two-way ANOVA with Bonferroni post-test was used to compare the % mortality of antioxidant dsRNA-treated groups over exposure time interval to the GFP dsRNA-treated control group. Significance was set at $P \leq 0.05$; $N = 5$.

Catalase neutralization of H_2O_2-mediated oxidative stress. To verify that H_2O_2 is the major oxidizing agent responsible for initiating sporocyst death, dsRNA-treated larvae were prepared as described above and exposed to 50 µM H_2O_2 containing 0.1%, 0.05%, 0.025%, 0.0125% of bovine catalase (Sigma-Aldrich). Additional control cultures containing H_2O_2 alone (positive killing control) and catalase alone (catalase control) were concurrently run. All parasites were incubated at 26°C, and larval death assessed at 0, 4, and 48 hr post-treatment, using propidium iodide (Invitrogen). For each dsRNA treatment, the mean percentage mortality between H_2O_2-exposed, H_2O_2 + catalase-exposed and unexposed parasites with time was compared using Two-way ANOVA with Bonferroni post-test and with significance set at $P < 0.05$ ($N = 6$).

Protective effect of antioxidants against *in vitro* hemocyte-mediated killing. To investigate the larval protective role of endogenous antioxidant molecules, sporocysts were treated with GPx-, Prx1/2-, GST26-, GST28-, SOD- and GFP-(control) dsRNAs for 7 days, followed by co-incubation with hemocytes of the susceptible NMRI strain of *Biomphalaria glabrata* in an *in vitro* cell-mediated cytotoxicity assay [5]. Because we wished to test for an effect of antioxidant knockdown on the efficacy of hemocyte-mediated killing, cells of the susceptible NMRI strain were used to determine if their basal level of killing efficiency could be significantly altered due to antioxidant knockdown. Eighteen snails (14–18 mm shell diameter) were used for each assay, in which the shell of each animal was dried, swab with 70% ethanol, and soaked in filter-sterilized "pond" water containing 60 µg/mL penicillin G, 50 µg/mL streptomycin and 25 µg/mL amphotericin B for 30 min. Snail shells were again

dried and swabbed with 70% ethanol before headfoot bleeding as described in Sminia and Barendsen [31]. Whole hemolymph was pooled in a Petri dish on ice to facilitate removal of any extraneous shell debris or mucus, and then transferred to sterile 15-mL centrifuge tubes containing an equal volume of ice-cold CBSS.

The cytotoxicity assay described by Hahn *et al.* [5] was used with some modifications. Approximately 500 µL of hemolymph was gently aliquoted in siliconized-eppendorf tubes containing an agarose plug (0.2% agarose) and 50 µL of 5% Ficoll (Sigma-Aldrich Inc.) in incomplete or I-Bge (24% Schneider's *Drosophila* medium, Invitrogen; 0.5% lactalbumin hydrolysate, Sigma-Aldrich; 7.2 mM galactose). Tubes were centrifuged at $20\times g$ for 17 min to isolate and concentrate hemocytes. Cell-free plasma and Ficoll were removed and discarded, followed by resuspension of hemocytes in sterile CBSS and redistribution in equal aliquots to wells of a 16 CultureWell™ Chambered Coverglass slide (Invitrogen) containing approximately 100 dsRNA-treated sporocysts in I-Bge medium. After 1 hr of co-cultivation at 26°C, 3 µL of propidium iodide (PI; 5 µg/mL) (Invitrogen) were added to a subset of wells and the total number of sporocysts (Nomarski DIC optics) and number of dead sporocysts, those exhibiting positive PI staining (PI+; epifluorescence microscopy), per treatment were counted in order to establish an initial mortality rate. Enumeration of total and dead sporocysts was again determined at 24 hr post-cultivation. The percentage of sporocysts killed after 24 hr of co-culture was calculated for groups of larvae treated with antioxidant dsRNAs and control GFP dsRNA, and compared according to the following formula [5]:

$$\%\,\text{sporocysts killed} = \left[{}^{(\text{d24 hr} - \text{d1 hr})}\!/\!_{(\text{T} - \text{d1 hr})} \right] \times 100,$$

where "d" = # dead sporocysts at the indicated time interval (1 or 24 hr) and "T" = total # sporocysts. Statistical analyses were performed using Student's *t*-test in which the % sporocyst death at 24 hr was compared between antioxidant dsRNA-treated and control GFP dsRNA-treated groups. Significance was set at $P \le 0.05$ (N = 4)

Results

Previous work in our lab has established a consistent and specific pattern of altered antioxidant transcript expression in primary sporocysts after 7 days of double-stranded (ds) RNA exposure [25]. Specifically, statistically significant knockdown of *S. mansoni* GST26, GST28, GPx, and Prx1/Prx2 transcript levels, and an unexpected robust increase in those of SOD were observed in dsRNA-treated larval populations. To further explore the functional relevance of these enzymes in this parasite model, we conducted experiments to determine how antioxidant dsRNA exposure affected gene expression at the protein level (for selected enzymes), and whether a functional association could be established between antioxidant gene knockdown and parasite survival in presence of stressors such as reactive oxygen species (H_2O_2) or encapsulating hemocytes.

To verify that specific dsRNA treatments had a predicted downregulating effect on sporocysts at the protein levels, Western blot analyses were performed on sporocysts treated with dsRNA for GST26, Prx1/2 and GFP (control) using antibodies specifically against *S. mansoni* GST26 and Prx1/2 [20]. In all experiments a crossreactive anti-α tubulin antibody served as a loading control. As shown in Figure 1, proteins extracted from GFP dsRNA-treated sporocysts (specificity control) presented two distinctive bands at ~26 and 55 kDa, corresponding to GST26 and α tubulin, respectively. However, although larvae treated with GST26 dsRNA also exhibited the 55 kDa α tubulin protein, little immunoreactivity was observed at 26 kDa, suggesting an RNAi-induced GST26 protein knockdown (Fig. 1A). Quantification of band intensities by scanning densitometry, using anti-α tubulin reactivity to normalize protein loads in both treatment samples, confirmed that GST26 protein levels were significantly reduced (by ~80%) in GST26 dsRNA-treated sporocysts compared to the nonspecific GFP dsRNA control group (Fig. 1C). Similarly, although not as dramatic, larval exposure to Prx1/2 dsRNA also exhibited a significant ~50% decrease in protein level compared to the GFP control treatment by semi-quantitative Western blot analysis (Figs. 1B and D).

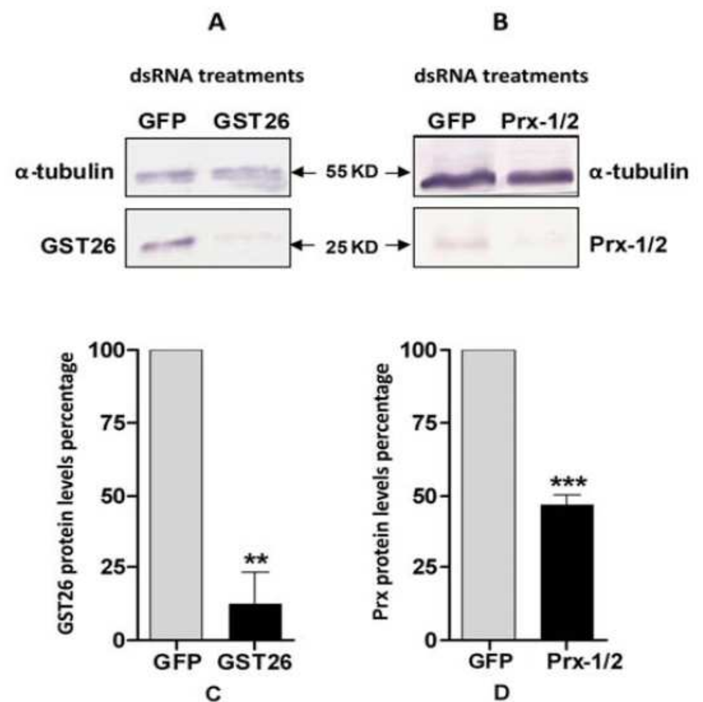

Figure 1. Western blot analyses of SDS-PAGE separated extracts of *Schistosoma mansoni* sporocysts. Larvae were cultured for 7 days in CBSS containing GFP, GST26, or Prx1/2 dsRNA, followed by probing with specific anti-GST26 (Fig. 1A), anti-Prx1 (Fig. 1B) or sample loading control anti-α tubulin antibodies. Using anti-α tubulin reactivity to normalize sample loads, scanning densitometry was used to quantify immunoreactive GST26 (Fig. 1C) and Prx1 (Fig. 1D) intensities in specific dsRNA-treated vs. GFP dsRNA control sporocyst groups. Both GST26 and Prx protein levels were significantly knocked down by 80% and 50%, respectively, when compared to the GFP dsRNA treatment. ** $P<0.01$; *** $P<0.001$; N = 3.
doi:10.1371/journal.pntd.0000550.g001

Consistent with Western blot analyses, *in situ* confocal observations of anti-GST26 localization in GST26 dsRNA-exposed and control GFP dsRNA-treated sporocysts revealed contrasting expressions of immunoreactivities. Anti-GST26 antibodies strongly reacted with endogeneous *S. mansoni* GST26 in sporocyst controls (Fig. 2A), but was much reduced in those treated with GST26 dsRNA (Fig. 2B), indicating a RNAi-mediated GST26 protein knockdown. Immunolocalization of anti-Prx1/2, however, revealed little difference in observed staining intensities between the GFP and Prx dsRNA-treated groups (Figs. 2C and 2D, respectively), except for a slight decrease in surface immunoreactivity in Prx-treated sporocysts. This also is consistent with the smaller knockdown effect of dsRNA exposure on Prx protein expression seen in immunoblot analysis (Fig. 1B).

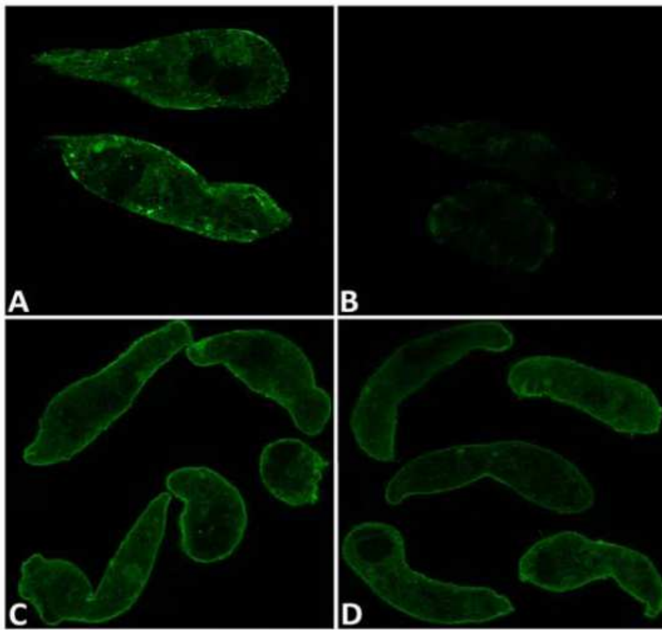

Figure 2. Representative confocal epifluorescent photomicrographs of *Schistosoma mansoni* sporocysts showing immunolocalization of anti-GST26 and anti-Prx1 antibodies after cultivation in medium containing GST26, Prx or control GFP dsRNA. Fluorescence specific to anti-GST26 reactivity (green) observed in GST26 dsRNA-treated larvae (Fig. 2B) was noticeably reduced compared to the nonspecific GFP dsRNA control sporocysts (Fig. 2A), consistent with the high protein knockdown (~80%) seen in Western blot analysis. By contrast, little difference in fluorescence levels was observed between the nonspecific GFP dsRNA control-treated (Fig. 2C) and Prx1/2 dsRNA-treated (Fig. 2D) sporocysts, reflecting the relatively small decrease (~50%) observed in immunoblot protein levels. N = 3.
doi:10.1371/journal.pntd.0000550.g002

In order to evaluate the effects of a potential loss of antioxidant activity in sporocysts due to dsRNA-induced antioxidant knockdown, we exposed groups of treated parasites to a range of hydrogen peroxide (H_2O_2) concentrations. In these preliminary tests 50 µM H_2O_2 was determined to represent a sublethal dosage under our experimental conditions (% larval death was not significantly different from control groups), whereas mortality rates significantly increased at 100 µM and higher H_2O_2 concentrations (data not shown). As shown in Figure 3, none of the dsRNA-treated sporocysts exhibited significant increases in H_2O_2-mediated mortality when compared to the GFP control treatments after 4 hr of exposure. However, at 24 and 48 hr sporocysts in all dsRNA-treatments, except the SOD dsRNA-exposed group, displayed significant increases in mortality with an average of 35% sporocyst death compared to 8% in control treatments after 24 hr, and 60 to 80% mortalities, compared to ~18% in control treatments, at 48 hr post treatments ($F_{dsRNA} = 28.21$, $P \leq 0.0001$; $F_{Time} = 84.71$, $P \leq 0.0001$, N = 4). In contrast to other antioxidant treatments, sporocysts exposed to SOD dsRNA exhibited a H_2O_2-mediated mortality rate similar to that of control treatments at all time points (Fig. 3). See Figure 3 legend for means comparisons using Bonferroni's post-test.

To confirm that sporocyst death was specifically due to H_2O_2 as an exogeneous oxidative stressor, we exposed dsRNA-treated sporocysts to 50 µM H_2O_2 in presence or absence of bovine catalase or to catalase only (no H_2O_2 control), and evaluated sporocysts mortality in all treatments after 48 hr. Overall ANOVA indicated a significant effect of dsRNA treatment and H_2O_2-exposure ($F_{dsRNA} = 7.44$, $P \leq 0.001$; $F_{Oxid} = 15.33$, $P \leq 0.0001$, N = 6). Within each treatment group, the percent mortalities for sporocysts exposed to GPx, GST26, GST28 and Prx1/2 dsRNAs were very similar when incubated in H_2O_2+catalase or catalase only (*t* values ranging from 0.23–1.74; all nonsignificant) (Fig. 4). These results are in contrast to the effects of exposure to H_2O_2 alone (positive killing control), in which mortality rates for sporocysts treated with the same antioxidant dsRNAs were significantly higher (ranging from 50–75%) when compared to 25% average sporocyst death in the catalase treatment groups (see Fig. 4 for Bonferroni's post-test comparisons). As previously observed, SOD dsRNA-treated larvae, again showed no difference in mortality rates between the different treatments, nor when compared to the control GFP dsRNA group.

Finally, in order to evaluate the effect of dsRNA antioxidant knockdown on snail hemocyte-sporocyst interactions *in vitro*, dsRNA-treated sporocysts were co-cultured with isolated hemocytes from the susceptible NMRI strain of *Biomphalaria glabrata*. After 24 hr of sporocyst-hemocytes incubation in an *in vitro* cell-mediated cytotoxicity assay [5], we observed that dsRNA knockdown of GST26 ($t = 2.50$, $P \leq 0.01$), GST28 ($P \leq 0.0461$) and Prx1/2 ($t = 3.17$, $P \leq 0.04$) resulted in small, but statistically significant increases in larval death, averaging ~20% compared to ~8% mortality in the GFP dsRNA control group (Fig. 5). Note that sporocysts treated with GPx dsRNA also showed an increase in mean mortality rate, but was not statistically significant when compared to the GFP control parasites. As observed in previous experiments, sporocysts treated with SOD dsRNA exhibited no difference in mortality compared to the GFP-treated control sample.

Discussion

Enzymes involved in cellular redox pathways, which include proteins with antioxidant activities, are believed to be essential components regulating *B. glabrata*/*S. mansoni* molecular interaction [1,2]. It is now well recognized that certain strains of *B. glabrata* snail immune cells or hemocytes produce substantial amounts of reactive oxygen [4,5] and nitrogen [6] species as a consequence of stimulation by known activators of ROS/RNS or when encountering *S. mansoni* sporocysts, and that sporocysts are exquisitely sensitive to ROS-mediated killing, especially to H_2O_2. Moreover, in a series of followup studies, Bayne and co-workers have implicated a Cu/Zn-superoxide dismutase (SOD1) as a key enzyme involved in oxidative killing activity by hemocytes of resistant (R) strains of *B. glabrata* snails. Their studies demonstrated that (1) SOD transcript expression and enzyme activity are higher in certain R vs. susceptible (S) snail hemocytes [32] and this correlates with greater H_2O_2 production in the R strain [33], (2) *B. glabrata* SOD1 is comprised of 3 alleles, of which one (B allele) is significantly associated with R snails [34], and (3) SOD1 B allelelic expression is higher in R hemocytes than those of the S strain [35]. Based on their findings it is suggested that snail strain differences in SOD hemocyte expression may be causally linked to the observed S and R strain phenotypes. Because SOD catalyzes the conversion of superoxide to cytotoxic H_2O_2 it is reasoned that upregulation of the SOD1 gene and its resultant heightening of SOD enzymatic activity in R hemocytes may represent a possible mechanism for the differential larval killing response by R vs. S snail hemocytes [2].

While snail hemocytes produce H_2O_2 as an anti-parasite effector molecule, evidence also strongly supports the presence of an active antioxidant system in early developing *S. mansoni* sporocysts [11–13]. Catalase gene homologues were not found in

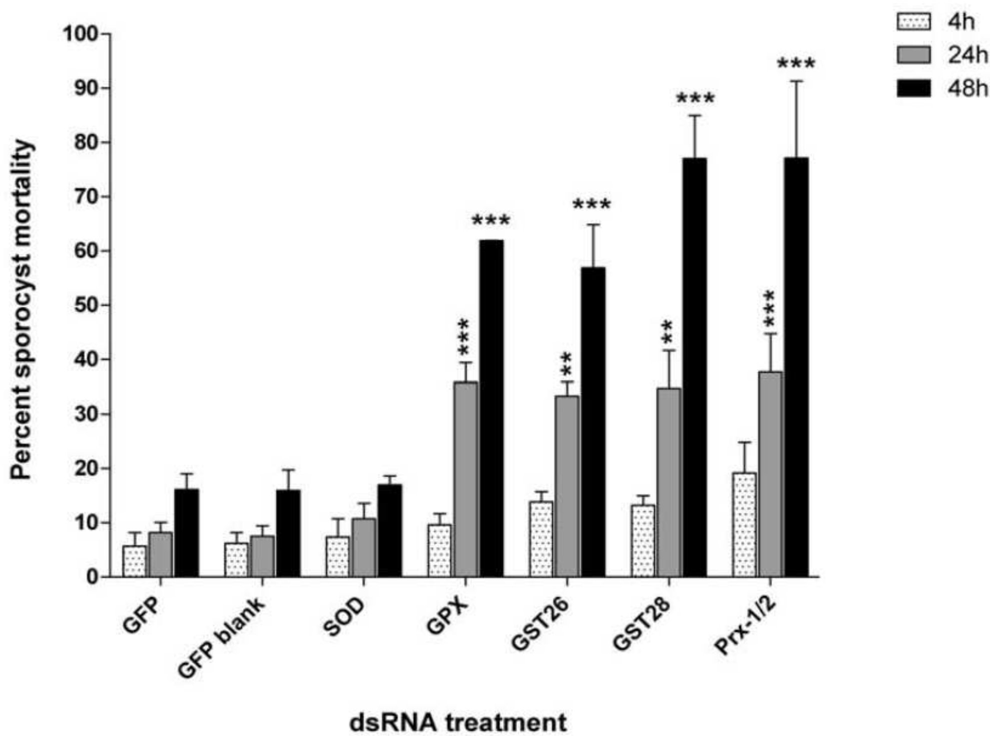

Figure 3. Graphic representation of the effect of exogenous H_2O_2 exposure on *Schistosoma mansoni* sporocysts following treatment with dsRNAs for GFP (specificity control), SOD, GPx, GST26, GST28 and Prx1/2. Double-stranded RNA-reated sporocysts were exposed to 50 µM H_2O_2 for 4, 24 and 48 hrs (stippled, grey and black bars, respectively). Knockdown of larval GPx, GST26, GST28 and Prx1/2 antioxidants increased sporocyst mortality after 24 and 48 hr under oxidative stress conditions when compared to GFP dsRNA-treated or no treatment controls. Note that sporocysts treated with SOD dsRNA showed no difference in susceptibility to H_2O_2 oxidation at any of the time points compared to controls. **$P<0.001$; ***$P<0.0001$; N = 4.
doi:10.1371/journal.pntd.0000550.g003

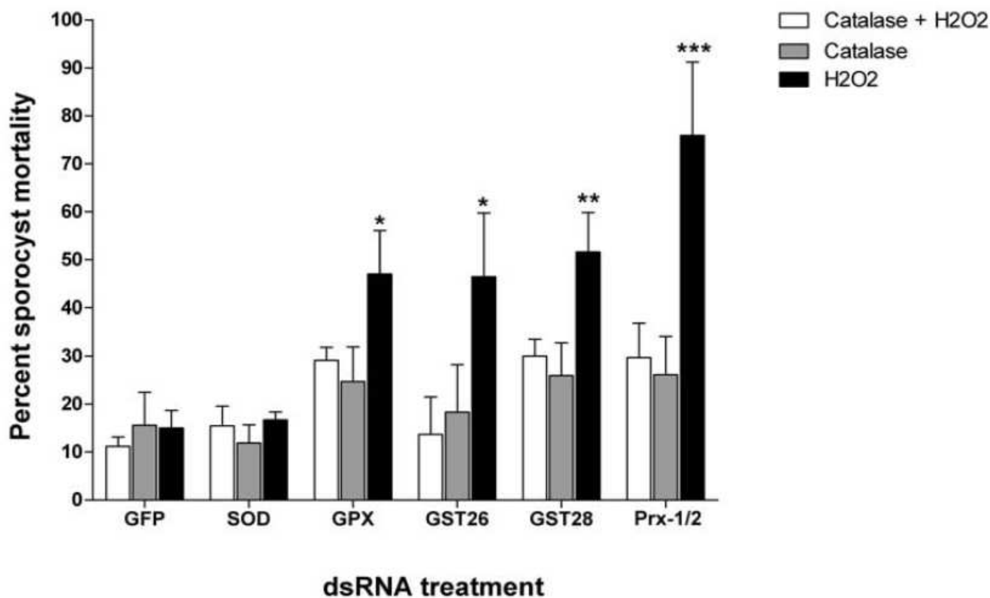

Figure 4. Effects of catalase on H_2O_2-mediate killing of GPx, GST26, GST28, SOD, Prx1/2 dsRNA-treated and control GFP dsRNA-treated *Schistosoma mansoni* sporocyst *in vitro*. After 7 days of dsRNA incubation sporocysts were exposed to H_2O_2 alone, catalase alone or catalase combined with H_2O_2 for 48 hr followed by evaluation of sporocyst death using propidium iodide staining. Significant increases in H_2O_2–mediated mortality was abrogated in the presence of bovine catalase [H_2O_2+catalase] showing that H_2O_2 was the primary source of larval killing in antioxidant dsRNA-treated sporocysts, with the exception of SOD, *$P<0.05$; **$P<0.001$; ***$P<0.0001$; N = 6.
doi:10.1371/journal.pntd.0000550.g004

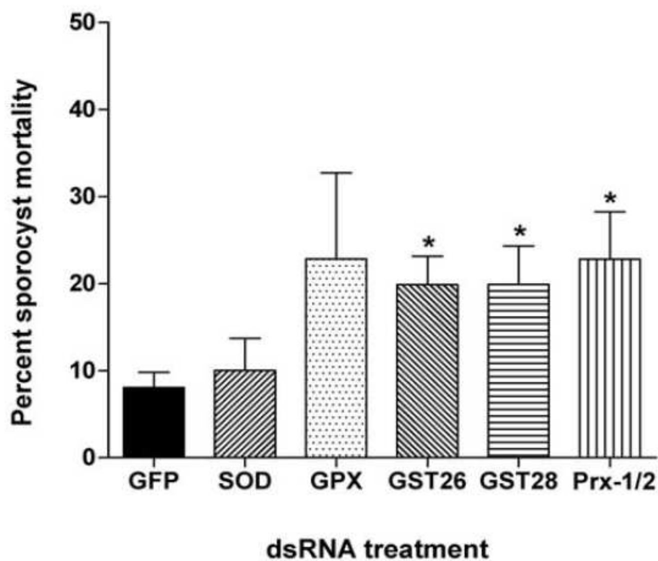

Figure 5. *In vitro* cell-mediated cytotoxicity (CMC) assay results. *Schistosoma mansoni* sporocysts, cultured for 7 days in medium containing antioxidant (GPx, Prx1/2, GST26 and 28, SOD) or control GFP dsRNA were incubated for 24 hr with plasma-free hemocytes from the susceptible NMRI snail strain of *Biomphalaria glabrata* followed by assessment of larval mortality by propidium iodide staining. Co-culture of GST26, GST28 and Prx1/2 dsRNA-treated sporocysts with snail hemocytes resulted in small, but significant increases in percent larval mortality when compared to GFP dsRNA controls. The GPx dsRNA-treatment exhibited a nonsignificant increase in larval killing, while mortality of SOD dsRNA-treated sporocysts showed no difference compared to GFP dsRNA controls. $^{*}P\leq0.04$; $N=4$.
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recent searches of the *S. mansoni* genomic and EST databases, and this is consistent with earlier findings [16,23,24] indicating that these parasites must possess alternative means for neutralizing H_2O_2 and other ROS. As clearly demonstrated in mammalian stages of *S. mansoni*, this is accomplished by a thiol-dependent redox system involving thioredoxin glutathione reductase (TGR) as the central enzyme driving redox reactions [36]. Similarly, early intramolluscan larval stages also express redox genes, including TGR, thioredoxin, Cu/Zn SOD, GPx, Prx and GST [7,11–13,37], and in the case of GPx [7] and Prx1 and 2 [11], sporocyst expression levels are dramatically increased in response to ROS exposure. In addition, Cu/Zn SOD, GST26 and 28 and Prx were recently identified in larval transformation proteins (LTP) released during *in vitro* transformation of miracidia to sporocysts, demonstrating not only the synthesis of these antioxidants by miracidia, but also their active release during larval infection [9,14]. Implied in these findings is the notion that antioxidant LTPs may be playing a potential protective role during early parasite development. This prospect of larval-protective antioxidants was given further credence by Vermeire and Yoshino [11] who demonstrated that Prx1/2 in LTP can function as scavengers of exogenous H_2O_2 suggesting the potential importance of excreted antioxidants as a sporocyst defense mechanisms.

In this study, we provide the first evidence for a functional role of the endogenous antioxidants GPx, Prx and GSTs in the survival of *S. mansoni* sporocysts confronted with exogenous oxidative stress. By successfully knocking down antioxidant transcript/protein levels using an RNAi-type approach, we were able to characterize the impact of introduced molecular H_2O_2 and presumed ROS produced during hemocyte encapsulation reactions on survival of intact primary sporocysts of *S. mansoni*. In a previous companion study that was run in parallel with the current experiments [25] we showed that larval treatment with double-stranded RNA (dsRNA) for all of the antioxidants, except SOD, produced a consistent, significant and specific transcript knockdown in sporocysts. In the present study, consistent with the transcript knockdown seen earlier, we demonstrated a dsRNA-associated decrease in GST 26 and Prx1/2 protein levels using specific antibodies in a semi-quantitative Western blot assay. This protein knockdown effect was supported by immunocytochemistry (ICC) in the case of GST26, but not as clearly for Prx. Importantly, the dsRNA-mediated decrease in GST26 and Prx protein content correlated well with significant increases in sporocyst mortality at 24 and 48 hr post-H_2O_2 exposure compared to the dsRNA control groups, implying a functional role for endogenous GST26 and Prx in the protection of primary sporocysts against external oxidative stress. Although lack of specific antibodies to the other antioxidants precluded a complete analysis of the other RNAi targeted genes used in this study, we continued to see a consistent correlation between dsRNA-induced decrease in transcript levels [25] and sporocyst survival patterns for larvae treated with GST28 and GPx dsRNA that were similar to those treated with GST26 and Prx1/2 dsRNAs. Indeed, compared to the untreated and GFP dsRNA controls, exposure of antioxidant dsRNA-treated sporocysts to a sublethal concentration of H_2O_2 *in vitro* resulted in dramatic decreases in parasite survival in all treatment groups except SOD, supporting the notion that GST28 and GPx, similar to Prx and GST26, also are capable of enhancing sporocyst survival in an oxidative environment.

These new findings are consistent with the extensive and ongoing work on the redox mechanism in the adult stage of *S. mansoni*, in which an active thiol-dependent redox maintenance system revolves around a thioredoxin glutathione reductase (TGR; [36]), a single enzyme that combines the activities of two enzymes, thioredoxin reductase and glutathione reductase, present in mammals [17]. Schistosome TGR is responsible for maintaining the reduced and active states of both thioredoxin (TR) and glutathione (GSH), allowing them to activate several Prxs and GPx, which in turn are capable of reducing H_2O_2 and other hydroperoxides [8]. Furthermore, in a more recent study, Sayed and coworkers [16] showed that Prx activity was essential to *S. mansoni* adult worm survival *in vitro*, further supporting the importance of maintaining a steady supply of this, and other antioxidant enzymes by *S. mansoni* adults. It appears that, like adult worms, early intramolluscan stages also must rely on robust endogenous system of antioxidant production that allows the parasite to overcome oxidative stress from both internal and external sources.

In addition to the antioxidant protective role of *S. mansoni* sporocysts in the presence of exogeneously introduced oxidative stress, we observed a similar survival pattern in dsRNA antioxidant-treated sporocysts that have come in contact with hemocytes from the susceptible NMRI *B. glabrata* strain. Our rationale for incorporating susceptible snail hemocytes in these experiments was to test the hypothesis that reducing the antioxidant capacity of sporocysts would increase their vulnerability to sublethal levels of ROS normally produced by NMRI snail hemocytes in *in vitro* cell-mediated cytotoxicity (CMC) assays [5,38]. In this *in vitro* biologically-relevant context, we demonstrated a significant protective role of Prx and GSTs in sporocysts during hemocyte interactions. Co-culture of plasma-free hemocytes from susceptible NMRI snails with Prx, GST26, and GST28 dsRNA-treated sporocysts induced an increase in sporocyst mortality (to ~20%) within 24 h of initial contact, when compared

to GFP dsRNA-treated control group (8%). GPx dsRNA-treated sporocysts also showed a comparable increase in hemocyte-mediated killing, but high variance in replicate values rendered the increase nonsignificant. Thus the protective role of GPx against hemocyte-mediated ROS attack still remains to be proven. Taken together, however, our overall results suggest that ROS production in susceptible snail hemocytes is capable of overpowering antioxidant-deficient parasites. Zelck and Janowsky [7] hypothesized that susceptible snails generate relatively small amount of ROS, which in turn may induce antioxidant production in schistosomes, effectively neutralizing snail-generated ROS. In this study, we have demonstrated that effectively reducing their antioxidant enzyme capacity, sporocyst survival, when confronted by a usually benign hemocyte challenge, is significantly reduced, thus supporting the critical importance of the endogenous antioxidant system in establishing viable larval infections within the susceptible snail host.

Finally, a major exception to our present finding of enhanced larval susceptibility to oxidative stress by redox proteins was signal peptide (SP) Cu/Zn SOD [39]. In this case Cu/Zn SOD dsRNA treatment consistently had no effect on parasite survival whether in the presence of sublethal H_2O_2 or encapsulating hemocytes. These differing effects of SOD dsRNA exposure may have been predicted as treated *S. mansoni* sporocysts consistently displayed extreme elevations, rather than knockdown in transcript levels [25], indicating a strong induction of SOD gene expression in these larval stages. At present, the signaling mechanisms involved in this response are not known although, as suggested by Zelck and Von Janowsky [7] and Vermeire and Yoshino [11], sporocysts may be sensing ROS levels (including H_2O_2) and responding by upregulating protective antioxidant proteins. It is speculated that larval treatement with SOD dsRNA may have caused an initial downregulation of SOD transcripts that then led to a compensatory triggering of SOD over-expression. However, as shown in other systems, small interfering dsRNA also can trigger activation of transcription [40] and, therefore, could also represent a likely mechanism [25]. Its unusual expression pattern not withstanding, results indicate that hyperexpression of the SOD gene in *S. mansoni* sporocysts appeared to have a "neutral" effect on dsRNA-treated larvae (i.e., an effect similar to control dsRNA treatment) (present study). This does not necessarily imply that SOD has no role to play in maintaining redox balance within sporocysts both internally or in response to exogenous ROS sources. However, the mechanisms by which this is accomplished are currently unknown and represent the subject of further followup investigations in our lab.

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Author Contributions

Conceived and designed the experiments: TPY. Performed the experiments: MMM ND. Analyzed the data: MMM ND GRF. Contributed reagents/materials/analysis tools: TPY. Wrote the paper: MMM ND TPY. Revisions of draft manuscripts: MMM ND GRF. Obtained fellowship support for MMM: GRF. Provided research funds: TPY.

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In vitro manipulation of gene expression in larval *Schistosoma*: a model for postgenomic approaches in Trematoda

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SUMMARY

With rapid developments in DNA and protein sequencing technologies, combined with powerful bioinformatics tools, a continued acceleration of gene identification in parasitic helminths is predicted, potentially leading to discovery of new drug and vaccine targets, enhanced diagnostics and insights into the complex biology underlying host-parasite interactions. For the schistosome blood flukes, with the recent completion of genome sequencing and comprehensive transcriptomic datasets, there has accumulated massive amounts of gene sequence data, for which, in the vast majority of cases, little is known about actual functions within the intact organism. In this review we attempt to bring together traditional *in vitro* cultivation approaches and recent emergent technologies of molecular genomics, transcriptomics and genetic manipulation to illustrate the considerable progress made in our understanding of trematode gene expression and function during development of the intramolluscan larval stages. Using several prominent trematode families (Schistosomatidae, Fasciolidae, Echinostomatidae), we have focused on the current status of *in vitro* larval isolation/cultivation as a source of valuable raw material supporting gene discovery efforts in model digeneans that include whole genome sequencing, transcript and protein expression profiling during larval development, and progress made in the *in vitro* manipulation of genes and their expression in larval trematodes using transgenic and RNA interference (RNAi) approaches.

Key words: Trematoda, larval stages, *Schistosoma*, *in vitro* culture, genome, transcriptome, gene manipulation, transgenesis, RNA interference.

INTRODUCTION

A well-recognized and highly conserved aspect of the life cycles of digenetic trematodes is their use of molluscs, mainly snails, as first intermediate hosts, and within which all species undergo a complex process of asexual reproduction. Infections of the molluscan host are initiated by entry of the ciliated miracidial stage where, once inside the host, they typically shed their ciliated epidermal plates, transforming to the primary sporocyst. Germinal cells within sporocysts then begin to divide forming multicellular embryos, each of which are destined to become either secondary (daughter) sporocysts or rediae (depending on the trematode species), thus constituting the next larval generation. This asexual sporocystogenic or redial-generating reproductive cycle may repeat itself several times, but eventually gives rise to the cercarial stage, which represents the next free-living stage responsible for transmitting the infection to the next host, either a second intermediate or a definitive host. Clearly the molluscan developmental phase of trematode life cycles is critically important, serving to greatly amplify the infective cercarial population that, in turn, dramatically increases the probability of successful transmission to the next host, even if the prevalence of infection within a given snail population is low (Basch, 1991).

As alluded to above, the intramolluscan phase of development is complex and, currently, little information is available regarding the underlying mechanisms regulating successful transition of the free-swimming miracidium to the parasitic primary sporocyst stage, or the biochemical and physiological requirements for establishing and maintaining an ongoing parasite infection. In general, digeneans exhibit very narrow ranges of snail host specificity, suggesting that the physiological parameters dictating larval survival may be quite different between

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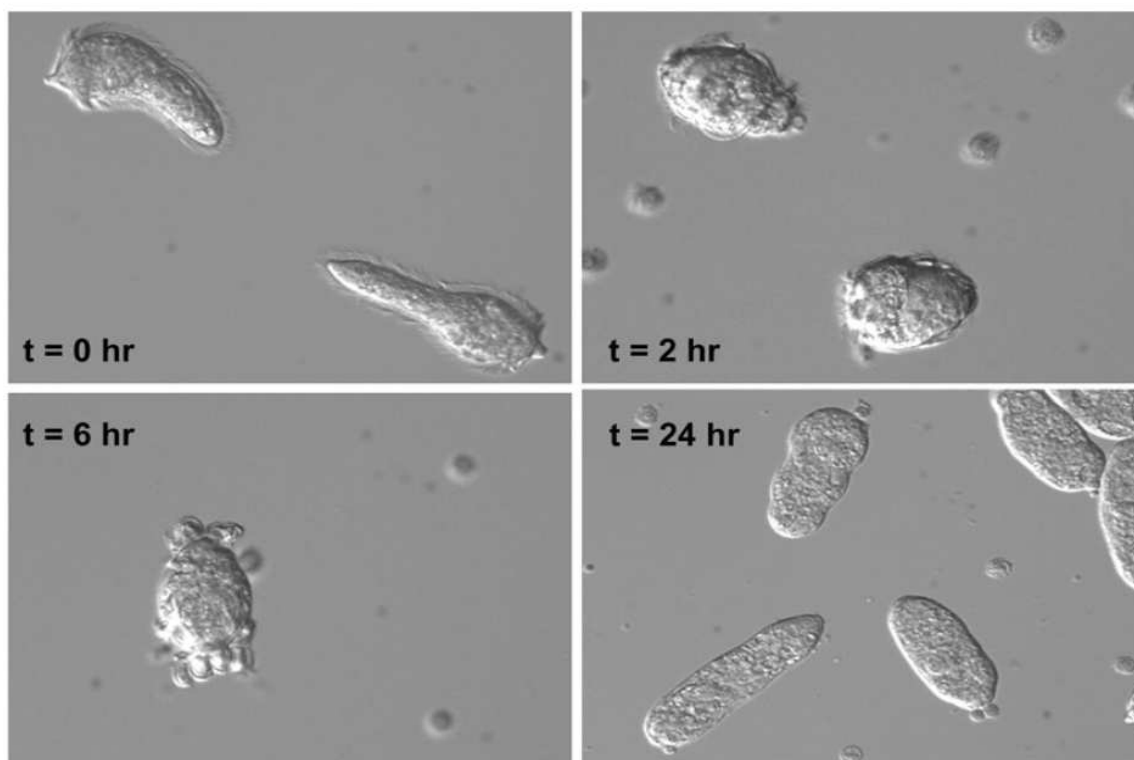

Fig. 1. *Schistosoma mansoni* miracidia (t=0 h) as they undergo morphological transformation to the primary sporocyst stage (t=24 h). Miracidia, cultured in isotonic snail saline (Chernin's balanced salt solution, CBSS; Chernin, 1963), first cease swimming, assume a rounded shape (t=2 h) as expansion of intercellular ridges force ciliated epidermal plates to round up and detach from the larval surface (t=6 h). 't' indicate the approximate time elapsed from initial introduction of miracidia into culture.

parasite species or their hosts. In the present review we attempt to summarize a growing body of research that focuses on the *in vitro* manipulation of early intramolluscan trematode stages, emphasizing the blood flukes *Schistosoma* spp. Topics include the current status of *in vitro* culture as an investigative tool, gene discovery in model digeneans, gene and protein expression profiling during *in vitro* larval development, and the development and application of larval transgenesis and RNA interference (RNAi) as functional genomic complements to gene discovery efforts. The results of these studies not only give important insights into fundamental questions about how host specificity is regulated and/or what physiological factors dictate infection success, but also provide a basis for the identification and possible targeting of critical molecular or biochemical pathways for disruption by chemical or biological interventions leading to termination of infections within the molluscan host and thus, transmission to humans or domestic animals.

In vitro *cultivation – essential tool for gene profiling and functional genomic investigations*

Earlier studies on the human blood flukes, *Schistosoma mansoni* (Yoshino and Laursen, 1995) and *S. japonicum* (Coustau *et al.* 1997; Coustau and Yoshino, 2000), revealed that exposure of freshly-hatched miracidia to snail isotonic saline alone, e.g. Chernin's balanced salt solution (CBSS; Chernin, 1963) would trigger the shedding of ciliated miracidial epidermal plates, and the concomitant formation of the primary (=mother) sporocyst tegument under *in vitro* conditions (Fig. 1). Thus, it appeared that the change in environmental osmolarity (from ~10 mOs/L in freshwater to ~140 mOs/L in snail saline=osmolarity of snail hemolymph) was sufficient to mimic the initial penetration and early larval development in the snail intermediate host. Other trematode species including echinostomes (*Echinostoma paraensei, E. caproni*) (Loker *et al.* 1992; Ataev *et al.* 1998), and the common liver fluke *Fasciola hepatica* (Gourbal *et al.* 2008) also appear to share in part this osmotic 'transformation signal', although under *in vitro* conditions, transformation may not involve the release or 'shedding' of ciliated epidermal plates typical of *S. mansoni* and *S. japonicum* miracidia. In fact, *E. caproni* cultivated *in vitro* for 24 h or more in serum-free medium or snail cell-conditioned medium, although ceasing swimming activity and losing some cilia from epidermal plates (Ataev *et al.* 1998), do not 'shed' their plates. Thus, it appears that the physico-chemical cues required by *E. caproni*, and possibly other *Echinostoma* spp. or fasciolids, for fully transforming to sporocysts

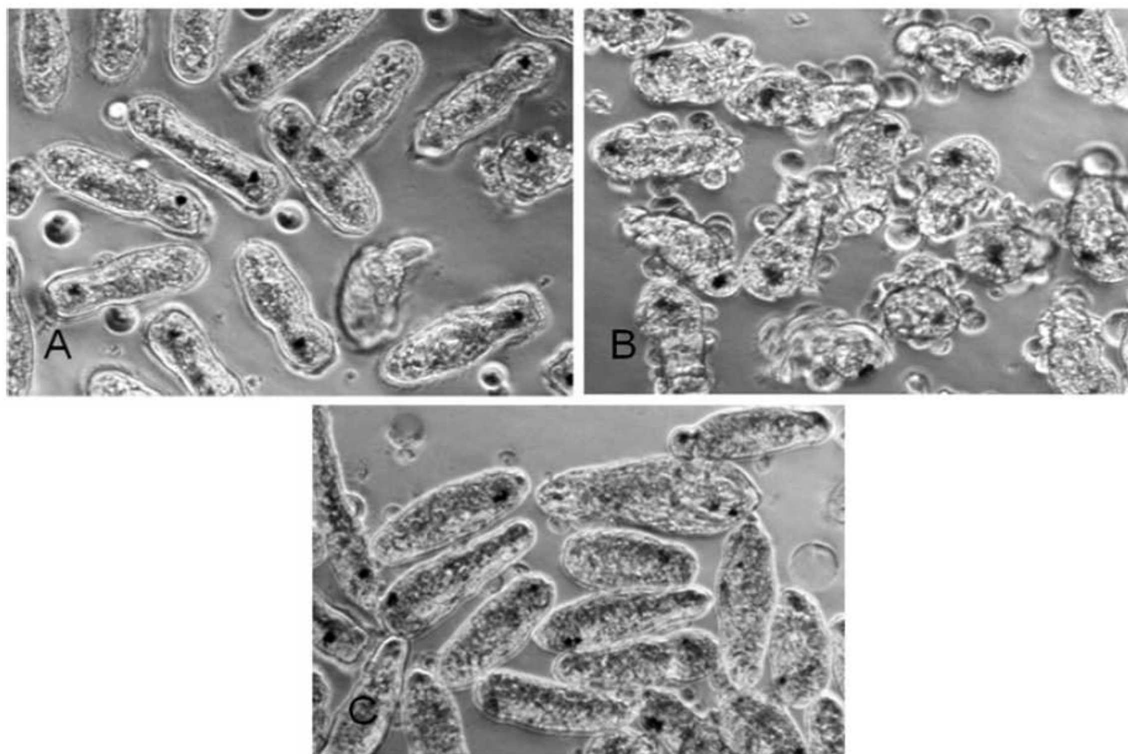

Fig. 2. *In vitro* cultivation of *Fascioloides magna* (Fasiciolidae) miracidia in untreated CBSS (Chernin's balanced salt solution; Chernin, 1963). Within 24 h, most miracidia cease swimming activity but the majority will not fully transform (i.e. will retain their ciliated epidermal plates (A). However, miracidia cultured in CBSS, pre-conditioned by cultivation with snail *Biomphalaria glabrata* embryonic (Bge) cells, are induced to transform (rounding and shedding of ciliated epidermal plates (B) to become fully-transformed primary sporocysts (C).

in vitro differ from the 'simple' osmotic cues of schistosomes.

The deer liver fluke *Fascioloides magna* provides an informative case to illustrate the complexities of miracidial transformation signals. Miracidia of *F. magna*, like *E. caproni*, do not spontaneously transform to primary sporocysts when incubated in snail ringers (CBSS) or serum-containing medium (Laursen and Yoshino, 1999). Although miracidia stop swimming within a few hours in culture, ciliated plates remained firmly attached to the majority of larvae at 24 h post-cultivation (Fig. 2A). However, when *F. magna* miracidia were cultured in CBSS that had been preconditioned by incubation with the *Biomphalaria glabrata* embryonic (Bge) cell line, miracidium-to-sporocyst transformation was readily induced (Fig. 2B, C). Moreover, treatment of the Bge cell-conditioned medium with heat (56 °C for 30 min; 100 °C for 10 min) or proteinase K significantly reduced the transformation-stimulating activity of the conditioned medium (Laursen and Yoshino, 1999). Based on these findings it appears that *F. magna* miracidia require an additional signal(s), likely a protein or protein-associated factor, from the snail host in addition to or in place of an osmotic signal in order to trigger transformation. Supporting these findings, Campbell and Todd (1955) reported that *F. magna* miracidial transformation *in vitro* (i.e. shedding of epidermal plates) only took place after miracidia had made physical contact with the snail, attempting to penetrate but failing, implying the requirement of a snail host molecular signal to initiate this process.

Clearly, the signals involved in early miracidium-to-sporocyst development are complex and undoubtedly differ between trematode groups. However, *in vitro* culture systems, while not mimicking precisely the environment within the natural host, do provide a means of tracking changes in gene expression that accompany early larval development that may provide critical insights into the role of specific genes or gene families in the establishment and maintenance of infections within the snail host. This is particularly true for *in vitro* systems capable of supporting transitions to the first intramolluscan parasitic stage (primary or mother sporocyst) and, in the case of schistosome species and *F. magna*, advanced development to subsequent daughter sporocyst (Yoshino and Laursen, 1995; Coustau *et al.* 1997; Ivanchenko *et al.* 1999) or redial (Laursen and Yoshino, 1999) stages. With the rapid expansion of gene/protein sequence databases for schistosomes and other trematode species, the incorporation of *in vitro* culture systems into experimental designs represents an invaluable tool for evaluating molecular changes linked to parasite development or for exploring gene function through manipulating transcript expression.

GENOMICS, TRANSCRIPTOMICS, AND PROTEOMICS OF LARVAL TREMATODES – SO MUCH DATA, SO LITTLE INFORMATION

Because of the recognized global importance of schistosomiasis as a significant public health concern, it is not surprising that the majority of molecular research has focused on *Schistosoma* spp.. In part due to the limited success of traditional methods in controlling schistosomiasis, the scientific community joined efforts in 1993 to begin generating genomic sequence data from *Schistosoma mansoni* (Franco *et al.* 1995) with funding support from the WHO/TDR (UNICEF-UNDOWorld Bank-WHO Special Programme for Research and Training in Tropical Diseases) (Oliveira *et al.* 2008). It was envisioned at the time that the genomic data generated would promote the acceleration of gene identification strategies and data analysis, which in turn could then be translated into new tools for fighting infection and disease including discovery of new drug and vaccine targets, improved diagnostics and clinical management approaches, and, importantly, tools for dissecting the basic biological underpinning of the complex host-parasite interactions (Franco *et al.* 2000; El-Sayed *et al.* 2004; Wilson *et al.* 2006; Brindley and Pearce, 2007; Hokke *et al.* 2007).

By means of whole-genome shotgun sequencing approaches the entire assembled genomes for *S. mansoni* and *S. japonicum* recently have been completed, revealing estimates of genome sizes of approximately 363 MB for *S. mansoni* and 397 MB per haploid genome for *S. japonicum*, and for both species, ~13 000 expressed transcripts, encoding ~11 000 genes (Haas *et al.* 2007; Berriman *et al.* 2009; The *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009). Genes are inserted into 7 pairs of autosomes and one pair of sex chromosomes (female = ZW, male = ZZ), each chromosome ranging in size from 18 to 73 MB (Simpson *et al.* 1982; LoVerde *et al.* 2004; http://lifecenter.sgst.cn/schistosoma/en/genomeProject.do; www.chgc.sh.cn/japonicum). Moreover, several transcriptomic analyses have been reported for *S. mansoni* (Franco *et al.* 1995, 1997; Santos *et al.* 1999; Verjovski-Almeida *et al.* 2003; Oliveira *et al.* 2004), and *S. japonicum* (Hu *et al.* 2003; Peng *et al.* 2003; McManus *et al.* 2004), contributing to a total of 205 892 expressed sequence tags (ESTs) from *S. mansoni* and 103 725 for *S. japonicum* in the dbEST database (dbEST; release 071009 – July 10, 2009), the vast majority of which are still unannotated. Given that schistosomes, like all other digenean species, exhibit complex life cycles involving multiple developmental stages, it is expected that at least 1000 genes would be uniquely expressed in a stage-specific manner (Wilson *et al.* 2006), although this estimate has yet to be experimentally verified. In terms of identifying genes expressed in the intramolluscan larval stages, transcriptomic analysis of *S. mansoni* has produced the most comprehensive datasets on gene expression in which 45 367 of 163 000 open-reading frame ESTs (ORESTs) were derived from miracidia, sporocysts (germballs) and cercariae (Verjovski-Almeida *et al.* 2003). For *S. japonicum*, 84 499 total ESTs were generated, but unfortunately only 569 ESTs were derived from miracidia (433) and cercariae (136), with the rest originating from non-molluscan parasite stages including adult worms and eggs (Peng *et al.* 2003; Liu *et al.* 2006).

With the exception of *S. mansoni* and S. *japonicum*, the accumulated genomic and transcript sequence data on other schistosome species or non-schistosome flukes are sparce indeed. For the third major human-infecting species of blood fluke, *S. haematobium*, dbEST lists only 6 ESTs, while GenBank entries (NCBI) total 127, with 21 sequences from cercariae and the rest from adult worms or unknown sources. *Fasciola hepatica* (common liver fluke) and *F. gigantica* (intestinal fluke), causative agents of fascioliasis in sheep and cattle (occasionally humans), are distributed worldwide and represent significant sources of economic losses in the livestock industry (McManus and Dalton, 2006). To date, there are only 324 and 149 nucleotide sequences in the NCBI gene database and 242 and 91 protein sequence entries in the NCBI protein database for *F. hepatica* and *F. gigantica*, respectively, and most of these originated from adult worm sources. Another model trematode that has received considerable attention are the echinostomes, which currently lists ~400 ESTs for *Echinostoma* spp. in the NCBI dbEST. Most of these are from the miracidium and primary sporocyst, but very few have been annotated.

Although there is an impressive amount of gene sequence data for schistosomes and databases for other non-schistosome species continue to grow, those genes specifically expressed during larval stage development, with the exception of cercariae, have been largely under-represented or unidentified in large genome or transcriptome investigations compared to adult worm-, schistosomula- and egg-derived sequences (Verjovski-Almeida *et al.* 2003; Peng *et al.* 2003; Liu *et al.* 2006). Bioinformatic searches of the NCBI's UNIGENE database revealed 10 219 and 9395 sequence clusters for *S. mansoni* and *S. japonicum*, respectively. Of total *S. mansoni* clusters, 3392 contain identified miracidial sequences, 3128 sporocyst cDNA sequences, and 2206 cercarial sequences, although many of the raw larval sequences are unknown/unannotated. For *S. japonicum*, only 352 and 137 sequence clusters contain cDNA sequences (transcripts) for miracidia and cercariae, respectively, and none for sporocysts (last update in the Unigene database 09/04/2008). Similarly few intramolluscan larval sequences are

specifically identified from other non-schistosome trematode species.

Despite the considerable progress made in completing the sequencing and assembly of the *S. mansoni* and *S. japonicum* genomes, gene identification and annotation are far from complete. During annotation of the *S. mansoni* and *S. japonicum* transcriptomes approximately 55% and 35%, respectively (Verjovski-Almeida *et al.* 2003; Hu *et al.* 2003) of sequences shared no similarity with known genes. Given that approximately half of putative genes are classified as unknown or predict hypothetical proteins based on possession of apparent ORFs and/or conserved domains, there is a critical need for continued efforts in identifying and annotating these 'unknown' genes with the future goal of assessing their expression and functional roles throughout parasite development. Because the intramolluscan larval stages, with the exception of cercariae, have generally been neglected as specific subjects of detailed gene analyses, an updating of gene sequence identification of, and expression within, the miracidium, primary sporocysts and secondary sporocysts are needed. Another limitation of the current schistosome transcriptomes is that expression profiles represent a composite of the whole organism and not genes expressed in specific tissues/organs within these complex multi-cellular animals. Consequently, although assumed functions of certain transcripts using computational analyses may provide hints as to possible cellular localization, the actual tissue in which specific genes are expressed cannot be predicted with accuracy, rendering the data much less useful in terms of aggregated information (Dillon *et al.* 2007).

Finally, protein analyses (proteomics) of early developing larvae are just beginning and amino acid sequence data are slowly accumulating in the NCBI database. Currently there are only ~46 entries for *S. mansoni* sporocysts in the schistosome protein db, and of those, 36 are mucins (~83% of the total) reported by Roger *et al.* (2008*a*). The same pattern is observed for other larval stages like miracidia and cercariae representing all schistosome species, in which only 13 miracidial and 62 cercarial sequences have been deposited to date. In *S. japonicum*, approximately 900 protein sequences were reported in miracidia by Liu *et al.* (2006). Overall, however, protein sequence data for molluscan schistosome larval forms is highly under-represented, further emphasizing the need for continued efforts to explore the larval proteome if we are to achieve a better understanding of the biology of these and other trematode groups. As detailed below, recent proteomic analyses of *in vitro*-cultured *S. mansoni* primary sporocysts and proteins released during miracidial transformation in culture represent significant additions to the larval protein database.

Regardless of the difficulties of manipulating and analysing the vast amounts of genomic and transcriptomic data in schistosomes, the mining of these data has stimulated the conduct of many spin-off investigations centering on intramolluscan larval stages including the study of SNPs and microsatellites (Valentim *et al.* 2009), large-scale profiling of larval gene expression by microarray analysis (Vermeire *et al.* 2006) and SAGE (Williams *et al.* 2007; Taft *et al.* 2009), stage-associated proteome analyses (Curwen *et al.* 2004, 2006; Knudsen *et al.* 2005; Roger *et al.* 2008*b*; Wu *et al.* 2009) and assessments of the larval glycome (Robijn *et al.* 2005; Lehr *et al.* 2008; Peterson *et al.* 2009). An update on the status of gene and protein expression during larval trematode development using stage-specific profiling approaches is discussed below.

GENE AND PROTEIN PROFILING DURING INTRAMOLLUSCAN LARVAL DEVELOPMENT

An important first step in evaluating the functional significance of gene expression is to identify genes whose expression is associated with specific developmental stages and localize the encoded protein products within parasite cells/tissues to help infer potential biological activities or roles. For early intramolluscan developmental stages (miracidia, sporocysts, germballs, cercariae) such gene expression profiling has been accomplished using several approaches. In each of these cases, *in vitro* cultivation systems have provided a critical means of identifying stage-associated expression of various molecules, from genes to carbohydrates, which may have functional significance for that particular stage of development.

In recent years, investigations of gene and protein expression in different schistosome intramolluscan larval stages have become increasingly common as more sophisticated questions are being asked regarding the regulation of larval development, growth and asexual reproduction. A number of molecular approaches, such as cDNA subtraction (Sargent and Dawid, 1983), suppression subtractive hybridization (SSH, Diatchenko *et al.* 1996) and mRNA differential display (Liang and Pardee, 1992), have been used in other systems to compare gene expression repertoires between different populations, and have now been applied to larval trematodes (e.g. Adema *et al.* 2000; Coppin *et al.* 2002; Nowak and Loker, 2005). These techniques principally evaluate transcript presence vs. absence and as such, generally reveal little in the way of quantification of gene expression between populations under study. Now with the accumulation of robust databases of gene sequences, especially for schistosome species, high-throughput methods for both qualitative and quantitative profiling of multiple transcript expression have made it possible to evaluate 'global' gene

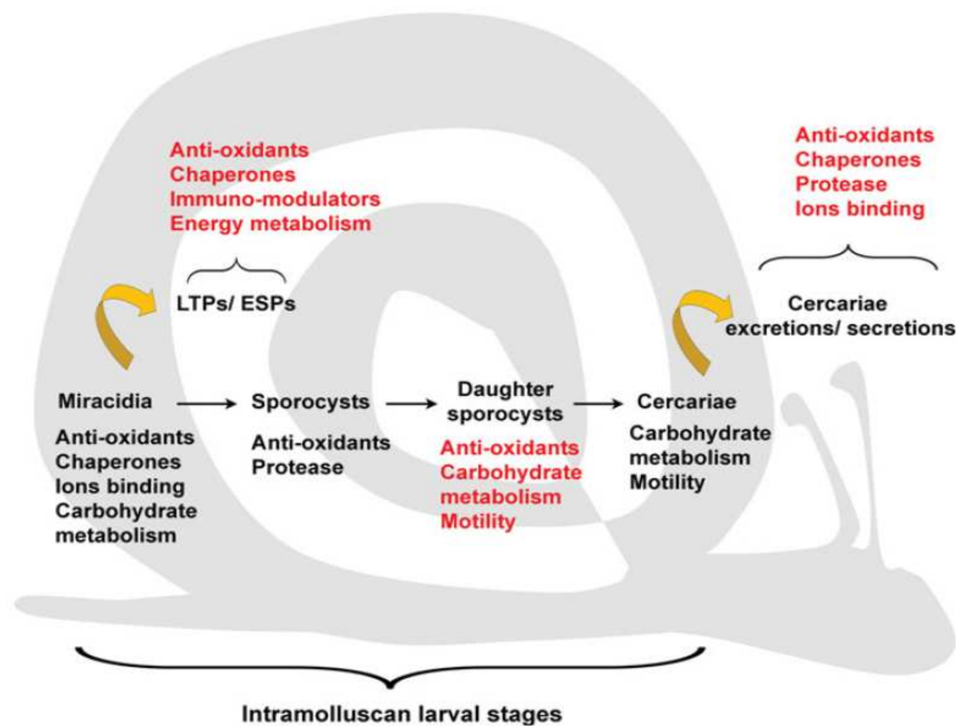

Fig. 3. Schematic representation of *Schistosoma* intramolluscan larval stages; miracidia, primary (mother) sporocysts, secondary (daughter) sporocysts and cercariae. For each larval stage, specific biological functions corresponding to predominant up-regulated transcripts (black lettering) or proteins (red lettering) are described. Functional categories of differentially-expressed excretory-secretory proteins/products (ESPs) and larval transformation proteins (LTPs) released inside the molluscan intermediate host during miracidium-to-sporocysts transformation, and cercarial ESPs release during and following emergence from the snail host are also listed (red lettering).

expression in stage-associated comparative analyses. Two such approaches, microarray analysis and serial analysis of gene expression (SAGE), illustrate how quantitative, multi-gene expression profiling can provide valuable insights into the regulation of early developing intramolluscan larval stages.

Microarray analyses

The cDNA/oligonucleotide microarray has recently served as an important tool for the rapid (high-throughput) quantitative analyses of gene expression patterns in the intramolluscan stages of schistosome development including miracidia, sporocysts and cercariae. Based on mRNA hybridization to its complementary cDNA template, two different cDNA populations (targets) are synthesized, usually with Cy-dye fluorescent-labeled nucleotides (cDNA populations labeled with different dyes), from RNA samples of interest. Recently, dye-labeled target cDNAs can be created from very small cell/tissue samples by incorporating high-fidelity amplified RNA (aRNA) (Petalidis *et al.* 2003; Tang *et al.* 2009), which has greatly facilitated gene expression comparisons between scarce or low-abundance tissues or between parasite stages. Typically, pair-wise comparisons of relative transcript abundances are made by simultaneous hybridization of labeled cDNA populations to an array of pre-selected known genes affixed or spotted to a glass slide or silicon chip, allowing for the quantification of relative gene expression patterns by detection and measurement of the intensity of the fluorescent labels hybridizing to each spotted gene. In the last several years, microarray techniques have been used to identify stage-specific expression patterns of transcripts in the different *S. mansoni* and *S. japonicum* intramolluscan larval stages including miracidia, primary sporocysts (Vermeire *et al.* 2006; Gobert *et al.* 2009), secondary sporocysts (germballs) (Jolly *et al.* 2007), cercariae (Fitzpatrick *et al.* 2005; Jolly *et al.* 2007; Gobert *et al.* 2009) and even gender-associated transcript expression pattern in *S. mansoni* cercariae (Fitzpatrick *et al.* 2008). A subset of the reported up-regulated transcripts is summarized in Supplemental Table 1, organized by sequence-derived biological functions based on gene ontology (GO) annotation. Although this table is only a qualitative compilation of gene undergoing expression changes, quantitative comparisons are presented in the referenced citations. Taken together, the changes in relative transcript levels associated with specific larval stages are interpreted to represent the parasite's response to the snail host environment, as well as its own changing needs for further growth and development. Indeed, there is a predominance of up-regulated transcripts related to specific biological functions for each of the intramolluscan larval stages (Fig. 3). For example

S. mansoni miracidia show enrichment in transcripts encoding proteins related to functions like refolding/chaperoning (p40, HSP40, HSP70, HSP90), energy production (triosephosphate isomerase, phosphoenolpyruvate carboxykinase PEPCK), motility and calcium binding (SME16, calcineurin and Ca binding dynein-like protein), primary sporocysts have higher gene expression levels for anti-oxidants (e.g. peroxiredoxins, GST26/28 and Cu/Zn SOD), proteases (cathepsin, elastase, preprocathepsin and haemoglobinase) and protein synthesis/degradation (40 S and L37a ribosomal proteins, ubiquitins), while cercariae are enriched in proteins associated with energy production (ATP-synthase, lipid-binding protein, NADH dehydrogenase 4/5/7, ATPase subunit 6) and motility transcripts (tubulin, actin, myosin light and heavy chain).

These latter findings of higher transcript levels involving motility and energy production transcripts in both schistosome miracidia and cercariae were not unexpected considering that both are highly motile with accompanying high energy requirements. Similarly, although anti-oxidant transcripts are observed throughout the different intramolluscan larval stages, their expression is higher in miracidia and both primary and secondary sporocysts, as these are the first larval stages to confront a potentially hostile oxidative haemolymph environment of the snail host (Bender *et al.* 2002), as well as possible encounter with the snail's immune defence system. In response to host reactive oxygen species (ROS), elevated anti-oxidant levels seen in early larvae suggest a potential role in protection against oxidative stress generated by the snail host, in addition to the need for larvae to maintain their own internal redox balance (Zelck and Von Janowsky, 2004; Vermeire and Yoshino, 2007). A similar transcript pattern is observed for Sm16, an anti-inflammatory glycoprotein (Ramaswamy *et al.* 1995), previously found in the cercarial secretory glands and believed to be involved in penetration into the mammalian host, as well as induction of apoptosis in responding immune cells (Curwen *et al.* 2006). Elevated steady-state Sj16 transcript levels in *S. japonicum* miracidia and sporocysts might suggest a similar role for this protein in host entry and/or defence against snail immune cells (Gobert *et al.* 2009). Sporocyst stages also appear to be significantly up-regulated in protease transcripts (cathepsin, elastase) and ribosomal genes. Proteases are believed to play an important role in the degrading of host tissue proteins, while elevated ribosomal proteins are indicative of high protein synthetic activity expected in these rapidly growing larval stages. These first multigene transcriptome analyses of different *S. mansoni* and *S. japonicum* intramolluscan stages provide valuable information supporting numerous hypotheses of the involvement of selected genes or gene families in parasite development, growth and survival within the snail host.

Gene expression in schistosome cercariae, although representing the 'end-product' of intramolluscan development, is of particular interest because sex-differentiation is presumed to occur during cercarial development within daughter sporocysts. Fitzpatrick *et al.* (2008) used a microarray referenced with genomic DNA to attempt a gender characterization of morphologically identical *S. mansoni* cercariae. By analyzing specific transcripts known to be gender-related, these authors successfully established specific transcriptional patterns distinguishing 'male' and 'female' cercariae. As would be expected, transcripts related to egg biology or to male germ cell development and reproduction were highly expressed in female and male cercariae, respectively. Moreover, genes encoding proteins involved in glucose transport, protein degradation (proteases) and immune response modulation dominate expression in 'male' cercariae, supporting the existing hypothesis that males are more capable when it comes to infection and survival within the mammalian host. The microarrays performed in *S. mansoni* and *S. japonicum* allowed the discovery of distinct patterns of gene expression that are assumed to reflect major biological functions for each of the specific intramolluscan larval stages. This suggests that regulation of transcriptional activity is highly dependent, not only on preset genetic programmes (e.g. those associated with sex-determination and activity), but certainly on the environment presented by the host. Microarray analyses allow a rapid, quantitative and simultaneous assessment of multiple gene expression in different populations at a specific developmental time points, or parasites maintained under different experimental conditions. However, one of the drawbacks of this technique is that construction of microarrays or the gene 'chips' themselves require prior knowledge of and sequence information for the genes of interest, thus limiting the study to the spectrum of genes for which sequence information is known.

Serial analysis of gene expression (SAGE)

An alternative to microarray analysis as an expression profiling tool is SAGE or serial analysis of gene expression (Velculescu *et al.* 1995). Unlike microarray, SAGE can be performed without prior pre-selection of specific transcripts, and allows for greater flexibility and potential transcripts coverage in its quantitative analysis of expressed transcripts. SAGE is based on similar principles as EST sequencing, with the additional notion that short tags (10–14 mers) of specific locations are sufficient to identify a specific transcript. In LongSAGE methods, tags are usually ~21 nt in length, a sequence length that is predicted through theoretical modelling to have a >99·8% chance of matching only once in a genome the size of humans (Saha *et al.* 2002).

To create SAGE libraries, copies of cDNA are synthesized from target mRNA previously trapped on polyT-coated beads. Once synthesized, bead-immobilized cDNAs undergo a series of specific digestions, resulting in short nucleotide sequences (tags) that, in turn, are bound end-to-end, generating concatemers of multiple tags that are finally cloned and sequenced. Given the availability of the complete transcriptome of the organism of interest, this analysis permits the identification of expressed transcripts (genes), and by enumerating the SAGE tags for a given transcript, a quantitative comparison of specific gene expression between multiple sample libraries also can be ascertained (Williams *et al.* 2007). Verification that differentially expressed tags in fact represent differential expression of the genes of interest can be determined by real-time q-PCR or semi-quantitative reverse transcriptase PCR. In addition, SAGE allows for the identification of antisense transcripts, which have been hypothesized to function in post-transcriptional gene regulation in vertebrate cells (Luther *et al.* 1998; Hastings *et al.* 2000). The inverse-proportionality of sense-to-antisense tag counts in larval *S. mansoni*, and overall high representation of antisense tags (35%) (Taft *et al.* 2009) is consistent with high anti-sense transcript content reported in other parasites (Patankar *et al.* 2001; Radke *et al.* 2005), and suggests a potentially novel mechanism for post-translational gene regulation in larval schistosomes (Gunasekera *et al.* 2004).

To date, only two LongSAGE analyses in *S. mansoni* have included intramolluscan larval stages; the first, by Williams *et al.* (2007), produced an average of 60 000 tags from each of the 10 stage-specific SAGE libraries, and more recently, a detailed follow-up analysis of 21 440 unique SAGE tags from 5 intramolluscan larval libraries reported by Taft *et al.* (2009). In both studies, 5 groups of *S. mansoni* larvae were analyzed; 1 miracidial library, and 4 different primary sporocyst libraries from larvae maintained in *in vitro* culture for 6 or 20 days (representing early and late developing primary sporocysts, respectively) in 2 separate media supplemented with or without *Biomphalaria glabrata* embryonic (Bge) cell pre-conditioned medium. Larval cultivation in the presence/absence of conditioned Bge cell medium was an attempt to mimic the snail's internal environment, and any resulting alterations in the parasite transcriptomic response. Although both SAGE studies identified numerous relevant transcripts specifically-associated with miracidial and sporocyst stages, the resulting transcript repertoire identified proteins that were highly consistent with previous microarray findings: namely a predominance of proteins/enzymes related to antioxidants, chaperones, egg antigens, carbohydrate metabolism and calcium-binding functions in miracidia, and anti-oxidant-, protease-, and ribosomal protein (protein synthesis)-related transcripts highly expressed in sporocysts. In general, this set of transcripts exhibiting enhanced expression appears to reflect parasite stages that are adjusting to abrupt physiological changes associated with the transition from a free-living to parasitic existence. By the same token, elevated steady-state transcript levels seen in sporocysts at 6 and 20 days of culture also were rich in growth and development-related genes, especially those involved in protein synthesis/metabolism (60 S acidic ribosomal protein P, 40 S ribosomal protein S19, elongation factor 1α, Golgi membrane protein, ubiquitin, HSP90 chaparonin) and energy production (ATP synthase B, ATPase) (Taft *et al.* 2009). Although not providing information as to the precise roles being played by the specific genes identified in these profiles, these studies can give an excellent impression of the major types of activities that are presumed to be important to larval stages at a particular point in their development.

Proteomic analyses

More information regarding the identity, functionality and origins of intramolluscan larval proteins would permit a better understanding of important physiological processes, in particular those associated with parasite defence and potential immune evasion mechanisms. A series of proteomic studies was recently performed focusing on: (1) miracidium-to-sporocyst transformation excretory-secretory (ES) proteins (Guillou *et al.* 2007; Gourbal *et al.* 2008), also termed larval transformation proteins (Wu *et al.* 2009); (2) protein differential expression between compatible and incompatible *S. mansoni* sporocysts (Roger *et al.* 2008*a*, *b*) and (3) proteins contained within or released by schistosome cercariae (Knudsen *et al.* 2005; Liu *et al.* 2006; Yang *et al.* 2009).

Using mass spectrometry (MS) techniques, recent studies have focused on identifying proteins released/secreted during *in vitro* miracidium-to-sporocyst transformation in several trematode species: *S. mansoni* (Guillou *et al.* 2007; Wu *et al.* 2009), *E. caproni* (Guillou *et al.* 2007) and *F. hepatica* (Gourbal *et al.* 2008). In their proteomic study, Wu *et al.* (2009) suggested a change in terminology from 'excretory-secretory proteins/products' (ESPs), commonly used to characterize proteins released during larval transformation in culture medium, to 'larval transformation proteins' (LTPs) in characterizing this group of molecules. The reason for the suggested change was based on their finding that approximatively 60% of the proteins identified as 'ES' transformation proteins lacked identifiable signal peptides or characteristics of non-classically-secreted proteins (Nickel, 2005), strongly suggesting that many proteins are being released by leakage or breakdown of ciliary epidermal plates during *in vitro* miracidia-to-sporocyst transformation. Interestingly,

however, despite the fact that these studies focused on miracidia-to-sporocyst transformation in three different species and used different methods for preparing samples prior to and during MS analyses, they all identified proteins with similar functions. For example, as in the previously described transcriptomic studies, chaperones/stress proteins, anti-oxidants, and calcium-binding proteins were well represented in the proteome of these digenean species. In addition, the presence of various proteases and protease inhibitors are indicative of active digestive/nutritional processes or, combined with the presence of anti-oxidants and immune modulators, suggest LTP involvement in the protection of the parasite against ROS generated from plasma or circulating immune cells (haemocytes). The presence of functional antioxidant enzymes and their activity (Vermeire and Yoshino, 2007; Wu *et al.* 2009) and proteases/protease inhibitors that are shared between invading miracidia and the snail host suggest at least two types of LTP-mediated functions; (1) aggressive counter-measures to the host's hostile environment or haemocyte responses or (2) more passive mechanisms whereby the parasite avoids host recognition and immune elimination by disrupting immune recognition or mimicking host molecular structures (Guillou *et al.* 2007; Wu *et al.* 2009).

Recent studies by Roger and colleagues (2008*a*, *b*, *c*), further support a passive interference hypothesis of host-parasite compatibility, as they identified differentially expressed sporocyst proteins between incompatible (IC, Guadeloupian) and compatible (C, Brazilian) *S. mansoni* strains. After clearly showing an antigenic variation between C and IC larvae, their differential proteomic study revealed an increase of a few proteins in C larval strain and the presence of 9 structurally-related, but highly polymorphic mucin proteins (Sm poMuc), differentially expressed between the 2 strains. Mucin-like proteins are believed to be involved in host-parasite interaction in various helminth species (Theodoropoulos *et al.* 2001), and are speculated to function as a potential smoke-screen or decoy against host defence (Roger *et al.* 2008*c*). In addition to differential expression poMuc in C and IC larvae, their presence in apical glands and in LTPs further support a potential protective role of these proteins in the intramolluscan stages of *S. mansoni*. As suggested earlier by Yoshino and Boswell (1986), the sharing of schistosome-specific glycotopes (Lehr *et al.* 2008; Peterson *et al.* 2009) also represents another type of mimetic mechanism in which larval stages express host-like molecules, thus avoiding recognition.

Acetabular gland contents and ESPs from *S. mansoni* cercariae and LTPs from transforming miracidia also exhibit similar expression patterns, with proteins belonging to the same predicted functional groups described previously (Appendix A; Supplementary Table 1). However, a few proteins appeared specific to cercarial ES, such as elastase, the 20·8/21·7 kDa Ca-binding proteins and HSP60, whereas *S. mansoni* LTPs were found strongly enriched in a variety of venom allergen-like proteins, suspected of being potentially involved in immunomodulation and larval infection. Both miracidial LTPs and cercarial ES proteins (released upon simulated invasion) clearly show a similar pattern in functionality, which can be explained by the fact that these two larval stages are responsible for active penetration and host entry, migration/movement within host tissues, maintaining energy production, and aggressive counter-measures against oxidative stress and immune reactivity. A similar case can be made for *S. japonicum* carcariae, in which UV treatment-induced abrogation of proteins involved in motility, energy metabolism and protein transport (chaparones) were shown to be important for parasite infection and survival (Yang *et al.* 2009). Indeed as shown by Liu *et al.* (2006), like *S. mansoni*, cercariae and miracidia of *S. japonicum* share a common proteome when it comes to expression of proteins involved in host entry (calpain, calreticulin), motility (α- and β-tubulins, dynein light chain, myosin heavy chain, actin), energy metabolism (ATP synthase, ADP/ATP carrier protein, MDH, GAPDH, PepCK, fructose bisphosphate aldolase) and stress responses (several HSP homologues, thioredoxin peroxidase).

Although cercarial ES proteins are believed to potentially originate from the secretory glands and cytosolic component of the tegument (Knudsen *et al.* 2005), the origins of miracidial LTPs have only recently been explored. Polyclonal antibodies generated against *S. mansoni* LTPs and poMuc in two different studies (Wu *et al.* 2009; Roger *et al.* 2008*b*, respectively), revealed differential immunolocalization patterns. Anti-LTPs antibodies showed no specific vesicle or gland cross-reactivity, but strong reactivity to ciliated epidermal plates of miracida and the sporocyst tegument. This supports the hypothesis that, similar to cercarial ES proteins, a subset of miracidial LTPs probably originate from the tegument. Anti-epidermal plate reactivity likely is a result of proteins released into LTP from plates undergoing lysis following larval detachment. Antibodies against poMuc, however, immunolocalized to miracidial and sporocyst apical glands, which are believed to be involved in the production of secretions essential to snail host invasion. Regardless of their specific function or localization, both the miracidium and the cercaria release an impressive variety of proteins whose main functions are likely targeted larval survival, defence, and/or development.

Over the past decade, there has been an impressive collection and archiving of gene and protein sequence data focused mainly on two schistosome species, *S. mansoni* and *S. japonicum*. Due to our current ability to culture the early intramolluscan stages of these digeneans we can now qualitatively

and/or quantitatively assess protein and transcript patterns across the intramolluscan larval stages, and as a result, are beginning to have a glimpse of the complex mechanisms used by trematode larvae during invasion, survival, and development within its intermediate host. With the next generation 454 pyrosequencing technology (Nordstrom *et al.* 2001; Vera *et al.* 2008) it is now possible to simultaneously evaluate multi-transcriptomic samples both qualitatively (gene discovery) and quantitatively (gene expression), and with the completion of genome sequencing, assembly and annotation of both *S. mansoni* and its snail intermediate host, *Biomphalaria*, ultra-sequencing using pyrosequencing methodologies will provide valuable insights into snail host-larval gene interactions ('interactome') (Barakat *et al.* 2009). Despite the creation of these ever-growing gene/protein databases, a full understanding of the functional role played by identified genes and gene products ultimately will depend on our ability to manipulate their expression within the living, intact parasite, and this represents the next critical challenge facing molecular helminthology investigators.

IN VITRO MANIPULATION OF GENE EXPRESSION AND FUNCTION IN INTRAMOLLUSCAN LARVAL STAGES

As amply demonstrated above, gene and protein discovery efforts have resulted in an ever-growing accumulation of molecular and biochemical data cataloguing numerous genes/gene products and their expression in various digenetic trematodes species, most notably the schistosomes, fasciolids and echinostomes. Moreover, for two of the major human fluke pathogens, *S. mansoni* and *S. japonicum*, life cycle stage-associated gene and protein expression profiling provides important clues as to the potential involvement of identified proteins or putative protein homologues in the myriad of molecular activities driving the development and/or maintenance of a given developmental stage. Unfortunately, this kind of information provides only limited understanding or insight into the actual functional role(s) of specifically-expressed genes or their protein products within the intact organism. Thus, effective approaches for predictably manipulating endogenous expression of specific genes within trematodes still represent a formidable barrier to exploring gene functions as they relate to the biology of these complex, multi-stage parasites and their interactions with their intermediate and definitive hosts.

Gene manipulation in Trematoda: development of methodologies

Methodologies for manipulating genes and their expression in flukes are still in its infancy, although significant progress is being made in the areas of transgenesis (i.e. introduction and expression of heterologous or homologous gene constructs using DNA plasmid vectors, DNA transposons, retrotransposons or replication-defective retroviruses) and RNA interference (RNAi). The work involving trematode transgenesis has focused almost exclusively on the schistosome blood flukes and has emphasized both snail and mammalian host stages of development as experimental models. Likewise, functional gene studies using RNAi also have emphasized the schistosomes, with a focus mainly on the mammalian stages of infection. Recent excellent reviews have summarized in detail the technologies underlying the construction, delivery and expression analyses of the variety of gene constructs used in demonstrating transient transfection and viral transformation within the *Schistosoma* spp. (Grevelding, 2006; Beckmann *et al.* 2007; Brindley and Pearce, 2007; Kalinna and Brindley, 2007; Mann *et al.* 2008; Han *et al.* 2009), and gene-specific knockdown in flukes by RNAi (Skelly, 2006; Geldhof *et al.* 2007; Kalinna and Brindley, 2007; Pearce and Freitas, 2008). In keeping with the theme of this review, the following discussion will focus on how these technologies have been applied specifically to evaluating gene function in the intramolluscan developmental stages of trematodes and the critical role being played by *in vitro* culture systems in the manipulation of specific larval genes.

Manipulation of gene expression in larval trematodes

Methods for the *in vitro* cultivation of intramolluscan larval stages, from the free-living miracidium to multi-generation sporocysts or rediae, have now evolved to the point where specific larval stages can be produced *in vitro* for use as potential targets of genetic manipulation, making it possible to begin addressing the role of specific genes during larval development. As pointed out previously, an added feature of transgenic approaches (silencing or over-expressing genes of interest) in the miracidium or sporocyst stages is the likelihood of effecting changes within embryonic germ cells, which then have the potential of being passed on to progeny (assuming expression is not deleterious) through the normal asexual reproductive process. Live, genetically-transformed miracidia or sporocysts carrying altered genes can then be transferred to compatible uninfected snails through natural infection or surgical transplantation, respectively. Although RNAi provides only transient knockdown effects, this method currently represents the only approach in which specific endogenous genes can be manipulated and larvae assessed for potential functional defects. Advances in double-stranded RNA delivery by transgenic approaches, as well as precautions in the set-up, execution and interpretation of findings in RNAi-type experiments also will be discussed.

Transgenesis in larval trematodes

Transgenesis, or the introduction and induction of expression of exogenous or foreign genes, has enjoyed considerable success in its application to trematode larval stages, particularly in the schistosomes. Although all stages of schistosomes to date have been subjects of attempted transfection, we will mainly focus this review on approaches involving the intramolluscan larval stages. Arguably, these stages represent the most desirable targets for transfection due to their ease of *in vitro* manipulation, the abundance of germinal cells readily accessible to transgene targeting while carrying the possibility for germline introduction and genomic integration, and finally the ability to propagate transgene-carrying progeny by natural infection or surgical transfer of transfected asexually-reproducing stages (miracidia or sporocysts) into suitable snail hosts (Kapp *et al.* 2003; Grevelding, 2006; Kalinna and Brindley, 2007).

The miracidium and *in vitro*-cultured primary sporocyst have attracted considerable attention as targets of transgenesis for reasons mentioned above. The earliest attempts to successfully deliver and express exogenous transcripts or transgenes utilized particle bombardment (biolistics) in which the gold particles coated with DNA encoding the green fluorescent protein (GFP) ORF flanked by *S. mansoni* HSP70 promoter and terminator sequences, were introduced into live *S. mansoni* sporocysts with a particle accelerator or 'gene gun' (Wippersteg *et al.* 2002*a*). The presence of GFP transcripts (by RT-PCR) in sporocyst extracts and GFP protein expression (by confocal fluorescent microscopy) provided evidence for transient larval transfection. Follow-up experiments showed that biolistic introduction of GFP flanked with promoter/termination sequences of the cysteine proteinase ER60, localized and expressed in lateral glands and cytons of the protonephridia, tissues with putative proteinase activity and known excretory-secretory function (Wippersteg *et al.* 2002*b*, 2003). These seminal studies showed the tractability of introducing and expressing foreign DNA in living sporocysts and opened up the possibility of tissue-specific targeting of transgene expression. The next important breakthrough in larval transgenesis was the demonstration that transgenes introduced into miracidia were expressed in developing sporocysts following natural infection of snails (Heyers *et al.* 2003). Biolistics was used to deliver an enhanced GFP (eGFP) construct flanked by an HSP70 promoter/terminator into *S. mansoni* miracidia, followed by infection of *B. glabrata* snails. Histological localization of gold particles near germinal cells in primary sporocysts of snails with 14-day-old infections and the finding of eGFP mRNA expression by nested PCR in infected, but not uninfected, snail tissues demonstrated the feasibility of transgene delivery and expression in miracidia, and importantly, the possibility of continuing propagation of germline-altered progeny (sporocysts, cercariae) and establishment of transgenic schistosome lines. This latter senario recently was eloquently demonstrated by Beckmann *et al.* (2007), who introduced plasmid GFP constructs driven by 1·5 kb promoter fragments of the *S. mansoni* actin gene (*Sm Act1*) into miracidia (F_0 generation) using biolistics, and detected GFP transcript expression through the F_0 and F_1 generations of the entire life cycle; F_0 miracidia $\rightarrow$ F_0 cercariae $\rightarrow$ F_0 adult worms generated in hamsters $\rightarrow$ F_1 miracidia from F_0 adults $\rightarrow$ F_1 cercariae $\rightarrow$ F_1 adults. Loss of transcript detection in F_2 stages indicated that, although clearly being carried in germline cells, the GFP transgene was not stably integrated, if at all, in the germinal cell genome (Beckmann *et al.* 2007).

A successful alternative approach for delivery and expression of foreign or altered genes in the various schistosome stages explores the use of transposons, retrotransposons and pseudo-typed retroviruses as schistosome-transducing agents (Mann *et al.* 2008). Applications to asexually developing larval stages have been limited, although work reported by Kines *et al.* (2006) appears to show promise. *In vitro*-cultured *S. mansoni* sporocysts were infected with *S. mansoni* promoter-flanked eGFP sequence incorporated into a Moloney murine leukaemia retroviral vector packaged into a non-replicative VSV-pseudotyped retrovirus. Larval exposure of the retroviral construct in polybrene resulted in infection and viral transduction of sporocysts as evidenced by localization of virions at the larval surface, integration of proviral sequences into sporocyst genomic DNA, and detection of reporter gene expression in virally-infected, cultured larvae. Whether or not retroviral transformation will prove to be a practical method for producing genetically-modified schistosome lines has yet to be determined. However, early results show considerable promise.

RNA interference as a functional genomics tool in trematodes

The post-transcriptional silencing of specific messenger RNA expression by RNA interference (RNAi) in parasitic helminths, including trematodes, is a significant advance in parasite postgenomics, as it represents the only approach to date for experimentally manipulating expression of specifically-targeted endogenous genes, thereby providing insight into putative gene function. Although there is some controversy as to whether helminth parasites, in particular parasitic nematodes, possess a fully-functional RNAi mechanism (Geldhof *et al.* 2007; Knox *et al.* 2007; Viney and Thompson, 2008), accumulating evidence for trematodes strongly supports a functional system for the regulation of mRNA expression by small interfering (si) RNA and/or microRNA

(Verjovski-Almeida *et al.* 2003; Xue *et al.* 2008; Krautz-Peterson and Skelly, 2008; Gomes *et al.* 2009). Because several recent reviews and primary reports cover the topic of RNAi applications in trematodes, mainly emphasizing the mammalian stages of schistosomes (Skelly, 2006; Geldhof *et al.* 2007; Kalinna and Brindley, 2007; Brindley and Pearce, 2007; Ndegwa *et al.* 2007; Morales *et al.* 2008; Pereira *et al.* 2008; Rinaldi *et al.* 2009; Krautz-Peterson *et al.* 2009 – in current special issue) and the liver fluke *Fasciola hepatica* (Rinaldi *et al.* 2008), we will focus our attention on its application to stages within the snail intermediate host; namely miracidia and primary sporocysts.

The possible existence of an RNAi-like mechanism in trematodes was first demonstrated in *S. mansoni*, initially in adult worms (Skelly *et al.* 2003) and then in the primary sporocyst (Boyle *et al.* 2003). In these studies, isolated cercariae and miracidia were treated *in vitro* with dsRNAs synthesized from transcripts of target genes by incubating (soaking) parasites for 6 days in dsRNA-containing media, during which time juvenile or larval stages transformed to their successive stages of development: cercariae→schistosomula and miracidia→sporocysts. These first attempts at silencing the cathepsin B gene in schistosomula (Skelly *et al.* 2003) and a glucose transporter (SGTP1) gene in primary sporocysts (Boyle *et al.* 2003) have now led to a number of follow-up studies investigating a variety of target transcripts, dsRNA/siRNA delivery systems and other experimental parameters aimed at optimizing the specificity and efficacy of gene knockdown (Skelly, 2006; Dinguirard and Yoshino, 2006; Krautz-Peterson *et al.* 2007; Ndegwa *et al.* 2007; Mourão *et al.* 2009). As a result of the convergence of technologies involving *in vitro* cultivation, stage-associated gene discovery/expression and gene manipulation through reverse-genetics approaches, we anticipate that functional genomic studies will not only continue in the schistosomes at an accelerating pace, but incorporate a diversity of other trematode species as well.

Assessing gene function in the development or activities of the miracidium of *S. mansoni* has been accomplished by introduction of gene-specific double-stranded (ds) RNA into eggs recovered from adult female worms or isolated from infected livers. Freitas *et al.* (2007) demonstrated that a *S. mansoni* Inhibin/Activin gene (*SmInAct*; member of the TGF-β signaling family) was a key regulator of miracidial embryogenesis by soaking eggs, newly deposited by cultured female worms, in *SmInAct* dsRNA and quantifying inhibition of larval development *in ovo*. A similar methodological approach was used to confirm previous pharmacological findings (Xu and Dresden, 1986) that *S. mansoni* leucine aminopeptidases (SmLAP) produced by miracidia *in ovo* were required for egg hatching. Rinaldi *et al.* (2009) exposed liver-isolated eggs to dsRNA of the two SmLAP isoforms (1 and 2) for 7 days at 37 C, after which time they were induced to hatch. Results demonstrated that egg hatching in the presence of SmLAP1 dsRNA, SmLAP2 dsRNA or both, was reduced by ~80% compared to both irrelevant dsRNA-treated and untreated control groups, and was comparable to groups treated with the LAP inhibitor bestatin. Concomitant with this hatching phenotype were significant reductions in specific LAP transcripts and enzymatic activity in dsRNA-treated eggs, indicating a clear relationship between LAP gene expression and hatching (Rinaldi *et al.* 2009). These studies not only provide a valuable method for screening potential drug targets for chemotherapeutic intervention, but also can be used for investigating the potential functions of genes involved in miracidial development, behaviour (e.g. ciliary activity, phototaxis), and larval transformation.

Application of RNAi methods to primary (mother) sporocyst stages of *Schistosoma* spp. also has taken advantage of *in vitro* culture methods that have permitted development of the miracidial to cercarial stages (Ivanchenko *et al.* 1999). As noted above, the first application of RNAi to sporocysts involved incubation (soaking) of freshly-isolated miracidia in snail ringers (CBSS) containing dsRNA and allowing them to transform to sporocysts followed by cultivation for 6 days in the presence of dsRNA (Boyle *et al.* 2003). Using dsRNA for the *S. mansoni* glucose transporter (SGTP1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), they demonstrated specific knockdown of SGTP1 transcripts upon exposure to SGTP1 dsRNA, using GAPDH dsRNA exposure as a specificity control. The gene knockdown effect was dependent on the presence of dsRNA during miracidial transformation and persisted in larvae maintained for up to 28-days in culture. Importantly, tritiated-glucose uptake by sporocysts treated with SGTP1 dsRNA, but not those exposed to GAPDH dsRNA, was significantly reduced, indicating a functional role of SGTP1, or a structurally related homologue (Skelly *et al.*, 1994), in sporocyst glucose acquisition. A second study, using essentially the same protocols as Boyle *et al.* (2003), examined the role of a sporocyst-expressed CD36-like scavenger receptor (SR) gene in modified (acetylated) low-density lipoprotein (LDL) binding to the sporocyst tegument and larval growth (Dinguirard and Yoshino, 2006). Consistent with previous findings, exposure of *S. mansoni* miracidia to SR dsRNA during *in vitro* transformation to sporocysts and incubation for 6-days, resulted in a significant decrease in SR transcripts, decreases in the prevalence and intensity of acetylated-LDL binding to sporocysts, and reduction in larval length. These examples illustrate the types of functional information that may be gained by applying

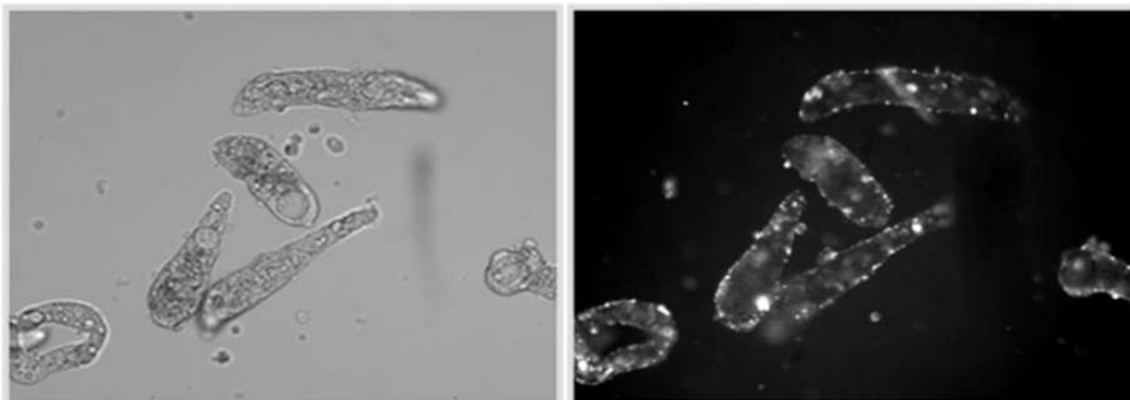

Fig. 4. Example of *Schistosoma mansoni* primary sporocysts subjected to square-wave electroporation in the presence of rhodamine-labeled double-stranded (ds)RNA. Localization of electroporated dsRNA is mainly tegumental in discrete 'patches' (right panel; epifluorescence). Intact sporocysts are shown in the left panel.

RNAi-type approaches to *in vitro* developing schistosome larvae.

Universality of gene-specific RNAi-like effects in cultured larval schistosomes

In our experience investigating dsRNA-mediated gene silencing we noted that not all genes employed in RNAi experiments yielded consistent and/or specific knockdowns. This prompted us to examine a wider range of different genes for their abilities to produce RNAi-like effects both at the transcriptional and phenotypic levels (Mourão *et al.* 2009). Briefly, *in vitro* transforming miracidia of *S. mansoni* were treated with dsRNAs (Boyle *et al.* 2003) generated from 32 genes expressed in primary sporocysts, including those encoding anti-oxidants, signaling proteins, transcription factors, metabolic enzymes and the like, and screened for morphological changes in larval phenotype. After 7 days in culture, a reduction in sporocyst size (length), similar to that reported by Dinguirard and Yoshino (2006), was the only morphological phenotype noted, and this was observed for 11 of the 32 genes examined. Of these 11 genes associated with the 'size' phenotype, 6 exhibited consistent knockdown in their steady-state transcript levels by real-time quantitative PCR, one (SOD) was highly elevated compared to the irrelevant GFP dsRNA-treatment control, while the remaining 4 phenotype-presenting genes were unchanged in transcript levels. The remaining 21 genes tested did not produce a consistent 'size' phenotype, although significant transcript knockdown was demonstrated for half of these genes (Mourão *et al.* 2009).

Results of this profiling study were enlightening as it pointed out several aspects of RNAi-type experiments incorporating larval schistosomes that require careful consideration: (1) *The choice of gene to be examined* – For reasons yet unknown transcript knockdown appears to be depend on the target gene or the specific gene segment being used to template dsRNA synthesis. Genes of interest should be pretested to ensure consistent, significant knockdown effects (Mourão *et al.* 2009). (2) *Off-target effects* – Some genes or gene segments may produce nonspecific or off-target effects that are not consistent with the predicted interaction with and/or degradation of specific target transcripts, but instead appear to affect phenotype-associated non-target genes. Off-target effects are one of the common unwanted side-effects of RNAi manipulation (Kulkarni *et al.* 2006), although steps can be taken to minimize these effects (Reynolds *et al.* 2004). (3) *Optimizing dsRNA delivery and timing* – Although we use miracidial incubation (soaking) in dsRNA for 6 days as part of our standard delivery protocol for gene knockdown in sporocysts, others have incorporated square-wave electroporation as effective alternative dsRNA delivery methods for mammalian stages of schistosomes (Correnti *et al.* 2005; Osman *et al.* 2006; Sayed *et al.* 2006; Krautz-Peterson *et al.* 2007; Morales *et al.* 2008; Zhao *et al.* 2008) and fasciolids (Rinaldi *et al.* 2008). In preliminary electroporation experiments we have found that over a range of delivered voltages (65–230 V) and capacitances (25–100 mF), 65–70% maximum sporocysts survival rates were attained at 72 h post-treatment resulting in an apparent consistency of dsRNA delivery (Fig. 4). Follow-up studies are now needed to determine if, in fact, specific gene silencing in surviving larvae is achieved by this approach and how knockdown rates and long-term larval viability compare to soaking or other delivery methods. Consistent with previous finding in schistosomula (Skelly *et al.* 2003), in general, sporocysts did not tolerate lipofection-type reagents used within recommended concentration ranges.

SUMMARY AND CONCLUDING REMARKS

With the dramatic advancements in DNA and amino acid sequencing technologies made over the last decade, combined with the powerful analytical tools

of bioinformatics, identification of genes and their products in diverse organisms, including model parasitic helminths, has become almost routine. However, it should come as no surprise that despite the rapid accumulation of gene/protein databases to date, our knowledge, and therefore understanding, of the role or function of identified genes/proteins is lagging far behind. This is especially evident in parasitic metazoans such as the digenetic trematodes, in which the entire genomes of selected species are only now completed. Although parasite expression profiling leading to the identification and activities of specific genes or gene networks can have immediate practical applications involving drug targeting or protective immune interventions (Brindley and Pearce, 2007; Pearce and Freitas, 2008; Han *et al.* 2009), experimental approaches for addressing fundamental questions of how genes may be functioning in the biology of parasites undergoing complex life cycle changes are urgently needed. As summarized in this review, part of this need is being met by the establishment of larval isolation and *in vitro* cultivation methods capable of generating and maintaining the major intramolluscan stages for trematodes of several prominent families (Schistosomatidae, Fasciolidae, Echinostomatidae), in combination with gene discovery efforts that have already yielded completed draft genomes of *S. mansoni* and *S. japonicum*, and ever-growing stage-associated transcriptomic and proteomic databases for these and other digenean species. The next major challenge for trematode biologists is to effectively combine these different approaches to investigating the functional role of expressed genes and their products in the maintenance, growth and development these parasites as they undergo morphological and physiological changes from one stage to the next. Recent exciting and promising research into gene transfer techniques (*in vitro* transgenesis and viral transduction) with the prospect of germline transfection, and reverse-genetic approaches using RNAi to manipulate endogenous gene function offer novel approaches for meeting this challenge as we truly advance into the post-genomics era.

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APPENDIX A:

Supplementary Table 1: Qualitative representation of predominant 'up-regulated' transcripts (vertical arrows) organized by specific biological functions in intramolluscan larval stages of *S. mansoni* miracidia, 4 day-sporocysts (mira and spo-4d, respectively; Vermeire *et al.* 2006), 6- and 20-day old sporocysts (spo-6d and spo-20d, respectively; Williams *et al.* 2006; Taft *et al.* 2009), secondary sporocysts and cercariae (2nd spo and cerc, respectively; Jolly *et al.* 2007), *S. japonicum* miracidia, sporocysts and cercariae (Gobert *et al.* 2009), and female (cerc-fem) and male (cerc-male) cercariae of *S. mansoni* (Fitzpatrick *et al.* 2008).

		S. mansoni						*S. japonicum*			*S. mansoni*	
Biological function	Example of proteins	mira	spo-4d	spo-6d	spo-20d	2d spo	cerc	mira	spo	cerc	cerc-female	cerc-male
Anti-oxidants	Glutathione peroxidase 1 (GPX)	↑		↑	↑							
	Peroxiredoxins (Prxs)		↑	↑	↑							
	Translationally-controlled tumor protein	↑	↑	↑	↑							
	Thioredoxin					↑				↑		
	GST26			↑	↑							
	GST28			↑	↑	↑						
	Cu/Zn SOD		↑	↑	↑							
	Sec SOD	↑										
	Mn SOD			↑								
	PRx1		↑	↑	↑							
	Prx2		↑		↑							
	Glycerol 3 phosphate dehydrogenase	↑				↑	↑					
Chaperone/heat shock protein	p40 egg antigen (HSP27)	↑										
	HSP90	↑				↑						
	HSP40/DnaJ	↑										
	HSP70/BiP	↑				↑						↑
	USP (universal stress protein-like)	↑										
	Cytochrome C copper chaperone									↑		
Carbohydrate metabolism/ energy production	Glycogen phosphorylase homologues	↑										
	Triose phosphate isomerase	↑						↑	↑	↑		
	Phosphoglucomutase	↑										
	Phosphoenol pyruvate carboxykinase	↑										
	Carboxykinase (PEPCK)	↑										
	Glycoprotein kappa-5	↑										
	ATP-synhase lipid binding protein					↑						
	High mobility group-like prot. 2					↑						
	Cytochrome b					↑						
	Cytochrome c		↑	↑	↑	↑	↑	↑	↑	↑		
	NADH dehydrogenase 4					↑↑	↑					
	NADH dehydrogenase 5/6					↑6	↑5/6			↑5		
	ATPase subunit6					↑↑	↑					
	LDH	↑										

Calcium interactive protein	SME16	↑										
	Calcineurin B	↑										
	Calreticulin		↑	↑	↑							
	Calmodulin		↑	↑	↑			↑	↑			
	8KD-Ca bp containing EF-hand domains	↑				↑						
	Calcium channel beta subunit 2	↑										
	Calcium ATPase	↑								↑		
	Calponin homologue							↑	↑	↑		
	16 Kd ca-binding protein	↑										
Protease	Cathepsin		↑C	↑	↑	↑					↑(L)	↑(L)
	Elastase		↑	↑	↑							
	Prepocathepsin		↑	↑	↑							
	Serine-enzyme							↑				↑
	Succinate dehydrogenase	↑										
	Esterase a									↑		
	Enolase					↑↑	↑					
Motility	Dynein light chain (Sj21·7 and Sm10)							↑	↑			
	Muscle glycogen phosphorylase -like	↑										
	Monocarboxylate transporter							↑				
	Plexin domain								↑			
	Pyruvate kinase								↑			
	UDP-glucose dehydrogenase								↑			
	Leu transmembrane prot 1								↑			
	Dynamin 1									↑		
	Actin bp 1									↑		
	Glutamine synthetase									↑		
	Sj16 anti inflamm							↑	↑			
	Tubulin beta chain					↑↑	↑					
	Actin-F					↑↑	↑			↑		↑
	Fibrillarin					↑↑	↑					
	Myosin light chain	↑				↑	↑					
	Myosin heavy chain					↑↑	↑					
Other	Polo-like kinase		↑	↑	↑							
	14.3.3 fatty acid binding protein		↑	↑	↑	↑						
	Stathmin-like protein spo1					↑↑	↑					
	ATP guanidokinase					↑	↑					
	Phosphofructokinase	↑										
	Endothelin B receptor									↑		
	Rho3	↑										
	Sm16 anti-inflammotory glycoprot.							↑↑	↑	↑		
	Flavohemoglobin											↑
	MGE,										↑	
	Egg secreted protein 3–6	↑									↑	
	Insulin-like peptide receptor							↑				

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