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Microarray based analysis of temperature and oxidative stress induced messenger RNA in *Schistosoma mansoni*

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Abstract

The body's defense against schistosome infection can take many forms. For example, upon developing acute schistosomiasis, patients often have fever coinciding with larval maturation, migration and early oviposition. As the infection becomes established, the parasite comes under oxidative stress generated by the host immune system. The most common treatment for schistosomiasis is the anti-helminthic drug praziquantel. Its effectiveness, however, is limited due to its inability to kill schistosomes 2 – 4 weeks post-infection. Clearly there is a need for new antischistosomal drugs. We hypothesize that gene products expressed as part of a protective response against heat and/or oxidative stress are potential therapeutic targets for future drug development. Using a 12,166 element oligonucleotide microarray to characterize *Schistosoma mansoni* genes induced by heat and oxidative stress we found that 1,878 *S. mansoni* elements were significantly induced by heat stress. These included previously reported heat-shock genes expressing homologs of HSP40, HSP70 and HSP86. One thousand and one elements were induced by oxidative stress including those expressing homologs of superoxide dismutase, glutathione peroxidase and aldehyde dehydrogenase. Seventy-two elements were common to both stressors and could potentially be exploited in the development of novel anti-schistosomal therapeutics.

Keywords

Schistosoma mansoni; temperature stress; oxidative stress; transcriptome

1. Introduction

Schistosomiasis is an intravascular infection that affects approximately 200 million people, 85% of whom live on the African continent [1]. The three species of schistosome most closely associated with the disease in humans are *Schistosoma mansoni*, *S. haematobium* and *S. japonicum*. Children are particularly prone to infection, often suffering stunted physical growth and mental development [2–4].

Upon infecting their definitive host, *S. mansoni* schistosomula migrate to the hepatic portal and mesenteric veins. Male and female worms pair about 28 to 35 days after infection, resulting in the release of an estimated 300 eggs per day [5]. Presently, the drug of choice for all forms

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of schistosomiasis is praziquantel. Its effectiveness has been shown to be biphasic, however, being unable to kill worms during the third and fourth weeks of a mouse infection [6–8]. In addition, a number of clinical and laboratory studies have suggested the emergence of praziquantel resistant parasites (reviewed by [9,10]). Although the interpretation of such studies remains controversial [11], the continued reliance on a single drug is, nonetheless, perturbing.

Once established, schistosome infections can be extremely long-lived. For example, Cross and colleagues have reported an active *S. mansoni* infection in a former Portuguese soldier 34 years after contracting the disease in Angola [12]. Similarly, Harris and colleagues reported 16 Polish patients with schistosomiasis in Western Australia, a non-endemic area, who had previously lived in refugee camps in East Africa in the early 1950's [13]. Three of these patients had been living in Western Australia for more than 31 years while 10 had been there for more than 20 years. Clearly, schistosomes have evolved very successful defense strategies to counter the host immune system.

With acute human schistosomiasis, fever may develop as soon as 2 weeks after initial infection, but this appears to have no discernable effect on worm survival [14]. In an analysis of 163,000 *S. mansoni* ESTs, Verjovski-Almeida and colleagues, were able to identify 23 assembled ESTs encoding heat-shock proteins [15]. This suggests that molecular mechanisms are present to deal with the rapid temperature transitions schistosomes must undergo throughout all stages of their lifecycle. Acute schistosomiasis is most easily identifiable in tourists and travelers and often resolves spontaneously. Chronic exposure of those living in endemic areas to schistosomes, however, generally leads to a much more severe pathology primarily associated with the parasite's eggs. An important component of the chronic anti-schistosomal response is due to reactive oxygen species (ROS), such as the superoxide radical anion. This is highly toxic to cells and is converted to H_2O_2 by superoxide dismutase [16]. H_2O_2 is also dangerous to cells as it can be converted into highly destructive hydroxyl radicals. In many species, H_2O_2 is removed from cells by catalase but this enzyme appears to be missing in *S. mansoni*. In addition to catalase, H_2O_2 can be removed from cells by glutathione and thioredoxin based systems. These pathways rely on glutathione reductase and thioredoxin reductase respectively to maintain glutathione and thioredoxin in their reduced state [17,18]. Alger and colleagues have provided strong evidence, however, that glutathione reductase and thioredoxin reductase have been replaced in *S. mansoni* by a single enzyme, thioredoxin glutathione reductase [19]. Interestingly, inhibition of this enzyme is able to kill schistosomes in culture and partially cure infected mice [20,21].

While the inhibition of thioredoxin glutathione reductase is a welcome step forward in the development of new anti-schistosomal therapeutics it is our belief that a more complete understanding of the molecular response of schistosomes to stressors such as heat and ROS will present new strategies for attacking them. In this paper we report the transcriptional response of *S. mansoni* to temperature and oxidative stress, and describe the induced transcripts that are unique to, and shared by, each stressor.

2. Materials and methods

2.1. Schistosoma mansoni

Schistosome infected mice and snails were supplied by Dr. Fred A. Lewis, NIAID Schistosomiasis Resource Center at the Biomedical Research Institute (Rockville, MD), through NIAID contract NO1-A1-30026. The abdomens of female SW mice were exposed to 125 *S. mansoni* PR-1 cercaria. Six weeks post exposure, mice were anesthetized and worms harvested by cardiac perfusion with enriched RPMI media (RPMI 1640 containing 20% fetal calf serum, 100 IU penicillin, 10 μg/mL streptomycin). Mice were subsequently euthanized

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by cervical dislocation. All animal experimentation complied with the policies, regulations and guidelines mandated by the Institutional Animal Care and Use Committee, University of New Mexico. Prior to all experiments, adult worms were allowed to recover overnight in enriched RPMI media at 37°C. This and all subsequent procedures that required worms to be maintained at 37 or 42°C (see below) were performed using a water jacketed incubator with 5% CO_2.

2.2. Stress experiments

Individual groups of 12 male and 12 female worms were placed into six Petri dishes containing 5 mL enriched RPMI media pre-warmed to 37°C. One dish was immediately harvested (0 h sample) by replacing the media with 600 μL of RLT buffer (Qiagen) containing 1 % β-ME (RLT/β-ME buffer) and homogenizing the worms (Kontes Glass Company). The homogenate was then frozen at −80°C until RNA was extracted as described below. The media in the remaining 5 dishes was replaced by 5 mL enriched RPMI media pre-warmed to 42°C and the dishes incubated at 42°C. Worms were then harvested as described above after 5, 15, 30, 60, and 240 min. A control group of 12 male and 12 female worms in 5 mL enriched RPMI media was maintained at 37°C and harvested after 4.0 h.

Oxidative stress was induced in schistosomes using H_2O_2 [22]. Individual groups of 12 male and 12 female worms were each placed into six Petri dishes containing 5 mL enriched RPMI media and H_2O_2 added to a final concentration of 100 μM. Worms were then incubated at 37°C and harvested after 0 (prior to the addition of H_2O_2), 5, 15, 30, 60, and 240 min as described above.

Each of these experiments was performed in duplicate.

2.3. Reference RNA

Six to seven weeks after infection of female SW mice, schistosomes were collected by cardiac perfusion to provide a universal reference RNA for the normalization of gene expression data in both the heat-shock and oxidative stress experiments. A universal reference RNA should provide a positive hybridization signal at as many elements on a microarray as possible [23]. To achieve this we mixed total RNA from untreated worms incubated at 37°C for 4.0 h (approximately 99% of the final amount) with that of worms incubated at 42°C for 4.0 h, along with worms treated with pathogen associated molecular patterns (PAMPs; 50 μg/mL lipopolysaccharide derived from *Escherichia coli*, 50 μg/mL peptidoglycan from *Staphylococcus aureus* and 50 μg/mL laminarin from *Laminaria digitata*) for 6.0 h at 37°C and a group treated with a sub-lethal dose of PZQ (50 μg/mL) for 0.5 h at 37°C.

2.4. RNA extraction

RNA was isolated from thawed worms using an RNeasy® Mini kit (Qiagen) according to the manufacturer’s instructions, eluted with 30 μL of RNase free distilled water (dH_2O) and quantified using a NanoDrop® ND-1000 spectrophotometer using ND-1000 3.3 software (NanoDrop Technologies). RNA integrity was evaluated using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano LabChip® Kit (Agilent Technologies).

2.5. cDNA synthesis, amplification, labeling and hybridization to microarrays

Complementary DNA was synthesized, amplified, labeled and hybridized using the modified SMART (Clontech) cDNA labeling protocol described by Petalidis and colleagues [24]. Briefly, 300 ng total RNA were mixed with 3 μL of 10 μM 3′ SMART CDS primer IIA (5′-AAGCAGTGGTATCAACGCAGAGTACT$_{30}$VN-3′) and 3 μL 10 μM template switching primer (5′-d(AAGCAGTGGTATCAACGCAGAGTACGC)r(GGG)-3′) and brought to a final volume of 15 μL with RNase free dH_2O. The template switching primer is a DNA:RNA hybrid

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where the last 3 bases are RNA. The reaction was then incubated at 72°C for 2 min and then placed on ice. Six microliters of 5× first-strand buffer (Clontech), 3 μL DTT (20 mM), 3 μL dNTPs (10 mM), and 3 μL and PowerScript™ reverse transcriptase (Clontech) were then added to the reaction and the mixture incubated at 42°C for 1.0 h to generate first strand cDNA. Second-strand cDNA was then amplified by mixing 15 μL of the first-strand cDNA reaction with 57 μL dH_2O, 10 μL 10× PCR buffer II (Applied Biosystems), 10 μL 25 mM $MgCl_2$, 2 μL 10 mM dNTPs, 4 μL 10 μM 5′ PCR primer (5′-AAGCAGTGGTATCAACGCAGAGT-3′) and 2 μl AmpliTaq® (40 U/μL). Amplification conditions were 95°C for 1 min for one cycle followed by 95°C for 5 s, 65°C for 5 s, and 68°C for 6 min for 15 cycles. Second strand cDNA was then purified using a QIAquick® PCR Purification Kit (Qiagen), quantified using a NanoDrop® ND-1000 spectrophotometer and labeled with Cy3/Cy5 d-CTPs (GE Healthcare-Amersham) using BioPrime® DNA Labeling System (Invitrogen). For labeling, 200 ng of second-strand cDNA suspended in 21 μL dH_2O was mixed with 20 μL of 2.5× random primer reaction buffer (Invitrogen) and incubated at 95°C for 5 min, then placed on ice. While on ice, 2 μL dH_2O, 5 μL low-C dNTP mix (5 mM dATP, 5 mM dGTP, 5 mM dTTP, 2 mM dCTP), 1 μL Cy3 or Cy5 dCTP and 1 μL Klenow enzyme (40 U/μL; Invitrogen) were mixed and incubated at 37°C for 2.0 h. The labeling reaction was stopped by adding 5 μL stop buffer (Invitrogen). For all experiments, reference samples were labeled with Cy3 and experimental samples with Cy5. Cy3 and Cy5 labeled probes were purified separately using an AutoSeq™G-50 Dye Terminator Removal Kit (GE Healthcare) and labeling efficiency quantified using a NanoDrop® ND-1000 spectrophotometer. Purified Cy3 and Cy5 labeled products were then pooled, ethanol precipitated, resuspended in 100 μL hybridization buffer (40 % formamide, 5× Denhardt’s, 5× SSC, 1 mM sodium pyrophosphate, 50 mM Tris (pH 7.4) and 0.1 % SDS) and incubated at 95°C for 5 min followed by 50°C for 5 min. Arrays were pre-hybridized for 3.0 h at 42°C in hybridization buffer prior to hybridization with labeled probes. Labeled cDNA was hybridized overnight at 42°C, to 7,066 *S. japonicum* and 12,166 *S. mansoni* element oligonucleotide arrays purchased from Agilent Technologies and described by Gobert and colleagues [25]. Only the *S. mansoni* elements were considered in the analyses.

2.6. Microarray scanning and analyses

Microarray slides were scanned with a GenePix® 4000B scanner (Axon Instruments) with GenePix® Pro 6.0 (Axon Instruments) software using a modified protocol as described by Aragon and colleagues [26]. A preloaded *S. mansoni* grid was used to align and identify array spots. Alignment diameter minimum and maximums were set at 50% and 200%, respectively. Nearest negative control spots were selected for background subtraction. Acuity version 4.0 (Axon Instruments) software was used in array analyses and arrays were normalized using a ratio of medians.

Microarray analysis was performed using samples derived from the 0 (untreated), 30, 60 and 240 min time points from both stress experiments. Raw expression data for each element was expressed as a ratio of the treatment (Cy5) to the common reference sample (Cy3). Expression ratios obtained for genes with the untreated samples were then subtracted from all sample ratios. Transcripts expressed ±1.0 $\log_2$, in both biological replicates, in either the 30 and 60 min, 60 and 240 min or 30, 60 and 240 min samples after this subtraction step were considered to be differentially expressed [27]. Unsupervised hierarchical clustering analyses were performed on all microarrays. All information is MIAME compliant (http://www.mged.org/Workgroups/MIAME/miame.html) and all data were submitted to Gene Express Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE11706.

The accession (TC) numbers associated with elements binding differentially expressed transcripts were used to obtain their associated sequences from the *S. mansoni* Genome Index

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(http://compbio.dfci.harvard.edu/tgi/tgipage.html). These sequences were then analyzed using the gene ontology program Blast2GO (http://www.blast2go.de/) [28] with a cut off of ≤ 1e-5. Sequences with ascribed functional annotations were further analyzed by blastx (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Elements with an E value greater than 1e-5 were considered as having no known database homolog.

2.7. Quantitative real-time PCR analysis

For quantitative real-time PCR (qRT-PCR) reactions, 250 ng of total RNA was reverse transcribed using TaqMan® Reverse Transcription Reagents (Applied Biosystems) with random hexamers as primers. Exogenous *Arabidopsis thaliana CAB* mRNA (Stratagene) was spiked into each RT reaction as a control. qRT-PCR reactions (95°C for 10 min followed by 40 cycles of 95°C for 15 s and 58°C for 1 min) were performed, in triplicate (technical replicates) for each sample from both biological replicates, using *Power* SYBR® Green PCR Master Mix (Applied Biosystems), forward and reverse primers (0.6 μM each), and a cDNA template (1–2 ng). Primer pairs were designed using Primer Express 2.0 (Applied Biosystems). Cycle threshold values for each reaction were determined using the ABI Prism 7000 SDS 1.1 software package (Applied Biosystems). The mean fold change was calculated using the $2^{-\Delta\Delta CT}$ method [29].

3. Results and discussion

3.1. Transcript induction after heat stress

S. mansoni worms, obtained 6 weeks after infection of a mouse host, were maintained at 42°C for 0.5, 1.0, and 4.0 h *in vitro* and changes in their transcriptome analyzed using an oligonucleotide microarray containing 12,166 unique *S. mansoni* elements. The significant induction of transcripts encoding 3 homologs of HSP40 (major egg antigen, TC16575), HSP70 (TC16542), and HSP86 (TC16552), known from previously published work to be induced in response to heat-shock was confirmed in this analysis [30–32]. This suggests that the treatment was successful in inducing the heat-shock stress response (Fig. 1A). HSP70 transcripts were induced significantly 30 min after the ambient temperature was raised to 42°C, while HSP40 and HSP86 transcripts were induced after 60 and 240 min respectively. At their peak, HSP40 and HSP70 transcript levels were both induced at least 40-fold and HSP86 at least 6-fold. Transcripts encoding the constitutively expressed β-tubulin (TC8581) and Ribo21 (TC17090) homologs remained stable during the period of heat-shock (Fig. 1A). In the case of HSP70, this is a significantly higher level of induction than that described by Neumann and colleagues who, using Northern blot hybridization analysis, reported a 5.1 and 7.1-fold increases in adult worm HSP70 mRNA after 3 and 6 h at 42°C respectively [32]. This paper also reported a 6.3-fold increase in HSP70 levels 6 h after the production of schistosomula by mechanical transformation in the absence of heat shock. Similarly, Chai and colleagues [33] have demonstrated using microarrays that a *S. japonicum* HSP70 homolog is induced 3.5-fold in lung schistosomula compared to adult parasites suggesting that the up-regulation of this gene may be associated with the induction of heat tolerance as the parasites had only been in the host 3 days and may still have been adjusting to the thermal shift or were reacting to host immune attack. Changes in the transcriptome of samples from untreated (0 min) worms and worms maintained at 37°C for 240 min were also analyzed. One percent of array elements were differentially regulated, well within the expected error range (data not shown). These elements were removed from our analyses.

Microarray expression data were validated for the transcripts described above using qRT-PCR. Transcript abundance was compared between control (37°C) and heat-shocked (42°C) worms (Fig. 1B). For these analyses, two additional time points (5 and 15 min post heat-shock) were included to judge how quickly the schistosomes were able to respond to the stressor. HSP70

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transcripts had greater than two-fold induction after 15 min, HSP40 after 30 min and HSP86 after 60 min, while β-tubulin and Ribo21 transcripts showed no change. The overall changes in the levels of expression of these 5 transcripts, as measured by microarray and qRT-PCR analyses, were closely matched.

The heat-shock experiment was performed on two separate occasions. There was a significant overlap of 1,878 elements when comparing the microarray elements reporting a significant induction in their bound transcript between these two biological replicates. (Fig. 2A). Gene ontology analysis of molecular function was determined for 825 of these elements (Fig. 2B). Genes with E values less than, or equal to 1e-5 were considered as having a known database homolog (See supplemental material. All supplemental material including a complete list of induced (and down-regulated) genes can be found at http://biology.unm.edu/Cunningham/Papers/SP.htm). The majority of genes were determined to be involved in 'binding' (46%), 'catalytic' (34%) and 'transporter' (7%) activities.

The 'binding' activity category, which encompasses 64% of all annotated *S. mansoni* genes, included those encoding HSP40 (TC16575), HSP70 (TC16542), HSP86 (TC16547) and an HSP70 associated DnaJ homolog (TC16732) [34,35]. Several genes encoding homologs of DNA break repair proteins were also included in this category. These proteins included MRE11A (TC11269), required for dsDNA break initiation and repair functions in yeast [36], Rad1 (TC13061) responsible for cell cycle checkpoint control in response to DNA damage and incomplete DNA replication [37] and endonuclease III-like protein 1 (TC19669) that functions in the repair of damaged DNA as a result of oxidative stress [38]. The 'catalytic' and 'transporter' categories included homologs of synovial apoptosis inhibitor (TC17764), whose expression renders cells resistant to cell death by apoptosis induced by endoplasmic reticulum (ER) related stress [39]; lactate dehydrogenase-B chain protein (TC16736) that is involved in anaerobic glycolysis and is induced in gastric mucosa of humans infected with *Helicobacter pylori* [40]; natural killer cell-enhancing factor B, which protects cells from oxidative stress [41]; double-strand DNA break repair protein Rad21 (TC8470), required for dsDNA break repair in yeast [42]; AMPactivated protein kinase (TC11310), induced in response to hypoxia, ischemia, and osmotic stress in cardiac and skeletal muscle tissue [43]; Derlin-2 (TC14077), induced in response to ER stress and ER associated degradation which is, itself, enhanced by heat-shock [44]; endoplasmic reticulum oxidoreductin-1 (TC18435), induced in response to heat, ethanol, and oxidative stressors in yeast [45]; DNA repair protein XRCC1 (TC8486), which functions in base excision repair and single-strand DNA break repair [46]; histone chaperone Asf1 (TC11916), which has a role in protecting against replicational stress [47] and P2X4 purinoceptor (TC9754) that has been shown to activate calcium ion influxes in sheer stressed endothelial cells [48].

3.2. Transcript induction after oxidative stress

Reactive oxygen species (ROS) pose a constant threat to all organisms living in an aerobic environment due to their ability to damage nucleic acids, proteins, and lipids. Such ROS are produced as a natural by-product of cellular respiration; however, schistosomes also encounter host generated oxygen radicals aimed at killing the parasite. To determine how the transcriptome of schistosomes changes in response to oxidative stress, worms were examined 30, 60 and 240 min after exposure to 100 μM H_2O_2 *in vitro*. The significant induction of 3 homologs known from previously published work to be induced by the presence of ROS was confirmed in this analysis suggesting that the treatment was successful in inducing the anti-oxidative stress response (Fig. 3A). These were extracellular superoxide dismutase (TC16777) which protects tissues against oxygen toxicity in a variety of organisms [49], glutathione peroxidase (TC10653) which possesses hydrogen-peroxide oxidoreductase activity [50] and aldehyde dehydrogenase (TC13692) which is required for detoxification of oxygen radicals

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[51]. In contrast, the expression of transcripts encoding β-tubulin (TC8581) and Ribo21 (TC17090) were unaffected. These results were confirmed by qRT-PCR (Fig. 3B) where all three anti-ROS transcripts were significantly induced after 15 min and were reproducible in both biological replicates for each of the elements examined. Interestingly, in an analysis of gender associated gene expression using a different microarray from that employed in this paper, Fitzpatrick and colleagues have demonstrated that 5 oligonucleotides representing exon regions of extracellular superoxide dismutase were highly enriched in adult female worms while 4 oligonucleotides encoding cytosolic superoxide dismutase were equally expressed between both sexes [52]. Glutathione peroxidase was also significantly enriched in female worms suggesting perhaps that this enzyme and cytosolic superoxide dismutase may play a role in detoxification of the by-products of hemoglobin digestion as females greatly increase red blood cell consumption as egg production begins. In an examination of strain- and gender-associated differences in gene expression, Moertel and colleagues [53] also found extracellular superoxide dismutase to be significantly up-regulated in Chinese strain *S. japonicum* adult female compared to adult male worms, however, this induction was absent when both sexes of Philippine strain *S. japonicum* were compared.

The oxidative stress experiment was performed on two separate occasions. A total of 1,001 microarray elements reported a significant increase in their bound transcript when both biological replicates were compared (Fig. 4A). Gene ontology was determined for 459 of these elements which fell into 25 categories (Fig. 4B). These included ‘antioxidant’, ‘transcription regulator’, and ‘response to stimulus’ activities. Genes with E values less than or equal to 1e–5 were considered as having a known database homolog (supplemental material) and some of these are discussed below. Fourteen transcripts were partitioned into the ‘antioxidant’ category. These included the superoxide dismutase, glutathione peroxidase and aldehyde dehydrogenase homologs described above, as well as L-2-hydroxyglutarate dehydrogenase (TC16490), an FAD-dependent enzyme catalyzing the oxidation of L-2-hydroxyglutarate to α-ketoglutarate [54]; ferredoxin $NADP^+$ reductase (TC8516), which is involved in the oxidative stress response in *E. coli* and *Salmonella enterica* [55] and thioredoxin glutathione reductase (TC7521), a flavoenzyme expressed by schistosomes that bridges two detoxification pathways crucial for the parasite survival in the host, and which has been suggested as a potential therapeutic target [20,21].

There were 29 transcripts categorized as active in the ‘regulation of transcription’ that increased in abundance during oxidative stress. These include transcripts encoding G protein-coupled receptor kinase type 2 (TC17610), which has been shown to be active during oxidative stress in rats [56]; nuclear receptor superfamily member TR4/TR2 [57]; retinoic acid receptor RXR (TC17352) that is a regulator of genes important for homeostasis and development [58]; an insulin receptor tyrosine kinase (TC14300) that has been shown to function during oxidative stress [59]; epidermal growth factor receptor (TC14983) that functions in cell differentiation and development [60]; a mediator of RNA polymerase II transcription (TC15461), which associates with core polymerase subunits to form the RNA polymerase II holoenzyme that is essential for transcriptional regulation [61]; the YY1 transcription factor (TC18482) that directs histone deacetylases and histone acetyltransferases for promoter regulation [62]; the SNF2 family N-terminal domain containing protein (TC9884) that is the catalytic subunit of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation [63]; a TAF13 RNA polymerase II, TATA box binding protein (TBP) (TC14686), involved in RNA polymerase II transcription initiation [64] and a transcriptional cofactor CA150 (TC7375) that regulates RNA Polymerase II elongation [65].

In the ‘response to stimulus’ category there were 51 transcripts that increased in abundance. These transcripts include those encoding DNA mismatch repair protein MSH2 (TC11333) that binds to DNA mismatches to initiate the mismatch repair process [66]; G/T mismatch-specific

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thymine DNA glycosylase (TC19118) that functions in excising thymine and uracil from G:T and G:U mismatches [67]; DNA-repair protein complementing *Xeroderma pigmentosum* group A protein (XPA) (TC11443) that functions during nucleotide excision repair [68]; a CREB-binding protein (TC13907) that modifies chromatin to an active relaxed state increasing transcription [69] and Rac GTPase, (TC7355) which has been shown to function in response to oxidative stress in fibroblast cells [70].

3.3. Transcripts induced in response to both heat and oxidative stress

Seventy-two transcripts were commonly induced in worms in response to both heat and oxidative stress. Forty-five of these had no known significant homologs. Of the remaining 27, there were several for which homologs have been previously reported to be active in stress responses (Table 1). A telomerase reverse transcriptase homolog (TC10026) is induced and translocated from the nucleus to the cytoplasm in human cells after oxidative stress [71]. It has been suggested that retinol dehydrogenase 13 (TC11759) protects mitochondria from oxidative stress in humans [72]. Yeast phosphoglycerate kinase (PGK; TC16651) has been shown to be induced following heat-shock, and to contain a heat-shock promoter element [73,74]. In addition, the *PGK* promoter has been shown to be induced following sub-lethal oxidative stress exposure [75]. Finally, alpha-glucosidase (TC16965) catalyzes the final step of glucose release during carbohydrate digestion in mammalian intestinal cells and serves as a target for blood glucose management in diabetic individuals [76].

It has been previously observed that some organisms use similar molecular mechanism to respond to heat-shock and oxidative stress. For example, both stressors induce the expression of a variety of heat-shock proteins such as DnaK (HSP70 homolog) and DnaJ (HSP40 homolog) in *E. coli* [77], HSP40 in human cell line SH-SY5Y [78], as well as HSP70 and HSP28 in human breast carcinoma MCF-7/ADR cells [79]. It is conceivable that ROS-damaged proteins might induce the heat-shock response in order to repair, or degrade any mis-folded, and/or non-functional molecules. Alternatively, oxidative stress has also been shown to play a major role in heat-shock induced cell death, most likely due to the thermal instability of antioxidant proteins [80]. Finally, the deletion of antioxidant proteins, such as superoxide dismutase, or catalase, makes cells more sensitive to the lethal effects of heat, while the over-expression of these enzymes significantly increases the ability of cells to survive heat-shock [81].

It has been suggested that the identification of schistosome proteins that may help counter the host immune response, such as heat-shock proteins and antioxidants, could be potential chemotherapeutic targets. For example, HSP40 (major egg antigen) has been proposed as an ‘anti-pathology’ schistosomal vaccine candidate due to its ability to stimulate IL-5 and IL-10 but not IL-4 and IL-13. Such a cytokine profile is associated with reduced collagen deposition, decreased fibrosis and granuloma inhibition formation which are hallmarks of the ultimately destructive host immune response against schistosome eggs [82]. Shalaby and colleagues [83] were able to achieve significant reductions in worm burden by DNA vaccination against enzymes such as superoxide dismutase and glutathione peroxidase. More recently, thioredoxin glutathione reductase, which has been shown to be essential for schistosome survival during oxidative stress, has been exploited as a target for the development of a new generation of anti-schistosomal compounds [20,21].

The work presented in this manuscript focused on the changes in gene expression that occur in both sexes in response to temperature and oxidative stress. We believe that the transcripts and proteins that are induced by these stressors should make excellent candidates as targets for future novel anti-schistosomal therapeutics. Interestingly, there is also some indication from the work of Fitzpatrick and colleagues [52] and Moertel and colleagues [53] that there may be differences in the capacity of adult male and female worms to respond to stress. This would

serve not only to highlight the dioecious nature of the adult parasite but also suggest another avenue of investigation to identify sex-specific therapeutics.

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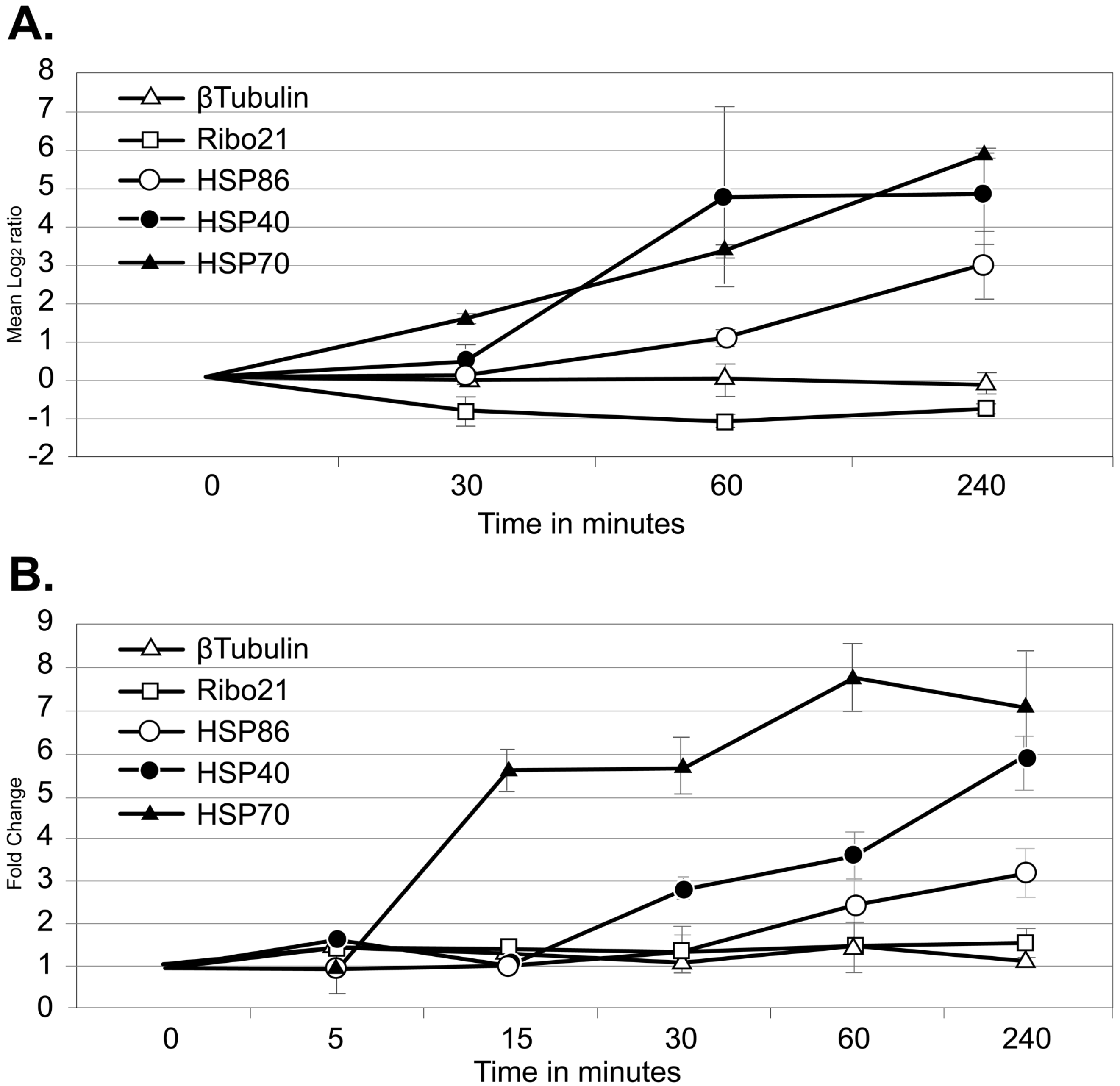

Figure 1.
Comparison of microarray data and qRT-PCR measurements for representative mRNAs from schistosomes exposed to heat-shock. **A.** Microarray expression data are plotted as mean $\log_2$ Cy5/Cy3 as a function of time after stress. Log_2 transformed data collected from array elements at 0, 0.5, 1.0, and 4.0 h were subtracted from $\log_2$ transformed data collected from 0 h arrays. Error bars represent the standard deviation of three measurements from biological replicates. $\text{Log}_2 > 1$ is considered a significant increase in transcript abundance. **B.** qRT-PCR data were plotted as fold change calculated using the $2^{-\Delta\Delta CT}$ method [29] over time after heat stress. Error bars represent the standard deviation of three measurements. Fold change > 1 indicates an increase in transcript abundance.

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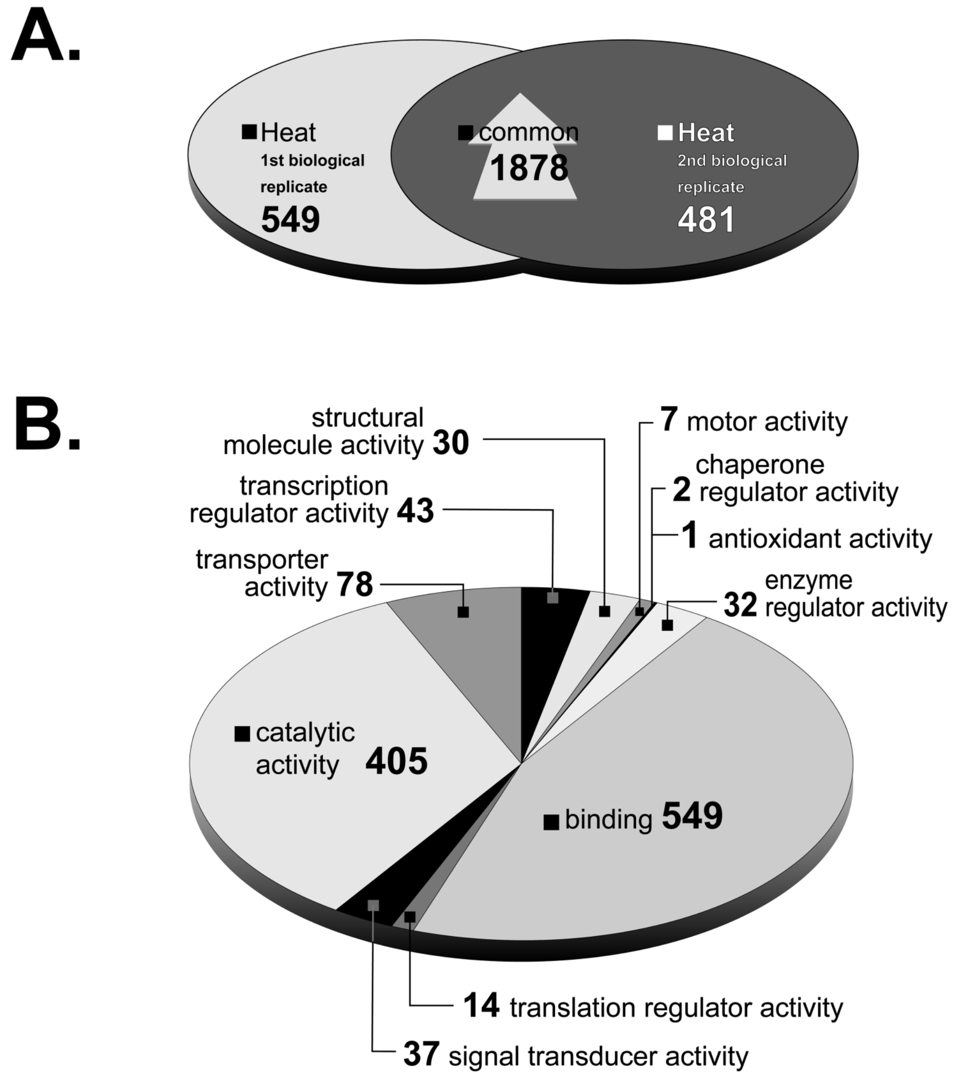

Figure 2.
A. Venn diagram of elements whose bound transcript increased after schistosomes were exposed to heat-shock. A total of 2,427 and 2,359 elements were up-regulated two-fold at two time points in the 1st and 2nd biological replicates respectively, 1,878 of which were shared by both experiments. *S. japonicum* probes were excluded from the analysis. **B.** Gene ontology annotation following heat-shock. Predicted molecular functions of 825 elements induced in response to heat-shock. *S. japonicum* elements were excluded from gene ontology analysis. Elements with multiple annotated functions were included and could be in more than one category.

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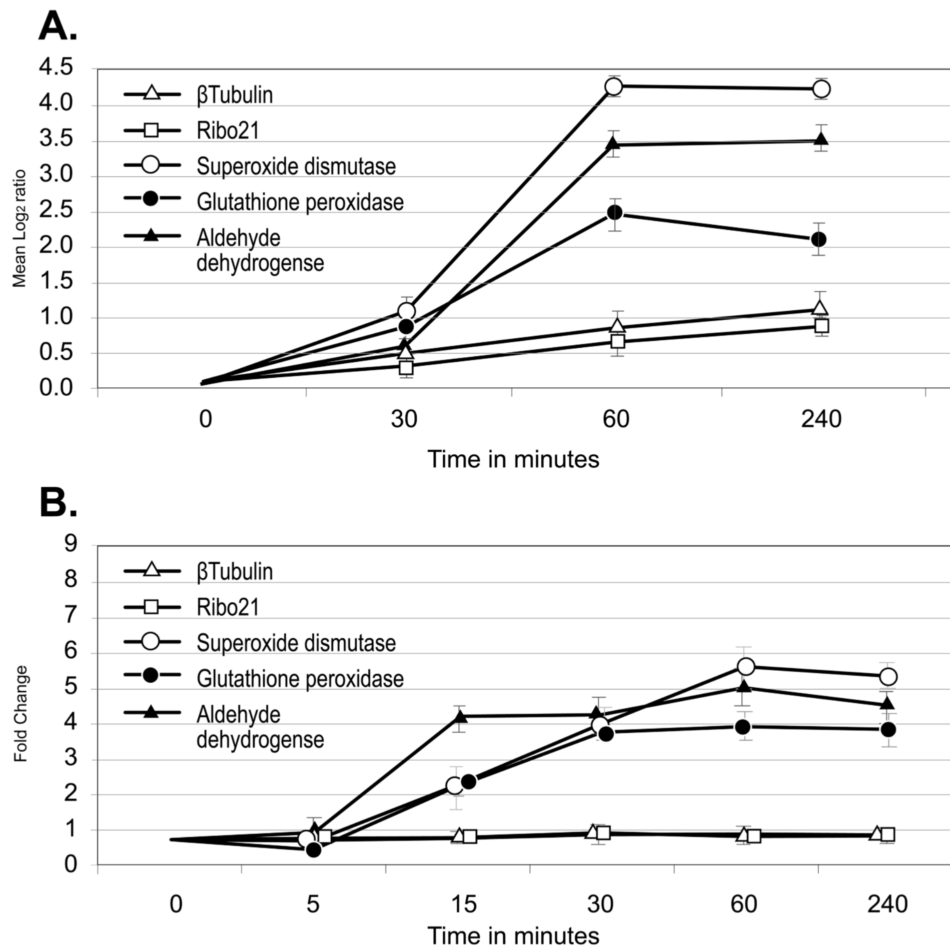

Figure 3.

Comparison of microarray data and quantitative RT-PCR measurements for representative mRNAs from schistosomes exposed to oxidative stress. **A.** Microarray expression data are plotted as mean $\log_2$ Cy5/Cy3 as a function of time after stress. Log_2 transformed data collected from array elements at 0, 0.5, 1.0, and 4.0 h were subtracted from $\log_2$ transformed data collected from 0 h arrays. Error bars represent the standard deviation of three measurements from biological replicates. $\text{Log}_2 > 1$ is considered a significant increase in transcript abundance. **B.** qRT-PCR results plotted as fold change calculated using the $2^{-\Delta\Delta CT}$ method [29] as a function of time after stress. Error bars represent the standard deviation of three measurements. Fold change > 1 indicates an increase in transcript abundance.

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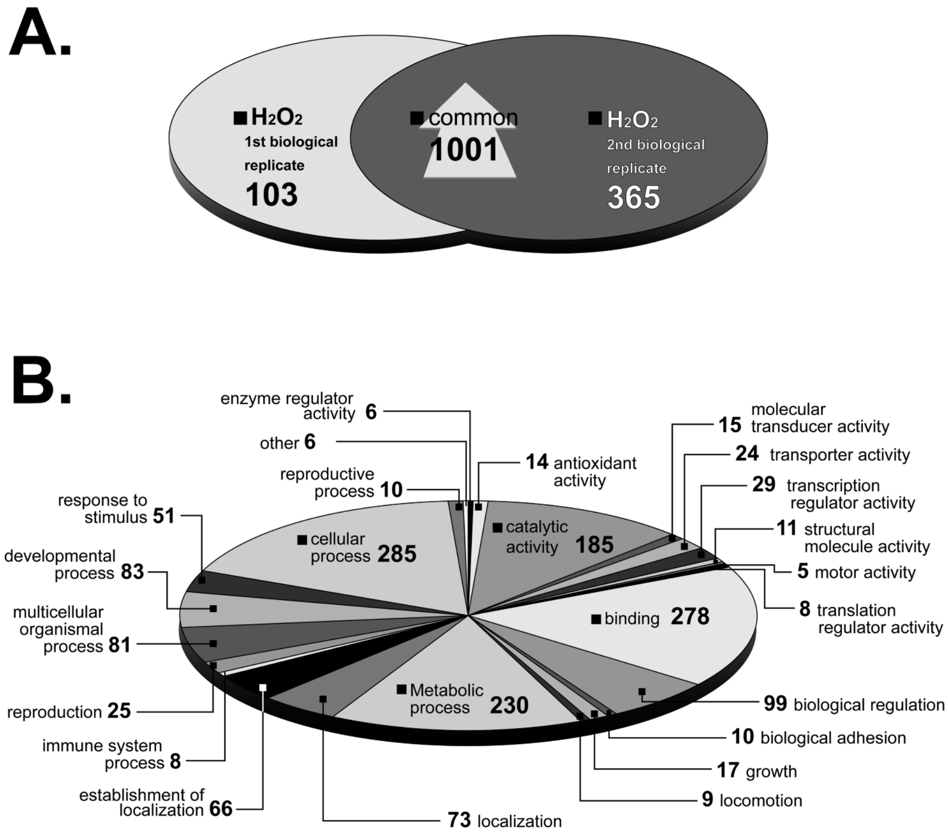

Figure 4.

A. Venn diagram of elements that increased after schistosomes were exposed to oxidative stress (100 μM H_2O_2). A total of 1,104 and 1,366 elements were induced two-fold in two time points in 1st and 2nd biological replicates respectively, 1,001 of which were found in common. *S. japonicum* probes were excluded from the analysis. **B.** Gene ontology following oxidative stress. Predicted molecular functions of 459 elements induced in response to oxidative stress. *S. japonicum* elements were excluded from gene ontology analysis. Elements with multiple annotated functions were included and could be in more than one category.

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Table 1

Commonly induced genes in response to heat and oxidative stress

Transcript*	Description
TC10026, TC11632, TC14826	Homolog to endonuclease-reverse transcriptase
TC10560	*S. mansoni* cathepsin B2 (smB2) protease
TC10640	Homolog to serine/threonine phosphatase 1 regulatory subunit 10
TC10763	Homolog to ankyrin 3 (ANK-3)
TC11295	Similar to yeast retrotransposable element
TC11425	Homolog to sulfite oxidase
TC11759	Homolog to retinol dehydrogenase
TC12705	Homolog to aspartate carbamoyltransferase, CAD protein
TC13469	Homolog to RNA-binding protein 40, snRNP 65
TC14047	Homolog to alpha-actinin, F-actin
TC14057	Homolog to serine/threonine-protein kinase PRP4
TC14202	Homolog to zinc finger protein
TC14204	Homolog to ATP-dependent RNA helicase DDX3Y
TC14312	Homolog to DNA replication licensing factor MCM2
TC16651	Homolog to phosphoglycerate kinase
TC16843	Homolog to plasminogen precursor
TC16965	Homolog to alpha-glucosidase
TC17032	Homolog to 1-AGP acyltransferase 2, (1-AGPAT 2)
TC17139	Homolog to PRPP synthetase-associated protein 2
TC18034	Homolog to mitochondrial dicarboxylate carrier
TC7461	Homolog to spliceosome RNA helicase BAT1, UAP56
TC7721	Homolog to oligosaccharyl transferase subunit STT3A
TC7745	Homolog to spliceosome-associated protein 155 (SAP 155)
TC8694	Homolog to high-affinity cationic amino acid transporter 1 (CAT-1)
TC9925	Homolog to aminopeptidase P3 (APP3)
45 transcripts	Unknown

*Transcript sequences were used to search Swissprot protein data base using NCBL blastx tool. Transcripts with blastx E values > 10^{-5} were not included.