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Differences in DNA methylation patterns in the Bg-hsp 70 DNA region

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Deoxyribonucleic acid (DNA) methylation is one of the widespread epigenetic modifications of genomic DNA, and has been postulated to be a predisposing influence on disease onset and infections. The ability to quantify differences in DNA methylation between the genomes of normal *vs*.stressed *Biomphalaria glabrata* would help to profile changes potentially linked to resistance to *Schistosoma mansoni* infection. Thus, this study sought to measure differences in cytosine DNA methylation of various *B. glabrata* tissues responding over time to either biological or physiological stresses (*S. mansoni* exposure *vs.* heat shock). Here, this study measured DNA methylation at the *B. glabrata*heat shock protein 70 (*Bg*- hsp 70) intragenic regionto profile the regions methylome with a simple and cost-effective method. The study found DNA hypomethylation of the *Bg*-hsp 70 region occurs in the snails in response to both stressors; heat shock and parasite exposure, however, overall DNA hypomethylation after heat shock was similar among the tissues examined. In contrast, DNA methylation remained suppressed for up to 5 h when snails were responding to stress from parasite exposure. In parasite exposed snails, the levels of *Bg*-hsp 70 methylation in whole body, head foot and ovotestis decreased from 30 min to 2 h, and the reduction persisted in the hepatopancreas for up to 5 h. The DNA hypomethylation of the *Bg*-hsp 70 intragenic region correlated negatively with the level of *Bg*-hsp 70 mRNA in infected snails but not in thermally stressed snails. From this study results, the study conclude that the hepatopancreas is the most active host organs in terms of differential DNA methylation events following parasite infection. Also, from these data the study postulate that different epigenetic mechanisms underlie, *Bg-hsp70* gene regulation in this snails while responding to stress due either to parasite exposure or heat shock.

Key words: DNA methylation, invertebrate, *Biomphalaria glabrata*, epigenome, *Schistosoma mansoni* infection, thermal stress.

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INTRODUCTION

Epigenetic modifications refer to changes in gene expression that occur without altering the underlying DNA sequence (Jaenisch and Bird, 2003; Richards, 2006). These changes are based on a series of molecular processes that can activate, reduce or completely disable the activity of genes (Bossdorf et al., 2008). DNA methylation is one of the key epigenetic modification in eukaryotic genomes and is crucial for regulation of gene expression during animal and plant development. This mechanism consists of a chemical modification of the genomic DNA that involves the addition of a methyl group to a nucleotide, usually the 5' carbon of the cytosine pyrimidine ring, by specific DNA methyltransferase (Lu et al., 2008; Mandrioli, 2007). Studies of DNA methylation are complicated by naturally occurring variation in methylation among biological entities: different cells, tissues and organisms may show divergent methylation patterns (Yi and Goodisman, 2009).

Since its original identification as an epigenetic regulator, cytosine methylation has been studied through numerous assays, including methylation-sensitive restriction enzyme mapping, deamination of un-methylated cytosines with sodium bisulfite, and enrichment with targeting antibodies (Harrison and Parle-McDermott, 2011; Laird, 2010). Studies during the past decade on the human methylome have increasingly focused on developing methodologies with single base pair resolution, since both regional and single CpG site methylation changes have been shown to have functional consequences (Gibney and Nolan, 2010; Michels et al., 2013; Xu et al., 2007). In addition, in most vertebrates studied, DNA methylation is predominantly limited to the CpG doublets that are present insubstantial numbers, either within CpG islands of promoters or at overlapping nearby transcription start sites, while the methylation in invertebrates is shown predominantly in the gene body Patterns of DNA methylation are highly variable among different animal taxa (Feng et al., 2010; Suzuki et al., 2007; Zemach et al., 2010). DNA methylation can generally be found in transposable elements and repetitive elements as well as in gene bodies in *Arabidopsis* (Cokus et al., 2008; Lister et al., 2008). Gene-body methylation was found to correlate with high expression level (Tran et al., 2005; Zemach et al., 2010; Zilberman et al., 2007) or alternatively, DNA methylation can define exons boundaries or regulate alternative splicing because it has been observed that exons are more highly methylated than intron (Feng et al., 2010; Naydenov et al., 2015).

Currently, studies on epigenetics in invertebrates are still lagging behind those that have been conducted on vertebrates. Therefore, the characterization of DNA methylation patterns from a broad spectrum of invertebrates would help advance the field of evolutionary epigenetics and provide much needed biological information about the effect of changing methylation patterns on animal environment adaptations. Mollusks are one of the most diverse and evolutionarily successful groups of invertebrates. They possess various body plans and adaptation strategies. Despite their species abundance and diverse geographical distribution, DNA methylation patterns in mollusks remain largely unexplored. A recent study showed that specific functional categories of genes were associated with different levels of DNA methylation in the Pacific oyster *Crassostrea gigas* (Gavery and Roberts, 2010). For example, genes involved in stress and environmental responses were prone to be hypo-methylated which might be advantageous for greater epigenetic flexibility and for higher regulatory control (Gavery and Roberts, 2010). Fneich et al. (2013), were the first to report DNA methylation in *Biomphalaria glabrata* snails (intermediate host of the human trematode parasite *Schistosoma mansoni*), and showed that the *B. glabrata* genome is methylated predominantly at CpG sites, as revealed by several techniques including restriction enzymatic digestion with isoschizomers, bisulfite conversion-based techniques, and liquid chromatography-tandem mass spectrometry (LC-MS) analysis.

This study focus was to evaluate the gene body methylation pattern of the hsp 70 region using the snail *B. glabrata* which is the intermediate host of the parasitic trematode *S. mansoni,* as a model organism responding to either biotic or abiotic stress. Heat shock proteins (Hsp) are highly conserved proteins whose expression is induced by different kinds of stresses. It has subsequently been shown that most Hsps have strong cytoprotective effects and behave as molecular chaperones for other cellular proteins. Inappropriate activation of signaling pathways could occur during acute or chronic stress as result of protein misfolding, protein aggregation or disruption of regulatory complexes. The action of chaperones, through their properties in protein homeostasis is thought to restore balance. Studies that compared the two highly homologous members of the Hsp 70 family; the constitutive Hsc 70 and the major stress-inducible Hsp 70, revealed that underphysiological conditions, the constitutive Hsc 70 is expressed at higher

levels than Hsp 70, but the synthesis of Hsp 70 is more rapid and accumulates in different subcellular compartments after stress (Ellis et al., 2000; Seidberg et al., 2003). Furthermore, in contrast to normal cells frequently overexpressed Hsp 70 in the cytocol, present Hsp 70 on their plasma membrane and actively release Hsp 70 (Multhoff et al., 1997; Vega et al., 2008).

In summary, the key actions of Hsp 70 depends on where the protein islocalized. In the cytosol, Hsp 70 functions by binding to client polypeptides in an ATP dependent manner to prevent aggregation of unfolded peptides and transports proteins (Kim et al., 2013; Mayer, 2013), regulates intercellular signaling and mediates antigen cross-presentation. As a membrane bound protein, Hsp 70 acts as a tumor-specific recognition structure for activated NK cells on the plasma membranes (Multhoff et al., 1999; Nylandsted et al., 2004) and mediates anti-apoptotic functions on the lysosomal membrane. Finally, extracellular free and lipid-bound Hsp 70 can induce both inflammatory and anti-inflammatory responses (De Maio, 2014; Henderson and Pockley, 2010).

Herein, this study examined changes in DNA methylation in varioussnail tissues (whole body, head foot, ovotestis and hepatopancreas) after either heat shock or following early exposure to a compatible strain of *S. mansoni.* By using an efficient, simple, cost-effective, fluorescence-based, and bisulfite-free procedure that required very small amount of DNA starting material, the study provide evidence, for the first time, of differences in DNA methylation patterns in the *Bg*-hsp 70 DNA region depending on the type of stressor; biotic or abiotic.

MATERIALS AND METHODS

Snails and stress conditions

In the study experiments, *B. glabrata* NMRI snail strain that is susceptibleto *S. mansoni* infection was used. This is a laboratory-maintained snail used at the Schistosomiasis Resource Center (Biomedical Research Institute, Rockville, MD). The snails (4 to 6 mm in diameter) were kept overnight in sterile water containing 100 µg/ml ampicillin. In total, twenty juvenile *B. glabrata* snails were used, which included biological triplicates and methodological triplicates. Snails were subjected to either thermal stress by incubation in pre-warmed (32°C) sterile water as previously described (Ittiprasert and Knight, 2012) for various time points: 15 min, 30 min, 1 h, 2 h and 5 h, or biologically stress by *S. mansoni* miracidia exposure (Ittiprasert et al., 2009). For parasite exposure, the snails were individually exposed to *S. mansoni* miracidia (10 miracidia/snail) at ambient temperature (~25°C) in 24 well plates containing filtered and aerated (at least 48 h) tap water for the various time points.

Snail tissue collection

Each snail was placed between 2 microscope slides and gently crushed to break the shell without damaging the internal tissue. Each crushed snail was transferred to a clean microscope slide after clearing all shell debris. Forceps and scalpels were used to dissect the whole body tissue or tissue of interest, namely the head foot, hepatopancreas and ovotestis. Dissected tissues were processed immediately for dual genomic DNA and total RNA extraction as described below.

Dual genomic DNA and total RNA isolation

To evaluate the effect of cytosine DNA methylation on the intragenic *B. glabrata* heat shock protein 70 region (*Bg*-hsp 70) and its effect on this gene'stranscription, the study isolated genomic DNA for methylation determination and total RNA for *Bg*-hsp70 transcript expression from the same tissue samples. Briefly, each tissue sample was homogenized in RNAzol® RT (Molecular Research Center, Inc., OH) as previously described (Ittiprasert and Knight, 2012). The sedimented DNA and proteins were precipitated with an equal volume of sterile distilled water (DW), and the supernatant was further processed for RNA recovery by isopropanol precipitation as per the manufacturer's instructions. The DNA from DNA/protein pellets was recovered using DNAzol® (Molecular Research Center, Inc., OH) and the DNA was recovered by the ethanol precipitation method as per manufacturer's instructions. Briefly, each DNA sample was homogenized in RNAzol®RT solution, then centrifuged at 10,000 x g to remove the tissue debris. By adding half volume of DW to the clear homogenate to allow DNA and protein precipitation. After centrifugation, the DNA/Protein pellet was resuspended in DNAzol®solution, while the top aqueous phase containing RNA was transferred into a new tube and an equal volume of isopropanol added. The RNA pellet was washed with 70% ethanol (EtOH) twice, and RNA was resuspended in nuclease-free water. For DNA purification, the pellet that was completely dissolved with DNAzol was used for DNA isolation by adding half volume of absolute EtOH. The DNA pellet after centrifugation and washing was resuspended in nuclease-free water. Solubilized DNA and RNA were suspended in nuclease-free water and kept frozen (-80°C) until required. Quantification of these nucleic acids was evaluated by NanoDrop 1000 Spectrophotometer (Thermo Scientific, DE).

Gene-specific DNA methylation assessment

Genomic DNA from whole body tissue, head foot, ovotestis and hepatopancreas were quantified and checked for purity ($OD_{260/280}$ ratio ~ 1.80) by NanoDrop 1000 spectrophotometer (Thermo Scientific, DE). The study used the One Step qMethyl™ Kit (Zymo Research, CA) to measure the level of methylated cytosines in dinucleotides from individual genomic DNA samples asper manufacturer's instruction (Gu et al., 2015; Rao et al., 2012; Sanders et al., 2014; Turanli et al., 2013; van Dijk et al., 2012). Because of this, the technique combines the basic techniques of DNA digestion and methylation-sensitive qPCR of a known sequence. So using this assay, the primer pairs were designed within the CpG rich of the *Bg-hsp 70* region according to the guidelines provided in the One Step qMethyl™ kit. This was accomplished by splitting the DNA to be analyzed into two tubes: a Test and Reference reaction. DNA in the 'Test reaction' was digested by Methyl Sensitive Restriction Enzymes (MSREs) provided with the kit, while the 'Reference reaction' had no MSREs. The DNA from both samples was amplified using real time PCR in the presence of SYTO® 9 fluorescent dye and then quantitated.

Cycle threshold (Ct) values for 'Test' and 'Reference' DNA samples will vary depending on methylation status, with large Ct differences being characteristic of non-methylated DNA. The reaction was set up in a single tube according to the manufacturer's instructions as follows: a final reaction volume of 10 ul contained either 2xtest reaction premix or 2x reference reaction premix, 1 ul (for each) of 10 uM forward and reverse primer, 3 ul of DNase/RNase-free water, and 5 ul of DNA (20 ng). The reaction sample was mixed by pipetting and both the test and reference reactions were performed in an ABI 7300 real time thermocycler. The thermal cycle was initiated with MSREs digestion at 37°C for 2 h, followed by an initial denaturation step at 95°C for 10 min and then 45 cycles of denaturation at 95°C for 30 s, 54°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 7 min. The melting analysis was also performed after final extension and prior to the hold step at 4°C. Ct values were determined using ABI 7300 SYBR®Green emission measurements of the SYTO® 9 fluorescent dye.

The *Bg*-hsp 70 specific primers flanking the CpG island region and containing 3 MSRE sites (following the manufacturer's primer design instructions and designed using Primer-Blast) were as follows: 1 *Hpa*IIssite and 2 *HpyCH*4IV sites; *Bg*-hsp 70_2316: 5'-gagaggcaccaccattccaa-3' and *Bg*-hsp 70_2538: 5'-gctgacgttgaggataccgt-3' (223 bpamplicon). The Ct values obtained are used to calculate delta Ct values [Ct(test)-Ct(reference)]. From this methylation percentages are calculated using Methylation% = 100 x $2^{-\text{deltaCt}}$, where the delta C_t is the average C_t value from the test reaction (contains MSREs) minus the average C_t value from the reference reaction (no MSREs). Percentage methylation was relative to each experiment (experimental samples compared to control samples). Each reaction was tested for reaction efficiency by measuring dilutions of fully methylated and un-methylated DNA samples provided with the kit (data not shown).

Bisulfite genomic sequencing of cytosine methylated heat shock protein 70 (hsp70) sequence

Bisulfite treatment of DNA and PCR

The pooled snail hepatopancreas DNA samples were modified with sodium bisulfite using EZ DNA Methylation-Direct™ Kit (Zymo Research, CA) by following the manufacturer's instructions. Briefly, 1 ug of DNA was first treated with bisulfite containing a C-T conversion reagent before incubating at 98°C for 8 min and at 64°C for 3.5 h. The modified DNA was purified using a Zymo-Spin™ IC column (provided with the kit) and stored at -20°C until used for downstream analyses. The bisulfite-treated DNA was quantified by NanoDrop 1000 Spectrophotometer using factor 33 to detect single stranded DNA (Holmes et al., 2014). The specific methylated primers for the *Bg*-hsp70 sequence were designed by the MethPrimer Program (http://www.urogene.org/methprimer/) (Li and Dahiya, 2002). For each PCR reaction, 50 g of bisulfite-treated DNA was amplified by specific methylated-*Bg*hsp 70 primers flanking CpG Islands 1, 2, 4, and 5 (Figure 1 and Table 1) and the GoTaq G2 Hot Start Master Mix (Promega, WI). PCR cycles were performed with the following conditions: hot start reaction at 95°C for 2 min, then 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s, followed by 10 min for the final extension. PCR fragments,153 bp and 198 bp, amplicons of primers CpG1/2 and CpG 4/5, respectively (Table 1), were separated on a 1.2% agarose gel. Methylation-specific PCR primers were designed using MethPrimer software (Li and Dahiya, 2002) and the complete gene sequence of *Bg*-hsp 70 with GenBank:AF025477.1 as the input sequence. Primers were selected using CpG island prediction with 50% as the GC setting. Six CpG islands were detected in the*Bg*-hsp70 gene, and two pairs of methylated specific *Bg*-hsp 70 region primer pairs were designed to target putative CpG islands 1, 2, 4, and 5 (Table 1). Two sets of primers, CpG_1/2 and CpG_4/5, were used in this study.

PCR fragment purification and TA cloning

The PCR amplicons at 153 bp and 198 bp from primers CpG_1/2 and CpG_4/5 were purified individually by GeneClean(MP Biomedical, LLC, OH)and cloned individually using a TOPO® TA Cloning® kit (Life Technologies, CA.). Five single colonies from each transformant were randomly picked using lysogeny broth (LB)/Ampicillin (Amp)/ 5-bromo-4-chloro-3indolyl-beta-D-galactopyranoside (Xgal)/ isopropyl beta-D-1-thiogalactopyranoside (IPTG) selection and the plasmid DNA was analyzed by direct sequencing. Amplicons from DNA methylation of the *Bg*-hsp 70 regions were separated by agarose gel electrophoresis (1.2% agarose/Tris-Borate-EDTA/ethidium bromide) and the single expected band was excised and purified with GeneClean® II Kits (MP Biomedical, LLC, OH) individually according to the manufacturer's instructions. The purified fragment was cloned. Five of each methylated *Bg*-hsp70 recombinants were picked for direct sequencing using the M13 primer (Eurofins Genomics, AL). The sequences were trimmed to remove vector contamination identified using Vector Screen software (http://www.ncbi.nlm.nih.gov/tools/vecscreen/), followed by further analysis and alignment by use of Serial Cloner Software v. 2.6 (http://serialbasics.free.fr/Serial_Cloner.html).

Real-time PCR

Total RNA was isolated, and *Bg*-hsp70 transcript expression was quantified by a fluorogenic method with 2x SYBR Green Master Mix using an ABI 7300 real time thermocycleras as previously described (Ittiprasert et al., 2009; Knight et al., 2009). Primers specific for *Bg*-hsp70 (Acc. no. AF025477.1) were designed spanning the coding DNA sequence (CDS) containing CpG islands and predicted DNA methylated locations within the *Bg* -hsp 70 region. The primer sequences were as follows: *Bg*-hsp70 forward: 5'-aggcgtcgacattcaggtcta- 3' and *Bg*-hsp 70 reverse: 5'-tggtgatgttgttggttttacca-3'. These primers amplify *Bg*-hsp70 at positions 2379-2578. The *Bg*-hsp70 mRNA levels of each sample were normalized to *B. glabrata* myoglobin value (arbitrarily assigned an abundance value of 1 as previously described (Dewilde et al., 1998; Raghavan et al., 2007). The *Bg*-hsp70 transcript fold change was analyzed by the comparative Ct method (Livak and Schmittgen, 2001), and normalized for endogenous *B. glabrata*myoglobin. The cytosine DNA methylation and real time PCR were performed independently 3 experiments and each sample run in triplicate. Data are presented as mean ± standard error of replicate experiments. For DNA methylation and real-time PCR data comparisons, the study used one-way ANOVA with *post hoc* Tukey's multiple comparison test and Student *t*-tests, respectively. Statistical analysis was performed using Microsoft Excel 2011 version 14.4.8with *P*-values less than 0.05 considered statistically significant.

Bioinformatic and statistical analysis

Analysis and identification of CpG islands and promoters in the *Bg*-hsp 70 gene region were performed using the online tools

Table 1. Predicted CpG islands of the *Bg*-hsp 70 gene and methylation-specific PCR primer sequences. The nucleotide code shown in 'bold and underlined' indicates the deoxycytosine position on the original genomic sequence that is converted to the deoxythymine on the bisulfite treated sequence.

CpG island	size (bp)	start-end	start-end	methylated-specific primer sequenes	primer binding sites
CpG_1	119	1213-1331	CpG-1/2-f:	5'-agtttacgacgttaagaggttgatc-3	1266-1418 (153 bp)
CpG-2	130	1377-1506	CpG-1/2-r	5'-tctaaaacgaaaaatttattctcgc-3'	-
CpG-3	106	1514-1619	-	-	no expected methylation site
CpG-4	113	2456-2568	CpG_4/5-f:	5'-tatcgacgttaacggtatttttaac-3'	2508-2705 (198 bp)
CpG-5	114	2583-2696	CpG_4/5-r:	5'-taattctccaactaattcctaaccg-3'	no expected methylation site
CpG-6	119	2718-2836	-	-	-

MethPrimer (Li and Dahiya, 2002) and Neural Network Promoter Prediction (Reese, 2001). To generate and analyze the DNA methylation patterns from bisulfite sequencing data of the clones with methylated *Bg*-hsp 70 fragments, the study used Meth Tools 2.0 software (Grunau et al., 2000). To study the association between stress-induced DNA hypomethylation of the *Bg*-hsp 70 region and *Bg*-hsp 70 transcript levels, Microsoft Excel 2011 version 14.4.8 to generate scatter plots and calculate correlation coefficients(r) was used. The correlation coefficient r measures the strength and direction of a linear relationship between two variables on a scatterplot. The value of r that is higher than 0.7 indicates a strong linear relationship (Ho, 2012).

RESULTS

Prediction of methylated cytidines (^{m}C) of the*Bg*-hsp 70 locus

This study investigated ^{m}C sites on predicted methylated sites of *Bg*-hsp 70 CpG islands using the input sequence AF025477.1. The study used MethPrimer Design Software (Cartharius et al., 2005) to predict CpG islands, and designed specific primers for methylated bisulfite converted DNA. The study results revealed that there were five predicted CpG islands on the *Bg*-hsp 70 gene, making a predicted total of six CpG sites (Figure 1 and Table 1). Out of these six sites, four (CpGs1, -2, -4 and -5) showed predicted ^{m}C, while the remaining two were predicted not to exhibit methylated sites. From these predictions, two primer sets were successfully designed and utilized for the present study. From direct sequencing of cloned amplicons, the study identified eight ^{m}C (on the positive strand) in the *Bg*-hsp 70 regions as summarized in Figure 1: 1272, 1275, 1290, 1395 and 1410 located on CpG1 and CpG2. The ^{m}C on CpG4 and CpG5 were found at 2511, 2514, 2520, 2532, 2634, 2636 and 2681 (Figure 1a). The two promoters located at 19 to 69 and 330 to 380 were predicted by Neural Network Promoter Prediction Software and no ^{m}C sites were found at these promoters (Figure 1a). For further analysis, the study analyzed the random picked recombinants, 5 clones from each TA cloning construction for CpG 1/CpG 2 (indicated as fragment A1 in Figure 1a) and CpG4/CpG5 fragments (indicated as fragment A2 in fig.1A) by MethTools 2.0 software. This software analyzed the DNA methylation data. The results (Figure 1b) showed that there were 71.43 and 70% of %5^{m}CpG on A1 (153 bp) and A2 (198 bp) fragments, respectively. The %5^{m}C on A1 and A2 fragments are 14.71 and 12.96%, respectively. In this study, there was no methylation outside CpG sites (Figure 1b).

Steady state cytosine methylationlevels of the *Bg*-hsp 70 region of *B. glabrata*

To investigate the baseline DNA methylation levels in three different tissues (head foot, ovotestis and hepatopancreas) and the whole bodyin non-stressed snails, 4 independent biological replicates, with 5 snails each was conducted. Using One Step qMethyl^{TM} kit (Zymo Research, CA) with *Bg*-hsp_2538 and *Bg*-hsp-2316 primers, the study found that the global methylation levels across tissues from control snails were quite similar,ranging from 15.11 to 17.98% (Figure 2).

Pattern of DNA hypomethylation between biological and physiological stresses in the *B. glabrata* epigenome are different

To determine differential patterns of *B. glabrata* DNA hypomethylation induced by biological versus physiological stresses, four independent experimental replicatesto study changes in DNA methylation patterns across three tissues (head foot, ovotestis and hepatopancreas) and the whole body induced by either biological or physiological stress was conducted. Five snails of each group were stressed either by heat shock at 32°C or by *S. mansoni* miracidia exposure, and tissues collected at various time points;0, 30 min, 2 h, and 5 h post exposure. Thermal and biological stress induced significant DNA hypomethylation of the CpG4 site after

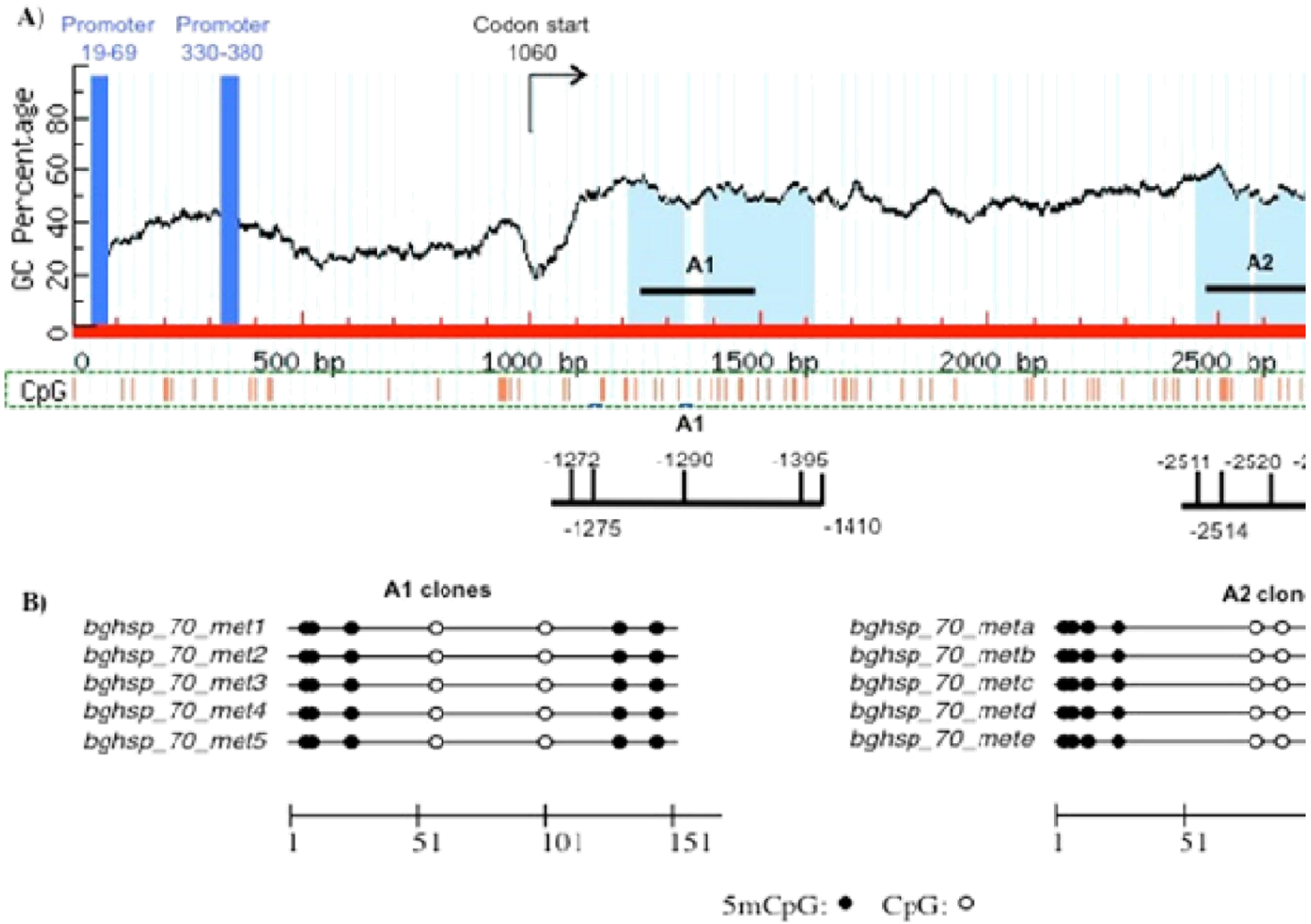

Figure 1. (A).Schematic of predicted CpG islands and promoters in the Bg-hsp 70 gene (~ 3Kb) region. The light blue region denotes the C the black lines marked by A1 and A2 indicate methylation sites. The red vertical lines in the green color box indicate the CpG sites and t and 330-380) that did not contain CpG sites. CpG methylation site in the A1 and A2 (Panel A). (B)The schematic representation of Cp (153bp) and 10 CpGs of A2 (198 bp). Clone fragments; bghsp_70_-met1, -met2, -met 3, -met 4 and –met 5 shownin the results from A1, metb, metc, metd, mete shown in the results from A2. Black and white circles corr espond to methylated and non-methylated CpGs, respectiv

30 min of treatment. The decrease in DNA methylation from heat shock treatment was found to be similar among these snails; a ~6 to 11% reduction was observed from 30 min to 2 h compared to non-stressed conditions (Figure 2, panels E-H). Following heat shock, however, DNA methylation returned to normal levels at 5 h even though the snails remained in a thermally stressed environment. Interestingly, hypomethylation events triggered by biological stress (in all the tissues) continued to accumulate for up to5 h, albeit with only ~2 to 9% increase in hypomethylated sites compared to controls (Figure 2, panels A to D). Reduction in the methylation of the *Bg*-hsp 70 gene in the hepatopancreas was found to be the most dramatic at 5 h post exposure (>13% increase in hypomethylated sites) (Figure 2, panel C). Beyond this time point post-exposure, the study found that individual snails exhibited differential patterns of DNA hypomethylation for up to 10 h, with a slow recovery to baseline levels after 24 h of parasite infection (data not shown).

Association of *Bg*-hsp 70 DNA hypomethylationwith *S. mansoni* miracidia exposure and effects on *Bg*-hsp 70 gene expression

At serial time points up to 5 h after parasite exposure, DNA hypomethylation of *Bg*-hsp 70 in parallel with *Bg*-hsp 70 mRNA levels was measured. In all tissue samples examined, the expression of *Bg*-hsp 70 transcripts increased in response to infection relative to levels in non-stressed tissues. The levels of *Bg*-hsp 70 transcript after 5 h of stress decreased slowly, recovering to normal levels within 12 h (data not shown). Figure 3, panel E shows the fold change of *Bg*-hsp 70 in the four tissues examined at various time points post-exposure to *S. mansoni*; the most significant differential expression of this transcript was detected in the hepatopancreas 2 to 5 h post-exposure with 8.32 and 9.35 fold change, respectively.

In parallel with *Bg*-hsp 70 transcript measurements, methylation levels in the hsp70 gene body region were also analyzed. Surprisingly, hsp 70 mRNA levels under heat stress conditions did not correlate with the methylation status of the hsp70 gene body region. For instance, whereas DNA hypomethylation levels after 30 min 2 h post-heat stress recovered to the non-stressed level at 5 h, the transcription of *Bg* -hsp 70 remained upregulated (~3 to 5.2 fold change, data not shown). Interestingly, with *S. mansoni* exposure as the stressor, a negative correlation between the methylation level of the *Bg*-hsp 70 locus and expression of its transcript was observed. Thus, as shown in Figure 3, panels A to D, the intergene DNA hypomethylation levels in the *Bg*-hsp 70 sequence correlated with its mRNA expression in all tissues examined: whole body (r= 0.70), head foot (r = 0.60), hepatopancreas (r = 0.91) and ovotestis (r = 0.99).

DISCUSSION

In this study, the percent of cytidine DNA methylation within the intragenic region of the *Bg*-hsp 70 locus was examined in different tissues; whole body, hepatopancreas, ovotestis and head foot of *B. glabrata* either after schistosome exposure (biological stress) or heat shock at 32°C (physiological stress) over time. The study results showed that hypomethylation of the *Bg*-hsp70 regions in snails undergoing these stresses were different at the time points examined, revealing changes in the ^{m}C patterns in the corresponding *Bg*-hsp 70 DNA sequences. From these results, the *B. glabrata* snails showed that DNA methylation on CpG sites only occurred within the intragenic region and not on the promoter sequence that had no CpG sites as predicted by MethPrimer software (Figure 1). This result is further confirmation of previous studies that showed in other invertebrates that methylation of cytosineresidues in DNA is mainly found in gene bodies but not in promoter regions as is the case in vertebrates (Laird, 2010; Regev et al., 1998).

Interestingly, the patterns of cytidine methylation observed in the snail while responding to either biotic or abiotic stress were different. For example, in the heat shocked snails results showed DNA hypomethylation occurred in all of the tissue samples examined with the same pattern of lower % DNA methylation level than what this study observed in non-stress snails (30 min to 2 h). However, the methylation level recovered, returning to the same basal level as in non-stressed snails within 5 h after heat shock (Figure 2).

In contrast, in the snails exposed to *S. mansoni* results showed the % DNA methylation continued to decrease only in the hepatopancreas (30 min to 5 h post parasite exposure), whilst in the other tissue samples (whole body, head foot and ovotestis) methylation began to increase at 5 h post exposure. This study represents the first descriptionof tissue-specific DNA methylation patterns in the schistosome-compatible snail host during the early phase of parasite exposure. In addition, these data support results from a previous study that showed that the hepatopancreas was the preferable organ for the localization of *Escherichia coli* infection in the snail (Deleury et al., 2012). It is possible that the hepatopancreas may also be the major organ that is most active in responses directed toward *S. Mansoni* miracidiae in the snail which is consistent with results from other host/pathogen studies.

In a previous study, the higher levels of mRNA was observed in the hepatopancreas, which is the site for the

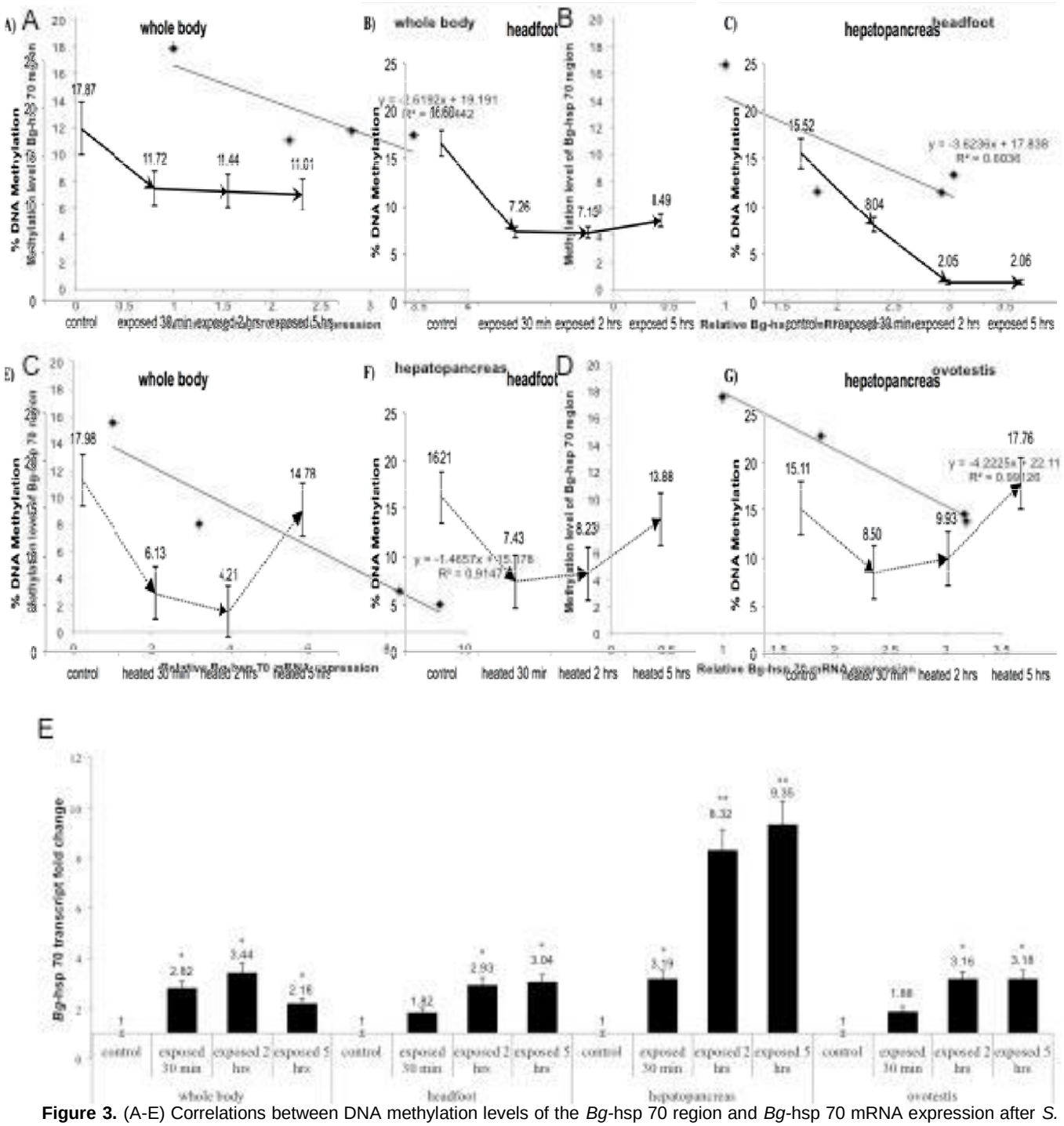

Figure 3. (A-E) Correlations between DNA methylation levels of the *Bg*-hsp 70 region and *Bg*-hsp 70 mRNA expression after *S. mansoni* exposure. A-D, correlations between *Bg*-hsp 70 hypomethylation and hsp 70 transcription levels in whole body tissue, head foot, hepatopancreas, and ovotestisare shown at different times post-parasite exposure. Y-axis indicates the percentage of DNA methylation and x-axis indicates the relative level of *Bg*-hsp 70 mRNA transcript expression for each organ. Figure 3E**,** mRNA expression levels of *Bg*-hsp 70 in different tissues at various times post-parasite exposure. Transcript level for all samples were assessed by quantitative RT-PCR and the relative expression of *Bg*-hsp 70 was calculated relative to *Bg*-myoglobin expression. Values are expressed as means +/- SD of the relative variation (fold induction) between exposed and non-exposed samples. *p<0.05; **, $p<0.01$.

differentiation of miracidiae into mother sporocysts. In the *S. mansoni-B.glabrata* interactions, the rapid change in the DNA methylation pattern of the hsp 70 locus, concurrent with an increase in the level of hsp 70 RNA, during the early phase of infection, supports the notion that a plastic and dynamic system becomes established early in the snail host helping it to cope with the selective constraints that are imposed by the parasite.

The parasite to survive, has evolved unparalleled phenotypic plasticity. This evidence is intimately linked to parasite survival transmission in the snail host especially in a changing host environment that requires rapid and profound alterations of their gene expression profiles (Gomez-Diaz et al., 2012), as shown here with hsp 70 gene expression in the early schistosome/snail interaction. Contrary to results obtained for epigenetic modulation in the snail host following early schistosome infection, heat shock (abiotic stress) affected the pattern of DNA methylation in the *B. glabrata* snails differently. Recovery to basal levels in all tissues examined occurred uniformly and within a shorter time frame than when the stimulus was from infection. These differences suggest that the epigenetic machinery regulating *Bg*-hsp 70 gene expression under conditions of abiotic stress has no tissue- or organelle – specificity.

It is possible that these differences may also reflect where the hsp 70 proteins is located in order for it to function optimally in the snail while it is responding to either thermal stress or to schistosomes. Thus, it is possible that under thermal stress conditions, the cytosolic hsp 70 may be the major effector molecule that is needed to control homeostasis in the snails preventing aggregation of unfolded peptides and transporting proteins. Whilst in the schistosome exposed snails, the parasite may elicit instead a specific tissue/ immune response via either a membrane bound and/or extracellular free, lipid-bound hsp 70 protein that induces either inflammation or anti-inflammation responses to support parasite to survival, development and differentiation to the sporocysts. This finding could be important for further studies in the snail vector and schistosome interaction about how biological stress affect

the phenotypic switching observed in *B. glabrata* snails (Ittiprasert and Knight, 2012).

Therefore, it is possible that the gene plasticity observed with epigenetic modulation after thermal stress in this snail can caused phenotypic change. However, in the susceptibility strain of B. glabrata changes to epigenetic marks after parasite exposure would favor host adaptation to support the parasite development and differentiation without phenotypic change. Strong linear negative correlations ($r > 0.7$) between the degree of intragenic (not promoter regions from this study) was also observed. *Bg*-hsp 70 DNA hypomethylation and *Bg*-hsp 70 mRNA transcript levels was induced after parasite exposure, but not between the magnitude of DNA hypomethylation and levels of *Bg*-hsp 70 transcript up-regulation from thermal stress. From previous reports of DNA methylation in invertebrates that display a wide range of DNA methylation from the absence of this mechanism in *Caenorhabditis elegan* to either low or moderate levels in the case of *D. melanogaster* invertebrates methylation is not limited to CpG regions but can also occur in other DNA coding regions or CpT islands. The study results confirmed the evidence showing that in invertebrates, major DNA methylation occurs only in the genebody rather than in promoter region(s) as in the case in many vertebrates (Bird, 2002; Colot and Rossignol, 1999; Elango and Yi, 2008; Mandrioli, 2004; Su et al., 2011) . However, this study lack considerable information about epigenetic modulation in *B. glabrata* (for examples, DNA methylation, histone modification and micro RNAs), especially involving responses to both biotic and abiotic stressors. The identification and elucidation of these mechanisms would be important to bridge the considerable information gap that currently exists in this subject in the schistosome and snails as well as the mammalian host interaction.

Conclusion

This study may conclude that the molecular mechanisms of the *B. glabrata* immune response to schistosomes differs from their adaptive response to thermal stress,and may be mediated by different transcription factors and/or another hsp 70 promoter(s) (Akerfelt et al., 2010; Nakai and Morimoto, 1993; Nakai et al., 1997; Sistonen et al., 1992; Stephanou and Latchman, 2011; Younis et al., 2011) or non-heat shock factor proteins (HSF) (Blatke et al., 2013; Madrigal-Matute et al., 2010; Stephanou and Latchman, 2011). Non-CpG forms of methylation, example, CpA, CpT, CpG, or histone or lysine methylation as reported in mammalians (Pinney, 2014) and invertebrates; *Apismellifera* and *Bombysmori* (Elango et al., 2009; Regev et al., 1998), should also be areas of considerations for studying epigenetics in *B. glabrata.* Thermal stress that induces epigenetic change and, therefore, genetic plasticity might cause the phenotype switching observed in *S. mansoni* parasite resistant snails exposed at elevated temperature (Ittiprasert and Knight, 2012; Knight et al., 2015).

In previous studies, *in* situ hybridization was used to show that there was non-random spatial repositioning of *Bg*-hsp 70 loci in early infected snails concurrent with *Bg*-hsp 70 transcription (Arican-Goktas et al., 2014; Knight et al., 2011). This have shown that in the *Bg*-hsp 70, locus of the *B. glabrata* snail model system, that a negative correlation exists between *Bg*-hsp 70 hypomethylation and up-regulation of the *Bg*-hsp 70 transcript level under

stressful conditions relative to biological stress. These data provides evidence, for the first time, that modulation of epigenetic marks in the snail host DNA plays a role in the stress-related regulation of the complex interaction of the schistosome parasite in its invertebrate snail host. These results will help to pave the way towards a better understanding of the role of epigenetic regulatory mechanisms and the involvement of stress in the innate immune response to schistosomes and environmental adaptation (thermal stress) in the snail host enabling us to develop novel vector based biological strategies to block transmission of schistosomiasis.

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Conflict of interest

The authors have none to declare.

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