Chapter 6: Real Time PCR Analysis of Gene Expression in Different Life Cycle Stages of *S. japonicum*.

6.1 Introduction

Expressed Sequence Tag (EST) sequencing approaches have proven to be a valuable resource for gene discovery in a number of parasites [111]. Through the use of microarrays, transcriptional data have highlighted many genes of interest for further characterisation [4]. Vermeire *et al*. [108] described oligonucleotide microarray gene expression profiling during the miracidium to mother sporocyst transformation in *S. mansoni*. Similarly, Chai M, McManus D P, McInnes R, **Moertel L**, Tran M, Loukas A, Jones M K and Gobert G N. [109] were able to show stage-associated gene expression between mature adult worms and lung schistosomula from amplified *S. japonicum* mRNAs. As discussed in Chapter 1 (section 1.1.2), paired adult schistosomes in the mammalian host produce eggs that hatch in fresh water, releasing free swimming miracidia. These then infect the intermediate host, a fresh water snail, transforming into mother sporocysts. The mother sporocysts produce daughter sporocysts by asexual reproduction, which in turn release cercariae that are released from the snail, which penetrate the skin of the mammalian host where they eventually develop into adult worms. These developmental phases are accompanied by many biochemical and molecular changes [108]. These changes have been monitored at the transcriptional level using microarrays to investigate differential gene expression in different schistosome life cycle stages [109].

In order to further address gene expressional differences between and within the Chinese and Philippine strains of *S. japonicum,* the differential expression of eight selected contigs were analysed in four key developmental life cycle stages (male and female adults, eggs, miracidia and cercariae) using real time PCR. Accordingly, primers designed on the selected contigs were used to probe the amplified complementary DNA (cDNA) templates from the various stages using real time PCR.

6.2 Materials and Methods

6.2.1 Selection of contigs

The lists of differentially expressed probes (p-value $\geq$0.001) generated in experiments outlined in Chapters 4 and 5 (sections 4.3.1 and 5.3.1, respectively) were further filtered to identify probes of interest. *S. mansoni* probes were not selected as target genes, as *S. japonicum* was the focus of this study. Although practical expressional data may be obtained from the *S. mansoni* probes the base mismatches between strains are unknown, and were not included. Furthermore, as the genome of *S. japonicum* is mostly uncharacterised, probes, including their assembled sequences that contained no or little sequence similarity to any known genes, and they were therefore not included in the final selection of contigs. All but one of these contigs were primarily identified in two groups; strain- and gender-associated gene expression (Table 6.1). Further to this, the probes were filtered by *in silico* analysis that included description and putative protein identification and a detailed investigation of the probe and assembled sequence. The probe contig 4696 was not differentially expressed in any of the microarray experiments. This probe alone was selected due to its annotation and adult stage life cycle expression as it may prove to be of biological importance in *S. japonicum*.

Table 6.1 Selected probes and their associated microarray fold expression.

		Strain (SJC or SJP)			Gender (Male or Female)	
Contig	**Description and associated "expect" (E) value**	**Mixed Sex**	**Male**	**Female**	**SJC**	**SJP**
Contig 3855	*S. japonicum* Sj-alpha-2 retroposon-like sequence (8.e-22)	SJC 11.10		SJC 7.71	F 14.07	
Contig 6725	Human DNA sequence from clone RP11-517P14 on chromosome 10 (0.27)	SJC 2.23			F 53.31	F 63.28
Contig 5299	*H. sapiens* PAC clone RP5-1140G11 from 14q24.3, complete sequence (0.29)	SJC 1.42	SJC 1.74	SJC 1.80		
Contig 8664	*S. japonicum* clone ZZD1474 mRNA sequence (0.0)			SJP 2.38	F 5.22	F 7.31
Contig 1966	*S. japonicum* clone ZZD209 mRNA sequence (1e-141)	SJC 4.63			F 2.97	M 3.49
Contig 7515	*S. japonicum* clone ZZD292 mRNA sequence (0.0)	SJC 3.74	SJC 3.25		M 16.76	M 18.61
Contig 8540	*M. musculus* chromosome 1, clone RP24-406F14, complete sequence (0.006)	SJC 3.70	SJC 9.06	SJC 10.34	M 4.61	M 4.64
Contig 4696	*S. japonicum* clone ZZD10 mRNA sequence (2e-41)	*Not differentially expressed*				

SJC, Chinese *S. japonicum*; SJP, Philippine *S. japonicum.* All probes were differentially expressed at p-value $\leq$0.001 excluding contig 4696. Expect (E) values were obtained by BLAST [169].

6.2.2 Isolation of SJC eggs and miracidia

Chinese *S. japonicum* (SJC) eggs and miracidia were isolated using a modified protocol as previously described [170]. Briefly, infected mouse livers were chopped finely and placed into a 50 ml Falcon tube together with 20 mg Collagenase B (Roche, Dee Why,

Australia), 10 mg penicillin (Sigma-Aldrich), 20 mg streptomycin (Sigma-Aldrich) and PBS to a total volume of 50 ml. The tube was sealed and placed on a platform rocker (Edwards instruments, Narellan, Australia) at 37˚C overnight. The tube was then spun in an Eppendorf 5810 R centrifuge (Eppendorf, North Ryde, Australia) at 800 g, 4˚C for 5 min, after which the supernatant was discarded and the pellet resuspended with 40 ml ice-cold PBS. The pellet was washed three times in order to remove any contaminants from the egg mixture. After the final wash, the pellet was resuspended in 25 ml PBS and passed through 250 µm and 150 µm copper sieves (Endecotts, London, England). The purified egg mixture was collected and centrifuged at 800 g, 4˚C for 5 min. After centrifugation, the supernatant was discarded and the pellet resuspended in 5 ml PBS. The egg mixture was then applied to a 25% Percoll column [40 ml 0.25 M sucrose (BDH, Kilsyth, Australia) 25% Percoll (Amersham, Baulkham Hills, Australia)] before centrifuging at 1800 g, 4˚C for 10 min. The supernatant was then discarded and the pellet washed three times with ice-cold PBS. The pellet was resuspended in 5 ml PBS before it was applied to a 33% Percoll column [20 ml 0.25 M sucrose, 33% Percoll] and again centrifuged at 1800 g, 4˚C for 10 min. After centrifugation the supernatant was discarded, and the pellet resuspended in 1 ml PBS and transferred to an microfuge tube. This tube was centrifuged at 800 g, 4˚C for 5 min before washing the remaining pellet with 1 ml PBS three times. This egg pellet was used either for total RNA or the isolation of miracidia.

In order to hatch and isolate miracidia, *S. japonicum* eggs were added to 5 ml of distilled water in a foil wrapped 10 ml beaker and exposed to a bright incandescent light. The majority of the miracidia hatched within 10 min, and the water containing the parasites collected every 30 min after the first hour for 3 h. The pooled water containing the miracidia was chilled on ice which caused the larvae to lose their motility

and sink to the bottom of the tube. The miracidia were then recovered by centrifugation at 1800 g, 4˚C for 10 min, after which the supernatant was visualised for miracidia before it was discarded. Finally, the miracidial pellet was resuspended in 1 ml of total RNA isolation reagent, TRIzol® (Invitrogen, Mount Waverley, Australia) then stored at -20˚C until the total RNA was isolated (see section 6.2.4).

6.2.3 Isolation of SJC cercariae

Forty *O. hupensis hupensis* snails kept under water were exposed to a bright light at room temperature for 3 h to release cercariae. Under these conditions, the majority of cercariae remain at the bottom of the beaker holding the snails, migrating over time to the surface of the water. Care was taken to reduce the movement of the beaker since cercariae will stick to glass or any object that comes in contact with them. Cercariae on the water surface were transferred to four 50 ml Falcon tubes. The tubes containing the cercariae were then centrifuged at 3000 g, 4˚C for 20 min, before the top 90% of water was discarded. The cercarial pellet was resuspended in the remaining water, transferred to two centrifuge tubes and centrifuged at 2000 g, 4˚C for 10 min. After discarding the supernatant the cercariae were re-suspended in 500 µl of TRIzol reagent.

6.2.4 Isolation of total RNA

The total RNAs from eggs, miracidia and cercariae were isolated using the protocols outlined in Chapter 4 section 4.2.2. The quality and quantity of the total RNA were assessed using a Bioanalyzer RNA Pico LabChip (Bioanalyzer) (Agilent, Santa Clara, USA).

6.2.5 Complementary DNA synthesis and amplification

Complementary DNA was synthesised from total RNA isolated from SJC eggs, miracidia, cercariae and adult male and female SJC and SJP worms (Chapter 4 section 4.2.2). Due to the limited amount of total RNA available, first strand cDNA was synthesised from individual 10 ng aliquots of total RNA using a Super "Switch Mechanism At 5′ end of RNA Template" (SMART) PCR cDNA synthesis Kit according to the manufacturer's instructions [171]. All the cDNA samples were amplified in an Eppendorf Mastercycler Gradient (Eppendorf) at 95˚C for 1 min followed by 20 cycles of 95˚C for 5 sec, 65˚C 5 sec, and 68˚C 5 min. The amplified samples were quantified by a ND-1000 spectrophotometer and stored in microfuge tubes at -20˚C until used.

6.2.6 Real Time PCR expression analysis of amplified cDNA

The expression of the selected genes of interest was analysed by real time PCR. The forward and reverse primers (Sigma-Aldrich) were designed from *S. japonicum* contigs (Table 6.2). All cDNA samples were diluted to 30 ng/µl and re-quantified by the ND-1000 spectrophotometer, and then 150 ng aliquots were combined with 10 µl SYBER® Green, 3 µl water (Sigma) and 2 µl (5 pmol) of forward and reverse primers in a 0.1 ml tube. All reactions were performed on a Rotor-Gene (3000) real time PCR (Corbett Research) and analysed by Rotor Gene 6 Software (Corbett Research). The real time PCR parameters were set by determination of primer melting temperature (58˚C) and addition of a melt curve (72˚ - 95˚C at 1˚C increments) to show primer viability. The gain of each experiment was set automatically by the software.

Table 6.2 Primers used for real time PCR to investigate gene expression in different schistosome life stages*.

Contig Name	Primer Pair Sequence (5'-3')	
	Forward	Reverse
Contig 3855	TTCGTCCTATTTGGGACTCATT	TGGCATAGCAATGCAAGTTC
Contig 6725	CCAAAGCTTCCAGGGTTACA	GGATGATCCAAACATGCTTTCT
Contig 5299	ATTCAGTCCCACTTCGTTGC	CCGGGATACCAATATATGCAC
Contig 8664	CAATTTATGAACGGGCAGGT	CGTGACAGTGACGGCAATAC
Contig 1966	AATGGTTGTCATGGTGGTGA	ACGTCTCGTAAGTCGGATTCA
Contig 7515	TGGTGCTAACTCATCCAACG	ACACACCGACATATTTTTGCAC
Contig 8540	CGATATCGTGAAACGGAACC	TGGATCTACATGCCTCCACA
Contig 4696	CAAACATTTGCTCTTCCAGTTG	CAGGACATATCGACTGGCATT

*For full details including product size, primer and probe position in the complete assembled sequence and putative protein description, see supplementary File 2.

6.2.7 PCR amplification of probe products

In order to show the presence of an expression product in real time PCR experiments where signal could not be detected, primers designed for Contig 4696 were used together with SJC male and female templates using an Eppendorf Mastercycler. The same total RNA from SJC males and SJC females, used for production of the amplified cDNA products, were combined with random hexamers (Invitrogen) to produce cDNA. Forward and reverse primers for Contig 4696 were combined together in a 0.5 ml PCR tube with GoTaq® DNA polymerase (Promega, Annandale, Australia). The tubes containing the Contig 4696 taq mix were then placed in a Eppendorf Mastercycler Gradient at 94˚C for 3 min followed by 36 cycles of 94˚C for 1 min, 58˚C 1.5 min and

72˚C 2 min. Following this, the products were visualised using a 1% Tris-acetate (TAE) gel.

6.3 Results and Discussion

Real time PCR was used to investigate gene expression patterns between and within the SJC and SJP strains of *S. japonicum* to identify stage specific expressional differences of selected genes of interest. As previously discussed in Chapter 4 (section 4.3) it is important to monitor differences in the quality of isolated total RNA samples, as this can compromise the measurement of gene expression. Extreme care was taken to ensure that all total RNA samples were of high quality and quantity (A*260*/A*280* nm $\geq$1.7 in nuclease-free water [127]) with minimal degradation, as recommended by Bustin and Nolan [125] (Supplementary File 1).

6.3.1 Real time PCR validation

An important step in real time PCR is the demonstration that all signals observed are a result of amplification of total RNA and not from contaminating DNA [172]. RNeasy silica membranes (QIAGEN, Doncaster, Australia) were used to remove the DNA in these experiments so that genomic contamination of total RNA samples was not an issue. All real time PCR experiments were undertaken in duplicate, the confidence threshold (CT) of the second set was normalised to the first set before evaluation (see Appendix B). The final copy and fold change values shown in Tables 6.3-6.9 were calculated from the mean of four or more original and normalised CT values. Although it is known from previous studies that real time PCR and microarrays may differ in expression values [173], the majority of data that were obtained indicated similar expression patterns to each other (see Tables 6.3-6.9). Previous studies have shown that

the 18s rRNA is a good choice as a house-keeping gene for normalisation of schistosome real time PCR experiments [107, 108]. Unfortunately, this house-keeping gene could not be used in these experiments due to the type of primer (which attaches to the poly A 3' end of the RNA template [171]) employed in first strand cDNA synthesis [155]. Additionally, the variation of GAPDH between male and female schistosome worms observed in previous studies made this gene unsuitable for use in the normalisation of the experiments described in this chapter. Therefore as a result, primary normalisation was calculated by careful quantification of sample cDNA templates. In future studies, additional house keeping genes may be discovered by microarray analysis, to reveal probes with similar expression patterns through out different life cycle stages.

6.3.2 Retrotransposons

As observed in the SJC and SJP strain comparisons described in Chapter 4 (section 4.3.2), one particular group of genes that were consistently up-regulated in SJC were retrotransposons [4]. It was hypothesised that variation in retrotransposon expression may reflect the fact that the SJC isolate used in the current study was obtained directly from the field and was probably under increased external selection pressures [135, 136] compared with the SJP isolate that has been maintained for a considerable period (over 14 years) in the laboratory at QIMR [4]. In order to further explore the differential expression of retrotransposons and to provide greater putative description, probe Contig 3855 [retrotransposon like sequence] was chosen for real time PCR experimental analysis (Table 6.3). The complete assembled sequence of the contig was subjected to BLAST (NCBI) which provided the same description of the original probe. Additionally, further analysis by 6-frame translation of the complete assembled

sequence was unable to show any putative protein regions that could be identified through BLAST analysis.

Table 6.3 Real-time PCR data for Contig 3855 [retrotransposon]*

Sample name	mean-CT	CN	Relative Fold Difference
SJP males	-	-	-
SJP females	24.29 ± 0.80	44357.59	1194.09
SJC males	25.17 ± 0.20	21884.80	589.13
SJC females	18.52 ± 0.17	4462763.84	120135.76
SJC eggs	26.19 ± 1.41	9711.59	261.43
SJC miracidia	28.86 ± 2.47	1147.80	30.90
SJC cercariae	33.16 ± 0.40	37.15	1.00

Microarray results	Strain/Sex up-regulated	Relative Fold Difference	p-value
Strain mixed sex	SJC	11.10	0
Strain females	SJC	7.71	0
SJC gender	F	14.07	0

*The real time PCR results show the fold differences between the *S. japonicum* Philippine (SJP) and *S. japonicum* Chinese (SJC) adult worms (both males (M) and females (F)), and SJC eggs, miracidia and cercariae for contig 3855. Fold differences shown are calculated from the lowest copy number (CN) of the sample list. All Contig 3855 CNs are calculated using the formula: CN = 10^ (-0.347 * mean-CT + 13.075) (A sample calculation is presented in **Appendix B**). All CTs shown are a mean of 4 or more replicates. The microarray results listed below each set of real time PCR data were generated from supplementary Tables 6-8, 11 and 12. The symbol (-) indicates the associated sample did not generate any products that were detectable by real time PCR and thus were not included in any calculation of fold change.

The real time PCR analysis of Contig 3855 confirmed the results of the microarray data (Table 6.3). The expression signals showed different expression patterns between the SJC life cycle stages examined but none in any of the SJP male

worm samples. As previously discussed (Chapter 4 section 4.3.2) retrotransposons respond to environmental stress by shaping genome size and stability through effects on retrotransposon replication rates [131, 132] The results may be indicative of subsequent shut down of these genes in the eggs, miracidia and cercariae. In contrast, previous studies on schistosomes and *Drosophila sp*. have showed that retrotransposons are always present scattered throughout the genome and in high copy number on both sex chromosomes in schistosomes [131, 174-176]. Therefore, the complete absence of signal from this probe in the SJP male worm real time PCR and SJP/SJC male microarray analysis suggest that this gene may not in fact be a retrotransposon [174]. In order to confirm this hypothesis, additional work will need to be undertaken on the characterisation of this probe including obtaining its complete DNA sequence.

6.3.3 Gender-specific probe expression

In Chapter 5 (section 5.3.1) microarray analysis identified 1,163/1,016 and 1,047/897 gender-specific probes (SJP/SJC: male/female respectively) that were differentially expressed between adult worms [4]. One of the differentially expressed probes was Contig 6725 [Human DNA sequence], highly up-regulated in female worms of both strains in microarray analysis. Data from real time PCR analysis confirmed this, showing there was a higher signal in both the SJC and SJP female worm samples (Table 6.4).

Table 6.4 Real-time PCR data for Contig 6725 [female protein]

Sample name	mean-CT	CN	Relative Fold Difference
SJP males	-	-	-
SJP females	24.98 ± 0.83	57.10	19.70
SJC males	17.61 ± 0.37	9815.84	3386.08
SJC females	14.89 ± 0.54	65165.44	22479.54
SJC eggs	29.26 ± 0.20	2.90	1.00
SJC miracidia	27.46 ± 1.21	10.14	3.50
SJC cercariae	-	-	-

Microarray results	Strain/Sex up-regulated	Relative Fold Difference	p-value
Strain mixed sex	SJC	2.23	7.92E-09
SJP gender	F	63.28	0
SJC gender	F	53.31	4.17E-37

For Table description please refer to Table 6.3 with the exception of the CN calculations; all Contig 6725 CNs were calculated using the formula: CN = 10^ (-0.303 * mean-CT + 9.327).

Further investigation of the complete assembled sequence for Contig 6725, including a 6-frame translation, identified a similarity to Fs800 [Female protein, *S. mansoni*] [177]. It is noteworthy that this probe is included on the microarray as TC 11954 (not differentially expressed in any of the *S. japonicum* microarray experiments undertaken in this thesis) but contains no nucleotide sequence similarity to Contig 6725. As expected from a gender-specific product, no significant variation could be detected between pools of SJC eggs and miracidia. Since there was signal detected from both eggs and miracidia, the absence of expression from cercariae may be the result of the domination of male cercariae after prolonged storage of *O. hupensis* snails at 4ºC prior to shedding [178].

The function of Fs800, which is found only in vitelline cells, has been described as being unrelated to eggshell formation but rather to other aspects of oogenesis [177]. As far as I am aware, there are no further details reported on this protein; consequently its complete function remains unclear. The results obtained in this study provide further expression characterisation of this probe, strengthening the evidence of sexual dimorphism which may assist in unravelling the biological complexities of *S. japonicum*.

6.3.4 Strain and gender differences in enzyme expression

As discussed in Chapter 4 (section 4.3.1) some probes showed strain-specific up-regulation in all microarray experiments. One of these probes, Contig 5299 [*H. sapiens* PAC clone RP5-1140G11 from 14q24.3], showed SJC up-regulation compared to SJP in all strain-associated microarray experiments undertaken. Two of the real time results confirmed this with the exception of a low SJC female signal (Table 6. 5). Additionally, a greater Contig 5299 real time PCR signal was noted in males compared to females, which was not evident in the microarray results. This was not reported because the Contig 5299 probe p-value in the gender-associated experiments was >0.001; consequently these data were not used in the production of the final Tables.

Table 6.5 Real-time PCR data for Contig 5299 [ADP-ART]

Sample name	mean-CT	CN	Relative Fold Difference
SJP males	13.89 ± 0.33	36618.96	4.37
SJP females	14.35 ± 0.46	27436.06	3.27
SJC males	13.06 ± 0.22	61650.63	7.35
SJC females	16.24 ± 0.27	8383.89	1.00
SJC eggs	16.12 ± 0.49	9007.99	1.07
SJC miracidia	14.60 ± 0.40	23452.87	2.80
SJC cercariae	14.06 ± 0.23	32918.48	3.93

Microarray results	Strain/Sex up-regulated	Relative Fold Difference	p-value
Strain mixed sex	SJC	1.42	0
Strain males	SJC	1.74	0
Strain females	SJC	1.80	0

For full Table description please refer to Table 6.3 with the exception of the CN calculations; All Contig 5299 CNs were calculated using the formula: CN = 10^ (-0.273 * mean-CT + 8.356).

The 6-frame translation and subsequent BLAST analysis of the predicted amino acid sequence from Contig 5299 identified an "adenosine diphosphate (ADP) ribosylglycohydrolase" (ART) conserved region. ADP-ribosylation is an enzyme-catalysed protein modification in which the ADP-ribose moiety is transferred from nicotinamide adenine dinucleotide (NAD^+) to a specific amino acid in a target protein [179]. In bacteria, ADP-ribosylation is a reversible post-transcriptional modification used to regulate endogenous protein functions [179]. Glowacki *et al*. [179] showed that most ART genes exhibit a tightly restricted expression pattern with relativity low transcript levels reflecting the regulatory function of the gene products. It has been shown that ARTs play an important function in bacterial virulence [179]. Additionally, previous studies have shown that SJC worms are more virulent than SJP [61], which may reflect the up-regulation of this enzyme as shown here. If this is also true in

schistosomes, then this protein may hold promise as a target for therapeutic intervention against SJC.

The probe Contig 8664 [*S. japonicum* clone ZZD1474 mRNA] identified through protein translation as an enzyme was up-regulated in SJP compared with SJC females (Table 6.6). This probe was also shown to be female-specific in microarray analysis, but not in real time analysis, the conflicting results possibly due to splice variance. BLAST identification of Contig 8664 amino acid translation showed the putative protein contained a conserved proton Adenosine 5′-triphosphatase (ATPase) subunit region. As discussed in Chapter 4 (section 4.3.4) the mode of action of praziquantel on schistosomes has not been fully elucidated, but it may be related to the disruption of Ca^{2+} homeostasis in adult worms [151]. Previous studies have shown that ATPases play an important role in the homeostatic control of calcium within schistosomes [180, 181]. Here, the expression of the ATPase was shown to be significantly up-regulated in adult worms compared to egg, miracidia and cercariae with the exception of SJC females (Table 6.6). Praziquantel is reportedly more effective against the SJC strain [61] and in males over females [182]; therefore the up-regulation of the ATPase in SJP females may show a link between the effectiveness of this drug and the activation of calcium intake. Additionally it may be presumed that the ineffectiveness of praziquantel on juvenile worms [40] may also be demonstrated by the down regulation of this probe in cercariae compared to the SJC /SJP male and SJP females.

Table 6.6 Real-time PCR data for Contig 8664 [ATPase]

Sample name	mean-CT	CN	Relative Fold Difference
SJP males	14.01 ± 0.66	22003.17	8.19
SJP females	13.46 ± 0.28	31803.87	11.84
SJC males	12.52 ± 0.24	59149.63	22.02
SJC females	16.60 ± 0.24	3965.46	1.48
SJC eggs	17.18 ± 0.65	2685.66	1.00
SJC miracidia	16.16 ± 0.45	5311.97	1.98
SJC cercariae	15.05 ± 0.23	11025.06	4.11

Microarray results	Strain/Sex up-regulated	Relative Fold Difference	p-value
Strain females	SJP	2.38	1.96E-26
SJP gender	F	7.31	8.79E-09
SJC gender	F	5.22	8.12E-24

For full Table description please refer to Table 6.3 with the exception of the CN calculations; all Contig 8664 CNs were calculated using the formula: CN = 10^ (-0.288 * mean-CT + 8.378).

The expression pattern of Contig 1966 [*S. japonicum* clone ZZD209 mRNA] in the microarray analysis shown here is noteworthy. This is because the gender-specific expression association was opposite between the SJP and SJC strains (Table 6.7). The real time PCR analysis (combined result of 4 separate experiments) confirmed this expression pattern, including a significant drop in signal from the adult worms to eggs and miracidia (Table 6.7). The protein BLAST analysis of the translated complete assembled sequence of Contig 1966 identified another conserved region of an enzyme, secreted cathepsin L 1.

Table 6.7 Real-time PCR data for Contig 1966 [cathepsin L 1]

Sample name	mean-CT	CN	Relative Fold Difference
SJP males	20.68 + 0.66	5277.68	50375.23
SJP females	22.10 ± 0.48	2045.48	19524.04
SJC males	20.06 ± 0.47	7972.47	76096.82
SJC females	19.23 ± 0.22	13856.14	132256.18
SJC eggs	36.49 ± 1.35	0.14	1.36
SJC miracidia	36.95 ± 1.82	0.10	1.00
SJC cercariae	22.94 + 0.60	1170.18	11169.28

Microarray results	Strain/Sex up-regulated	Relative Fold Difference	p-value
Strain mixed sex	SJC	4.63	0
SJP gender	M	3.49	3.00E-05
SJC gender	F	2.97	1.71E-06

For full Table description, please refer to Table 6.3 with the exception of the CN calculations; all Contig 1966 CNs were calculated using the formula: CN = 10^ (-0.289 * mean-CT + 9.698).

Schistosomes express at least two cathepsin L proteinases, SmCL1 and SmCL2 (both identified in *S. mansoni*) [183]. Cathepsin L1 is used in the metabolism of haemoglobin by the adult female worms, whereas L2 is located around the uterus and the gynaecophoric canal [184]. The male association shown by the putative cathepsin in SJP reported here is unusual as previous studies have shown that cathepsin L1 is expressed in female schistosomes and is not associated with copy number polymorphism [15, 111, 185, 186]. In order to further elucidate to the possible function of the contig, the primer product and probe section of the complete assembled sequence were translated and subjected to BLAST. This revealed that this soluble protein contained a peptidase CIA conserved region C1, that is a subfamily composed of cysteine peptidases similar to papain, including the mammalian cathepsins B, C, F, H, L, K, O, S, V, X and W [169]. Of potential significance, an unexpectedly high signal was detected in cercariae (Table 6.7). The microarray used here contains several

cathepsin L derivatives which were differentially expressed in variable patterns throughout all microarray experiments. Chai M, McManus D P, McInnes R, **Moertel L**, Tran M, Loukas A, Jones M K and Gobert G N. [109] reported several cathepsins were up-regulated in adult worms when compared to lung-stage schistosomula using this microarray. An investigation of Contig 1966 in the supplementary material of Chai M, McManus D P, McInnes R, **Moertel L**, Tran M, Loukas A, Jones M K and Gobert G N. [109] showed this probe was not differentially expressed at a p-value $\leq$0.001. Additional investigation of the original FE Tables showed that there was hybridization from Contig 1966 (GEO [109]) but because the p-value was >0.001 the data were not used. Further expression experiments and localisation of Contig 1966 may provide a greater understanding of the biological role of cathepsins in schistosomes.

6.3.5 Tetraspanins

Recently, Tran *et al*. [187] reported the discovery of protective tetraspanin antigens located on the surface of *S. mansoni*. Antibodies raised against the two tetraspanins *Sm-tsp-1* and *Sm-tsp-2* were able to produce significant protection against schistosomes in mice with up to 29-53% reduction in adult worm burdens and 69-75% reduction in faecal egg counts at p-values <0.0001 for both molecules [187]. The microarray analysis reported here showed the up-regulation of two unnamed probes, Contig 7515 [*S. japonicum* clone ZZD292] and Contig 8540 [*M. musculus* chromosome 1, clone RP24-406F14] in SJC adult worms. These probes were also up-regulated in adult males when compared to females in both SJC and SJP (Table 6.8). The BLAST search of putative protein identification showed that both probes potentially encode unique tetraspanin conserved regions. These domains were not repeated in any of the complete microarray assembled sequences reported here (Supplementary Table 1).

Real time PCR results confirmed the microarray results for contig 8540 and two of the four Contig 7515 samples (Table 6.8). The SJP and SJC standard error of mean-CT values for Contig 7515 (Table 6.8 (A)) overlapped each other which may have accounted for the inconsistencies reported between the real time PCR and microarray results. Previous characterisation of a family of tetraspanins from schistosomes revealed strain-specific differences between the Chinese and Philippine strain *S. japonicum* genomic sequences [188]. This may be the reason for the up-regulation observed in SJC adult worms in the microarray and real time PCR analysis reported here. The two putative tetraspanins represented by Contigs 7515 and 8540 have not been previously studied in detail. Further sequence analysis and subsequent comparison of the SJC and SJP products may provide an explanation of the observed differential expression.

Table 6.8 Real-time PCR data for two putative tetraspanins Contigs (A) 7515 and (B) 8540

(A) Sample name	mean-CT	CN	Relative Fold Difference
SJP males	07.86 ± 4.25	7982.97	3811.05
SJP females	20.04 ± 2.76	100.43	47.94
SJC males	13.32 ± 1.53	1123.44	536.32
SJC females	17.87 ± 2.00	219.05	104.57
SJC eggs	21.48 ± 3.56	59.76	28.53
SJC miracidia	30.81 ± 1.87	2.09	1.00
SJC cercariae	25.47 ± 0.12	14.28	6.82

(A) Microarray results	Strain/Sex up-regulated	Relative Fold Difference	p-value
Strain mixed sex	SJC	3.74	0
Strain males	SJC	3.25	3.45E-20
SJP gender	M	18.61	0
SJC gender	M	16.76	2.48E-25

(B) Sample name	mean-CT	CN	Relative Fold Difference
SJP males	14.44 ± 0.40	10214.73	13.01
SJP females	18.54 ± 0.66	785.05	1.00
SJC males	10.44 ± 0.57	125039.72	159.28
SJC females	16.42 ± 0.46	2962.80	3.77
SJC eggs	16.74 ± 2.59	2426.82	3.09
SJC miracidia	17.91 ± 5.29	1164.75	1.48
SJC cercariae	12.93 ± 0.12	26223.25	33.40

(B) Microarray results	Strain/Sex up-regulated	Relative Fold Difference	p-value
Strain mixed sex	SJC	3.70	1.76E-18
Males	SJC	9.06	5.93E-13
Females	SJC	10.34	1.41E-19
SJP gender	M	4.64	1.21E-06
SJC gender	M	4.61	4.22E-33

For full Table description please refer to Table 6.3 with the exception of the CN calculations; all Contig (A) 7515 CNs were calculated using the formula: CN = 10^ (-0.156 * mean-CT +5.128), where as all Contig (B) 8540 CNs were calculated using the formula: CN = 10^ (-0.272 * mean-CT + 7.937).

The real time PCR signal from the cercariae may reflect the shed of male dominated parasites referred to above (section 6.3.3) for both Contig 7515 and Contig 8540. The significant differential expression shown here between male and female worms may represent some unreported localisation or splice variation of these putative tetraspanins. This expression pattern is similar to another Contig 5872 [*S. mansoni* CD63-like protein Sm-TSP-2 mRNA, complete cds] also up regulated in male worms compared with females; 2.97 and 4.27 fold for SJP and SJC, respectively (Supplementary Table 11 and 12). Male and female worms of the same strain were used in gender-associated experiments; thus any base mismatch would not promote a change in differential expression. Since tetraspanins are now regarded as important vaccine targets in *S. mansoni* [187], it is important that further study undertake similar vaccine/challenge experiments with *S. japonicum*, to localise and reveal their precise biological function.

6.3.6 A house-keeping gene of interest

The majority of the probes reported here are differentially expressed in adult SJC and SJP worms. In contrast, some that showed no differential expression had sequence similarity to biologically important schistosome genes. One of these probes, Contig 4696 [*S. japonicum* clone ZZD10], showed nucleotide sequence similarity to *S. mansoni* insulin receptor tyrosine kinase 3 (RTK-3) mRNA. Real time PCR analysis showed a significant down-regulation of signal from SJC miracidia compared to the other stages, whilst there was no signal detected with the SJC female template (Table 6.9).

Table 6.9 Real-time PCR data for Contig 4696 [insulin receptor]

Sample name	mean-CT	CN	Relative Fold Difference
SJP males	25.38 ± 0.99	50369.94	6.76
SJP females	25.87 ± 0.89	40681.99	5.46
SJC males	23.99 ± 0.26	92334.18	12.40
SJC females	-	-	-
SJC eggs	28.25 + 0.85	14425.14	1.94
SJC miracidia	29.77 ± 0.49	7447.28	1.00
SJC cercariae	23.38 ± 0.66	120526.84	16.18

For full Table description please refer to Table 6.3 with the exception of the CN calculations; all Contig 4696 CNs were calculated using the formula: CN = 10^ (-0.189 * mean-CT + 9.499). Notably, Contig 4696 was not differentially expressed in any of the microarray experiments listed here (p-value ≤0.001).

In a variety of eukaryotes, insulin-related peptides regulate key processes such as control of growth, reproduction, metabolism and ageing [189]. This regulation is activated by the binding of insulin to the receptor tyrosine kinases which are located in the surface of target cells [189]. In schistosomes the activation of this receptor has been shown to increase oxygen and glucose uptake in schistosomula, in addition to increasing the *in vitro* viability of cercariae, when insulin is added to their incubation medium [190, 191]. The presence of insulin receptors on cercariae is perhaps related to the preparation for immediate uptake of glucose upon penetration of the mammalian host [192]. Once the cercariae have penetrated the skin they must take up host glucose to supplant their depleted reserves of glycogen in order to transform into schistosomula [192]. This activation of absorption may therefore be promoted by the presence of insulin in host blood. The up-regulation of the Contig 4696 probe in shed cercariae compared to eggs and miracidia may reflect biochemical changes associated with preparation of the schistosome for penetration and rapid development in the mammalian host. Additionally, the up-regulation of this probe in adult worms, except SJC females

where no signal was detectable, may also reflect increased glucose requirements in this stage. The fact the SJC females produced no signal contrasts with a previous report of higher expression patterns in females compared with male worms [191]. A Contig 4696 product in both the male and female worm templates is shown (Figure 6.1); thus if a higher concentration of template was used in real time PCR a signal would have been detected in all samples. This was not possible in these experiments because of the low amount of starting material available. Considering this, the real time data shown here represent signal from a low concentration of PCR template. Therefore, a signal from a lowly expressed product may not be amplified by this method but does show relative expression from different templates. Since the insulin receptor is such an important regulatory molecule, the expression profile shown here may be important in the understanding of its role in the biology of schistosomes. Further studies on the expression and functional biology of this protein are clearly warranted.

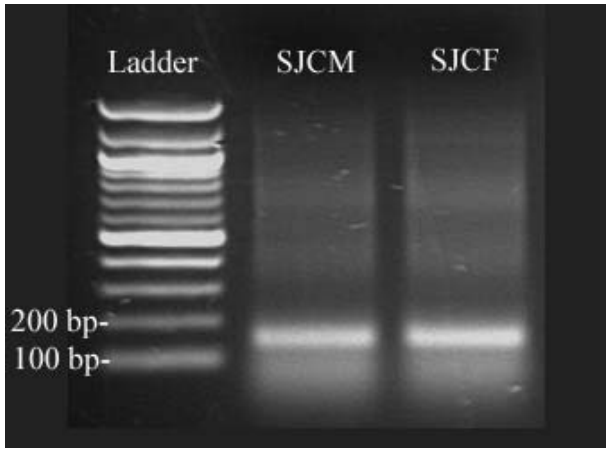

Figure 6.1 PCR amplification product of Contig 4696

The amplified PCR product of Contig 4696 resulting from 1 µg of SJC male (M) and female (F) cDNA templates on a 1% TAE gel (90 V for 50 min).

6.3.7 Summary

This chapter describes the comparative differential expression of a selected group of genes from adult worms and several developmental life stages of *S. japonicum*. The work described here shows the first use of real time PCR analysis to investigate differential expression of these genes selected by microarray analysis. The analysis indicated variable gene expression patterns throughout the schistosome life cycle and has demonstrated the practical use of template amplification for real time analysis. The eight target contigs described here serve as a basis for further investigation of differential gene expression during the schistosome life cycle and for studying sexual dimorphism between adult worms. Continual refinement and annotation of the microarray will aid in furthering such studies that will build on the gene expression profiles described here.