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**Differential gene expression, including *Sjfs800*, in *Schistosoma japonicum* females
before, during, and after male-female pairing**

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23

24 **Abstract**

25 Schistosomiasis is a prevalent but neglected tropical disease caused by parasitic
 26 trematodes of the genus *Schistosoma*, with the primary disease-causing species being
 27 *S. haematobium*, *S. mansoni*, and *S. japonicum*. Male-female pairing of schistosomes
 28 is necessary for sexual maturity and the production of a large number of eggs, which
 29 are primarily responsible for schistosomiasis dissemination and pathology. Here, we
 30 used microarray hybridization, bioinformatics, quantitative PCR, in situ hybridization,
 31 and gene silencing assays to identify genes that play critical roles in *S. japonicum*
 32 reproduction biology, particularly in vitellarium development, a process that affects
 33 male-female pairing, sexual maturation, and subsequent egg production. Microarray
 34 hybridization analyses generated a comprehensive set of genes differentially
 35 transcribed before and after male-female pairing. Although the transcript profiles of
 36 females were similar 16 and 18 days after host infection, marked gene expression
 37 changes were observed at 24 days. The 30 most abundantly transcribed genes on day
 38 24 included those associated with vitellarium development. Among these, genes for
 39 female-specific 800 (*fs800*), *eggshell precursor protein*, and superoxide dismutase
 40 (*cu-zn-SOD*) were substantially upregulated. Our in situ hybridization results in
 41 female *S. japonicum* indicated that *cu-zn-SOD* mRNA was highest in the ovary and
 42 vitellarium, *eggshell precursor protein* mRNA was expressed in the ovary, ootype,
 43 and vitellarium, and *Sjfs800* mRNA was observed only in the vitellarium, localized in
 44 mature vitelline cells. Knocking down the *Sjfs800* gene in female *S. japonicum* by

approximately 60% reduced the number of mature vitelline cells, decreased rates of pairing and oviposition, and decreased the number of eggs produced in each male-female pairing by about 50%. These results indicate that *Sjfs800* is essential for vitellarium development and egg production in *S. japonicum* and suggest that *Sjfs800* regulation may provide a novel approach for the prevention or treatment of schistosomiasis.

Author Summary

Schistosomiasis is a common but largely unstudied tropical disease caused by parasitic trematodes of the genus *Schistosoma*. The eggs of schistosomes are responsible for schistosomiasis transmission and pathology, and the production of these eggs is dependent on the pairing of females and males. In this study, we determined which genes in *Schistosoma japonicum* females were differentially expressed before and after pairing with males, identifying the 30 most abundantly expressed of these genes. Among these 30 genes, we further characterized those in female *S. japonicum* that were upregulated after pairing and that were related to reproduction and vitellarium development, a process that affects male-female pairing, sexual maturation, and subsequent egg production. We identified three such genes, *S. japonicum* female-specific 800 (*Sjfs800*), *eggshell precursor protein*, and superoxide dismutase, and confirmed that the mRNAs for these genes were primarily localized in reproductive structures. By using gene silencing techniques to reduce the amount of *Sjfs800* mRNA in females by about 60%, we determined that *Sjfs800* plays a key role

in development of the vitellarium and egg production. This finding suggests that regulation of *Sjfs800* may provide a novel approach to reduce egg counts and thus aid in the prevention or treatment of schistosomiasis.

Introduction

Schistosomiasis, also known as bilharzia, is a tropical disease caused by parasitic trematodes of the genus *Schistosoma*. Although it is one of the most prevalent tropical infectious diseases, with more than 240 million people in 78 countries infected and approximately 800 million people at risk, schistosomiasis has been drastically understudied [1-3]. The primary disease-causing species of *Schistosoma* are *S. haematobium*, *S. mansoni*, and *S. japonicum*, the latter of which is distributed in China, Indonesia, and the Philippines [1-3]. Disease burden assessments for schistosomiasis, based on the extent of end-organ damage and the associated morbidities related to malnutrition and chronic inflammation, indicate that the annual number of disability-adjusted life years lost is approximately 70 million [4]. Current control of schistosomiasis depends largely on a single drug, praziquantel; however, reliance on a single drug produces a precarious situation. Indeed, some studies have shown that isolates of schistosomes have reduced susceptibility to praziquantel [5-7]. Thus, additional novel strategies are urgently needed to prevent and control schistosomiasis.

S. japonicum has a complex developmental cycle that involves an aquatic snail as an intermediate host and a mammalian definitive host. In contrast to other trematode

species, these parasites are unique in that males and females need to pair to continue development. Pairing of schistosome females and males promotes female reproductive system maturation and the production of eggs, which are a primary means of schistosomiasis transmission and immunopathological lesions [8-10]. Maturation and maintenance of normal reproductive function in female *S. mansoni* require permanent pairing with the male. During pairing, germ cells in the reproductive organ differentiate into oocytes or vitellocytes, and some chemical or tactile stimulus exchange occurs between the male and female, leading to a cascade of changes during the pairing process [11-16]. However, the effects on female reproductive system development and the molecular mechanisms underpinning male-female pairing have not been completely determined, leaving myriad questions that require further study.

Ongoing work in our laboratory has indicated that during the development of *S. japonicum*, no male-female pairing occurs up to 16 days after the host is infected. Some pairing occurs 17 days post infection (dpi), and pairing is common 18 dpi. Paired females begin laying eggs approximately 24 dpi. Therefore, in the present study, to identify genes that likely contribute to pairing and reproduction, we used microarray technology to determine differential gene expression in females 16, 18, and 24 dpi. We identified genes that play critical roles in the development of the vitellarium and in the production of eggs, providing a clearer understanding of gene regulation before and after male-female pairing in the *S. japonicum* female and insights on schistosome reproduction biology.

Materials and methods

Ethical statement

All procedures performed on animals within this study were conducted using animal husbandry guidelines approved by the Animal Ethics Committee of Anhui Medical University (LLSC20140060).

Animals and parasites

Freshly shed wild-type cercariae of *S. japonicum* were harvested from infected *Oncomelania hupensis* that were purchased from the Hunan Institute of Parasitic Diseases in Yueyang, China. Female Kunming mice (6–8 weeks old) and New Zealand rabbits (4 months old) were obtained from the Laboratory Animal Center of Anhui Medical University. New Zealand rabbits and female Kunming mice were infected with 1000 or 50 cercariae, respectively, via the skin of the abdomen. After 16, 18, 24, 28, or 42 dpi the worms were washed out the hepatic portal vein using perfusion techniques. Male and female worms were manually separated. In order to collect *S. japonicum* eggs, liver tissues from rabbits 6 weeks post infection were homogenized and then subjected to consecutive fractional filtration. The filtrate was centrifuged. The supernatant and the tissue-containing layers were removed, leaving the egg-containing layer, which was diluted in 1.2% saline and passed through a nylon net (300 mesh, i.e., 300 holes per inch). All parasite samples were soaked in RNAlater (Invitrogen, Thermo Scientific, USA) and stored at –80°C until they were used for total RNA extraction.

RNA extraction, amplification, and labeling

Total RNA was extracted and purified using RNeasy micro kit (QIAGEN, #74004, GmBH, Germany) following the manufacturer's instructions, and the overall RNA quality was assessed using denaturing gel electrophoresis (Agilent Technologies, Santa Clara, CA, USA). Total RNA was amplified and labeled using a Low Input Quick Amp Labeling Kit, one-color (Agilent Technologies, #5190-2305), following the manufacturer's instructions. Labeled cRNA was purified using a RNeasy Mini Kit (QIAGEN, #74106).

Microarray construction and hybridization and subsequent data analysis

A schistosome genome-wide microarray was used for profiling gene expression in *S. japonicum* female 16, 18, and 24 dpi. Microarrays were printed on the Agilent custom Schistosoma 4 × 44K chip (design ID: 048766). There were eight specimens; thus, eight chips in total were needed. Each slide was hybridized with 1.65 µg of Cy3-labeled cRNA using a Gene Expression Hybridization Kit (Agilent Technologies, #5188-5242) with a Hybridization Oven (Agilent Technologies, #G2545A) according to the manufacturer's instructions. After 17 hours of hybridization had elapsed, the slides were washed in staining dishes (Thermo Shandon, #121, Waltham, MA, USA) with a Gene Expression Wash Buffer Kit (Agilent Technologies, #5188-5327), following the manufacturers' instructions. All of these aforementioned procedures were performed by Biotechnology Shanghai China. The slides were scanned with an Agilent Microarray Scanner (Agilent Technologies, #G2565CA) using the following default settings: dye channel, green; scan resolution,

5 μ m; PhotoMultiplier Tube, 100% and 10%, 16 bit. Data were extracted with Feature Extraction software, version 10.7 (Agilent Technologies). Raw data were normalized using the Quantile algorithm, GeneSpring software, version 11.0 (Agilent Technologies). Outlier probes were identified, and their contribution was reduced at the reported gene expression level. The expression value of a gene was a weighted average of all forward or reverse probe sets when both background correction and quantile normalization were performed.

Bioinformatics analysis

The mRNA and expressed sequence tag transcripts highly enriched in *S. japonicum* 16, 18, and 24 dpi were retrieved from the National Center for Biotechnology Information Entrez Gene database (<http://www.ncbi.nlm.nih.gov/gene>) based on fold change ($FC = \text{Signal A} / \text{Signal B}$) values ($FC \geq 2$, three biological replicates; $FC \geq 3$, two biological replicates). Student's *t*-test was used to determine genes differentially expressed between one stage and the other ($p < 0.05$). All analyses were conducted using the online SBC Analysis System of Shanghai Biotechnology Corporation (<http://sas.shbio.com>).

Quantitative PCR (qPCR)

Thirteen genes whose expression levels were increased and two genes whose expression levels were decreased in females after pairing relative to those levels before pairing were selected for validation using qPCR. Total RNA (500 ng) from the females was reverse transcribed into first-strand cDNA using a PrimeScript RT Reagent Kit (TaKaRa & Clontech, #RR037A, Japan) according to the manufacturer's

instructions. Each 10 µL of PCR reaction contained 10 µL of 2× SYBR Premix Ex Taq II, 1 µL of cDNA, 1.6 µL of the forward and reverse primer pair, 0.4 µL of 50× ROX Reference Dye, and 7 µL of sterile water. The PCR cycling conditions were as follows: 95°C for 30 s, followed by 40 cycles of 30 s denaturation at 95°C and 1 min annealing and extension at 60°C. A dissociation step (95°C for 15 s, 60°C for 1 min, and 95°C for 15 s) was performed to confirm the amplification specificity for each gene. A reliable reference gene for transcriptomic analysis of *S. japonicum*, proteasome 26S subunit, non-ATPase 4 (*PSMD4*), was used as a control gene in the assays. The PCR primers were designed using Primer Premier 5 software (Table 1). PCR reactions were performed in technical triplicates using the StepOnePlus Real-Time PCR System (Applied Biosystems). The relative expression level of each gene was analyzed using SDS 1.4 software (Applied Biosystems).

Table 1. Quantitative PCR Primers (5' to 3').

Gene Name	Gene ID	Sense Primer	Anti-sense Primer
unknown	226471383	ATTCACCACAACCCACT	TATTGCCACCTCCACTT
trematode eggshell synthesis	226473101	ATCCACGTCTTACCAGA	AGCAGCGATACTACCTT
superoxide dismutase	226474963	CCATTCAACATGCGTCC	CTGCCTTCATCTGGATTCTT
keratin9	226477615	ACAGCTACGGAAATGC	CAATAATAGGAGGGTGTC
prostatic spermine-binding protein	226477985	GATTATGCTGATGTGAG	CGTTTGATTCTGTCTA
myoglobin 1	226487201	TCAGGGACTTGATGCTA	CAACTGGTCGAGTTCTAT

annexin	226469397	TCCATTCGGTTTACTCT	TTCAGCAATGTCCCTAG
cytochrome b	56046805	AAGAAGTTGGTGGTGG	GTGCCTTTGAGGTTGTCC
female-specific protein 800	60600619	TGGAAACGAAAGTGAG	CTGGAATTGAAAGGACC
tetraspanin-1	56757404	AAGGTAAAGGTGGTAGC	CAATGAATGCCGATAAG
tyrosinase precursor	56757710	TGGTGTTTGTTTCCCTA	CTCTATTACCACCTCTTTGA
putative transmembrane protein	56757833	TAGTTGGGAGACTTTGC	TACATTTCGGATTGCTG
multivesicular body protein 5	226467851	TTGCTTCAAGGCGGCT	CCGTGTTGTTTATTGGGAC
unknown gene	226477693	TGGTGGTCCAGATTGTT	CATAGTAGTCATTTCCGTAG
gshell precursor protein	226478597	ATGACTACAACCTCCGACTAC	CTCTGACATCTAAACGACCA

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192 Total RNAs from eggs, cercariae, schistosomula, and females 24 and 42 dpi were
193 extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions.
194 The total RNA concentration and purity were measured using a NanoDrop 2000
195 (Thermo Fisher). Quantitative PCR was performed as described above using primer
196 (Table 1) combinations to amplify gene transcripts of *S. japonicum* female-specific
197 800 (*fs800*), superoxide dismutase (*cu-zn-SOD*), and *eggshell precursor protein*.

198 In situ hybridization

199 Riboprobes were synthesized according to previously published methods [17].
200 Briefly, probes were synthesized from restriction enzyme-digested DNA according to
201 the orientation of the insert in pSPT18 using a DIG RNA Labeling Kit (SP6/T7)
202 (Roche, #111750251910, Germany) labeled with digoxigenin. For whole-mount in

situ hybridization, trematodes were fixed in 4% paraformaldehyde for 45 min and dehydrated in methanol. Following being bleached in 6% hydrogen peroxide in methanol to prevent tanning of the vitellaria, trematodes were permeabilized using proteinase k (TaKaRa & Clontech, #9034), incubated with prehybridization buffer (50% deionized formamide, 5× saline sodium citrate, 1 mg/mL yeast RNA, 1% Tween 20) for 2 h and then hybridized (prehybridization buffer with 10% dextran sulfate) with a riboprobe at 56°C for 20 h. Excess riboprobe was removed by washing in 2× and 0.2× saline sodium citrate, followed by blocking in blocking reagent (Roche, #11175041910). The bound riboprobe was detected after incubation of trematodes in antidigoxigenin alkaline phosphatase–conjugated antibody (Roche) diluted 1:5000 in blocking reagent overnight at 4°C. The unbound antibody was removed by washing in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, and 0.1% Tween 20 at pH 7.5) for 4 h in twelve changes of buffer. After being washed, specimens were incubated in detection buffer (0.1 M Tris-HCl and 0.1 M NaCl at pH 9.5). Hybridization signals were detected by adding 200 µL of NBT/BCIP in detection buffer. After development, trematodes were washed in PBS then de-stained with 100% ethanol. Trematodes were mounted in 80% glycerol, and then microscopy and digital image capture were performed using an Olympus DP73 microscope.

RNA interference

The siRNAs (21 base pairs) were designed using the *Sjfs800* mRNA sequence (GenBank accession No., FN313803.1) with the Thermo Fisher website software (<https://rnaidesigner.thermofisher.com>) and chemically synthesized by Shanghai

GenePharma Co., Ltd. (China). Three siRNA sequences (siRNA1–3) that shared no
homology with any other *S. japonicum* gene based on an online analysis with BLAST
(National Center for Biotechnology Information) and the scrambled siRNA sequence
are shown in Supplementary Table 1. The results of RNAi were tested using qPCR as
previously described [18]. Briefly, Kunming mice were challenged percutaneously
with 60–70 cercariae and were humanely killed 28 dpi. The worms were obtained by
portal perfusion using RPMI 1640 medium at 37°C and were then incubated in
24-well plates (15 pairs/well) containing 1 mL of complete Basch medium, with half
of the medium exchanged every day. The medium was supplemented with 10 KU/mL
penicillin, 10 mg/ml streptomycin, 250 µg/mL amphotericin B (Sangon Biotech,
#B13540732, China), and 10% fetal bovine serum (Gibco, #10091148, Thermo
Scientific, USA). Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) was
used to transfect worms with one of the three *Sjfs800*-specific siRNAs, at a final
concentration of 100 nM, or RNAase-free water (mock, no siRNA). The
gene-silencing effect of each siRNA was determined using qPCR at the end of a 72-h
cultivation period. The worms were then transfected with either the siRNA that was
found to be most efficient or with the scrambled siRNA (control). The transfected
worms were cultivated for up to 10 days and transfected again with siRNA1 on the
fourth and seventh days; half of the medium was exchanged every day. After 10 days,
all the eggs in the medium were collected and counted using light microscopy. Adult
male-female pairings were also observed and counted. The siRNA silencing effects
and morphological changes were measured using qPCR and confocal laser scanning

microscopy (Leica TCS SP5, Germany), respectively.

Confocal laser scanning microscopy

The confocal laser scanning microscopy procedure has been described previously [19]. After 10 days of *Sjfs800*-specific siRNA1 treatment, the male-female paired couples were separated manually and fixed separately in a solution of 95% alcohol, 3% formaldehyde, and 2% glacial acetic acid. The trematodes were then stained in 2.5% hydrochloric carmine (Ourchem, #71009382, China) for 16 h at 37°C and de-stained in 70% acidic ethanol. After dehydration in an ethanol series for 1 min in each concentration, parasites were cleared for 1 min each in 50% xylene diluted in ethanol and 100% xylene, and then whole-mounted with neutral balsam (Sinopharm Chemical #36645, China) on glass slides. The reproductive organ morphology of the trematodes was examined using confocal laser scanning microscopy, with a 470-nm longpass filter and a 488-nm He/Ne laser under reflection mode.

Statistical analysis

Results were acquired from triplicate values representing three independent experiments with identical conditions. One-way analysis of variance and independent-sample *t* tests were used for data analysis with the SPSS, version 17.0, statistics software package. Data are expressed as the mean \pm SEM, and $p < 0.05$ or $p < 0.01$ were deemed statistically significant.

Results

Microarray screening of differentially expressed genes in female *S. japonicum*

before, during, and after pairing with males

The results of microarray analyses, the most comprehensive and informative probe assay design to date, indicated that after removing the duplicates, signal intensities were upregulated ($FC \geq 2$), for 132 sequences during the pairing stage and for 198 sequences in paired female trematodes. Many mRNA transcripts were differentially expressed before, during and after pairing, with most of these genes elevated in expression after pairing. The most highly increased gene products after pairing were associated primarily with oxygen metabolism, the metabolic machinery of egg production, and vitellarium development. The 30 differentially expressed genes with highest expression levels after pairing are given in Table 2.

279

280 Table 2. The Top 30 Differentially Expressed Genes in Female *S. japonicum* after Male-Female Pairing Relative to Levels Prior to Pairing.

281

ProbeName	Gene ID	Protein ID	P-values	Foldchange	Description
CUST_14058_PI428956223	226474963	FN316039.1	0.001295	574.7126437	Extracellular superoxide dismutase [Cu-Zn]
CUST_13165_PI428956223	226473101	FN315505.1	0.021586	462.9629297	Trematode Eggshell Synthesis
CUST_21221_PI428956223	257207815	FN327835.1	0.020981	393.7007874	Protein matches (BLink) hsp40 subfamily A members 124
CUST_15298_PI428956223	226477615	FN316795.1	0.01517	294.9852507	keratin 9
CUST_15773_PI428956223	226478597	FN317063.1	0.035783	289.017341	eeggshell precursor

CUST_449_PI428956223	56758543	AY815680.1	0.041391	283.286119	protein
					similar to B61146
					hypothetical protein
					(eggshell gene region)
CUST_15773_PI428956223	226477693	FN316810.1	0.017062	252.525522	unknown
CUST_21010_PI428956223	257207472	FN327620.1	0.029122	157.9778831	1atypical protein kinase C
CUST_25908_PI428956223	257215731	FN330801.1	0.026122	149.7000599	DNA damage-responsive
CUST_12207_PI428956223	226471111	FN314905.1	0.04503	145.7725948	protein 48
CUST_4027_PI428956223	60600619	AY810878.1	0.038204	85.68980291	serine/threonine kinase-1
CUST_7243_PI428956223	56756748	AY814814.1	0.03629	71.73601148	Female-specific protein
CUST_21060_PI428956223	257207522	FN327670.1	0.013621	44.98353526	800
					similar to histidine kinase
					DhkM

						heterogeneous	nuclear
						ribonucleoprotein	
CUST_991_PI428956223	226469397	FN314445.1	0.018024	39.06715587	ESG-1 protein precursor		
CUST_384_PI428962778	7644645	AW736781.1	0.037435	36.43282441	hemoglobin	subunit	
						alpha-1/2	
CUST_15482_PI428956223	226477985	FN316955.1	0.035783	35.52397869	Prostatic		
						spermine-binding	protein
						precursor	
CUST_11368_PI428956223	226469397	FN314445.1	0.026528	30.82413089	annexin		
CUST_5987_PI428956223	56753799	AY813365.1	0.039938	27.21671965	transmembrane		9
						superfamily member	
CUST_15466_PI428956223	226477953	FN316939.1	0.000743	23.10313701	unknown		
CUST_7706_PI428956223	56757833	AY815325.1	0.02429	22.54660519	putative epiplakin 1		

CUST_7411_PI428956223	56757114	AY814997.1	0.025618	22.38696546	Contains	repetitive
CUST_999_PI428962772	56046805	CV738262.1	0.048425	22.00714633	sequences	
CUST_713_PI428956223	60601043	AY811090.1	0.019922	21.02855408	cytochrome b	
CUST_3699_PI428956223	60599676	AY810494.1	0.002295	17.84851112	venom	allergen-like
CUST_17613_PI428956223	226482651	FN318196.1	0.040935	8.663126238	(VAL) 7 protein	
					similar to	labial-like
					protein	
					PRP4	pre-mRNA
					processing factor	4
					homolog B	
CUST_17613_PI428956223	2290411	U92488.1	0.037768	8.470548849	cyclophilin A	
CUST_1037_PI428956223	22164072	AF412216.1	0.04818	8.237366919	Vacuolar	proton
					translocating ATPase	116

kDa subunit a isoform1

Bioinformatics analysis of differentially expressed genes

To determine the potential function of these upregulated genes, the Gene Ontology (GO) functional categories were assessed. In the biological process GO category, the genes involved in metabolic and biosynthetic processes were more active in females during and after pairing than before pairing, indicating that nutritional acquisition is more crucial for paired than unpaired female trematodes, which is likely a reflection of the oviposition status. In the molecular function GO category, the genes involved in hydrolase activity were more active in females during and after pairing than before pairing. In the cellular component GO category, gene products localized to membrane-bound organelles were more enriched in females after pairing than before or during pairing.

Confirmation of differentially expressed genes by qPCR analysis

Fifteen differentially expressed genes were selected for confirmation using qPCR. The analysis for each gene was repeated three times, and *PSMD4* was used as the housekeeping gene. After normalization, the relative changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method. The expression levels of two genes, *annexin* and *tetraspanin-1*, were not consistent between the qPCR and microarray analyses. The results for the remaining thirteen gene expression levels were consistent across qPCR and microarray analyses (Fig 1).

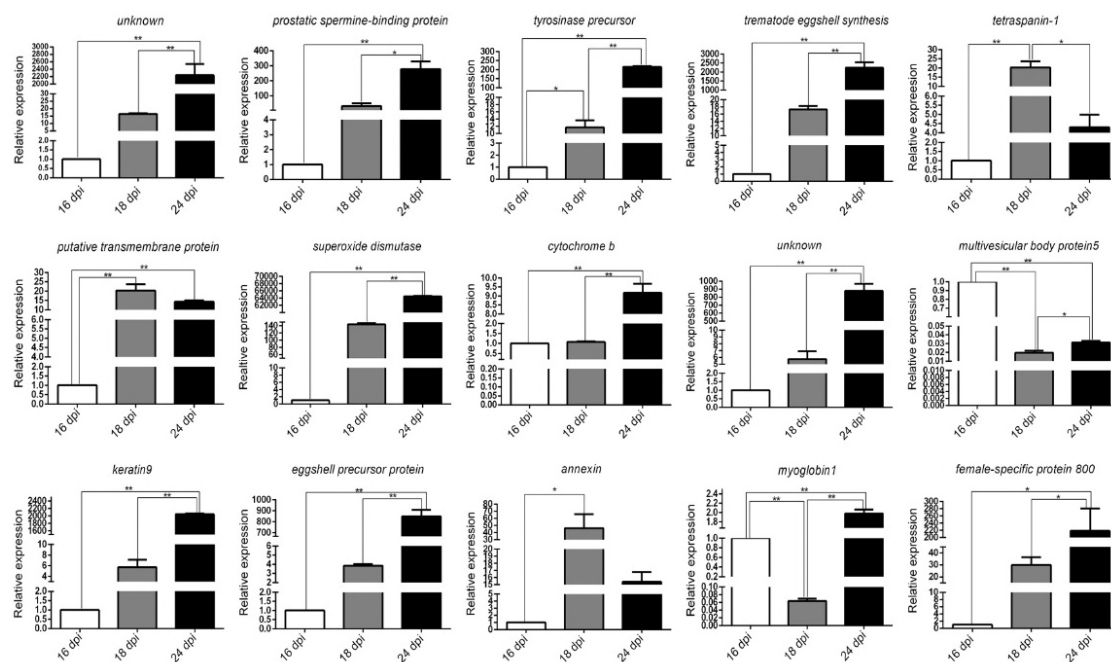


Fig 1. Validation of the Microarray Analysis Results by qPCR.

Data are normalized to the internal housekeeping control *PSMD4*. Expression levels of 15 selected genes were determined using the comparative method ($2^{-\Delta\Delta Ct}$). Data are expressed as the mean \pm SEM of three independent experiments ($n = 3$); independent-sample t test, * $p < 0.05$, ** $p < 0.001$.

Expression of *Sjfs800*, *cu-zn-SOD*, and *eggshell precursor protein* in the eggs, cercariae, and schistosomula of females 24 and 42 dpi

The gene expression levels for *Sjfs800*, *cu-zn-SOD*, and *eggshell precursor protein* were determined using qPCR of the eggs, cercariae, schistosomula (at 16 dpi), and female worms 24 and 42 dpi. As above, the results were normalized to the housekeeping gene *PSMD4*, and the relative expression was then determined using the $2^{-\Delta\Delta Ct}$ method. We found three genes that were highly expressed in females 42 dpi, and *eggshell precursor protein* and superoxide dismutase were also highly expressed

in females 24 dpi. The expression levels of *Sjfs800* were low in the eggs, cercariae, and schistosomula (at 16 dpi) and modestly increased in female worms at 24 dpi, with a further increase at 42 dpi (Fig 2). These results suggested that these genes may be associated with the differentiation and development of *S. japonicum*.

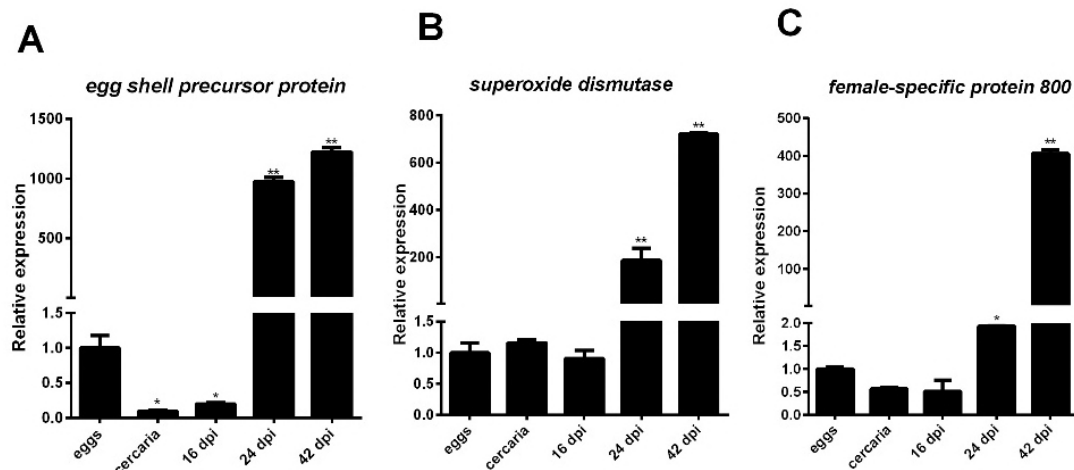


Fig 2. Relative mRNA Expression Levels of Female-Specific Protein 800, Superoxide Dismutase, and Eggshell Precursor Protein at Various Developmental Stages of *S. japonicum* as Measured by qPCR.

The eggs, cercariae, schistosomula (at 16 dpi), and female trematodes 24 and 42 dpi were analyzed (n = 3; * $p < 0.05$, ** $p < 0.001$, compared with the egg group).

Localization by in situ hybridization of *Sjfs800*, *cu-zn-SOD*, and *eggshell precursor protein* transcripts in female *S. japonicum*

A situ hybridization analysis showed the transcriptional activities of *cu-zn-SOD*, *eggshell precursor protein*, and *Sjfs800* in female *S. japonicum* 24 dpi (Fig 3).

Although *cu-zn-SOD* mRNA was located throughout much of the whole body, the

signal intensity in the ovary and vitellarium was markedly stronger than that in other parts. *Eggshell precursor protein* was substantially expressed in the ovary and ootype and was also found in the vitellarium. The expression of *Sjfs800* mRNA was observed only in the vitellarium.

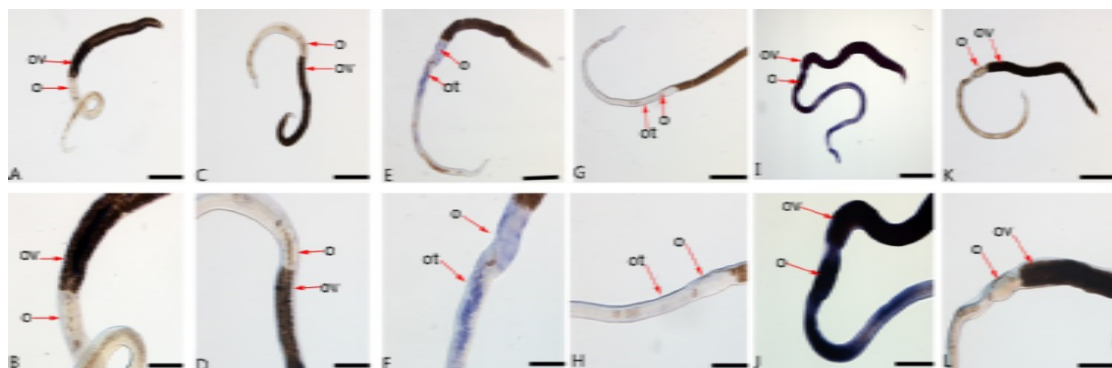


Fig 3. Localization of *Sjfs800*, *eggshell precursor protein*, and *cu-zn-SOD* Transcripts in Female *S. japonicum* 28 dpi.

Images are of whole-mount in situ hybridization results using DIG-labeled antisense (A, B, E, F, I, J) and sense (C, D, G, H, K, L) RNA probes. All three transcripts (A, B, C, D: *Sjfs800*; E, F, G, H: *eggshell precursor protein*; I, J, L, K: *cu-zn-SOD*) were detected. Abundant transcription of *Sjfs800* was observed in the vitellarium; transcription of *eggshell precursor protein* was observed in the ovary and ootype; and abundant transcription of *cu-zn-SOD* was observed in the ovary and the vitellarium. Abbreviations: ov, ovary; vt, vitellarium; ot, ootype. Scale bars: top row, 200 μm; bottom row, 100 μm.

qPCR analysis of *Sjfs800* mRNA levels after *Sjfs800*-specific siRNA transfection in trematodes 28 dpi

The *Sjfs800* mRNA levels were analyzed by qPCR to determine the effects of the

Sjfs800-specific siRNA transfection. First, the paired 28-dpi worms were transfected with one of the three siRNAs (siRNA1–3) targeting *Sjfs800*. After 3 days' cultivation, *Sjfs800* gene transcript levels were determined by qPCR. The reduction in *Sjfs800* transcription level following transfection with siRNA1 was 60% of that in the mock transfected group and negative control group, which was the highest efficiency among the three siRNAs tested. Thus, *Sjfs800*-specific siRNA1 was used in the ensuing experiments. worms were transfected with *Sjfs800*-specific siRNA1 or scrambled siRNA in vitro. The results of the qPCR analysis indicated that compared with the scrambled siRNA control group, the *Sjfs800*-specific siRNA1–treated group showed an approximately 60% reduction in *Sjfs800* mRNA levels on the tenth day later, and this experiment was repeated three times (Fig 4).

Effects of *Sjfs800* knockdown on pairing rate, egg production, and reproductive organ development

The number of male-female paired worms was counted on the tenth day to determine the effect of knocking down the *Sjfs800* gene on the pairing rate. We found that the pairing rate in the *Sjfs800*-specific siRNA1 transfected group was significantly lower than that in the scrambled siRNA transfected group. In addition, the number of eggs collected in the medium and counted using light microscopy was reduced approximately 50% in the *Sjfs800*-specific siRNA1 transfected group compared with that in the scrambled siRNA transfected group (Fig 4).

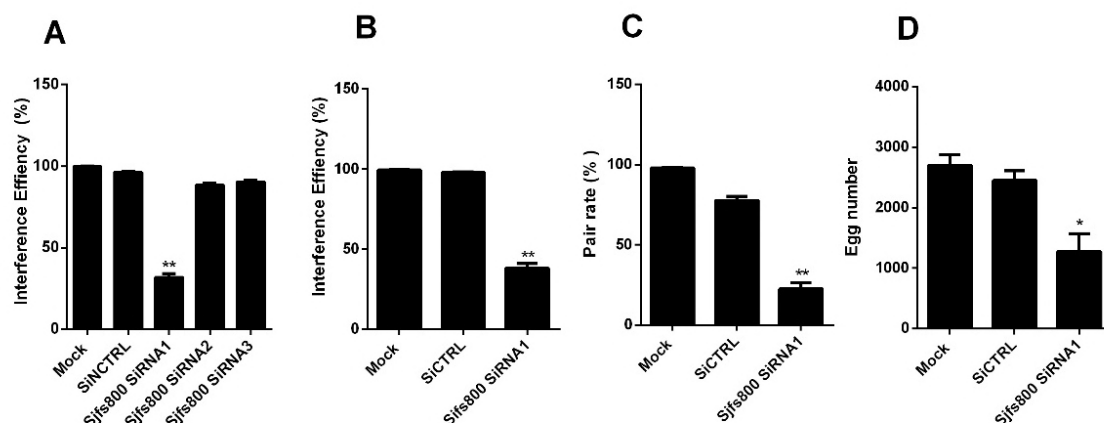


Fig 4. Effects of *Sjfs800*-specific siRNA Transfection.

A, To determine the *Sjfs800*-specific siRNA with the best interference efficiency in vitro as measured using qPCR, 28-dpi worms were transfected with one of three siRNAs (siRNA1–3) and were harvested 3 days later. The qPCR results showed the effects of siRNA1 were reduced by nearly 60% compared with those in mock-transfected group. B, Effects of siRNA1 on *Sjfs800* mRNA levels in worms were tested 10 days later. *Sjfs800* mRNA levels were normalized to the endogenous control *SjPSMD4*. The qPCR analysis results showed that the *Sjfs800* mRNA levels in the group transfected with *Sjfs800* siRNA1 were reduced by approximately 60% compared with those in the scrambled siRNA-transfected group. C, The male-female pairing rate of worms transfected with *Sjfs800* siRNA1 was reduced by approximately 70% compared with the rate in the scrambled siRNA-treated group. D, The eggs in the culture medium were collected on the tenth day and counted using light microscopy. SiNCTRL represents negative control siRNA group; SiCTRL represents scrambled siRNA.

The morphologic changes in the vitellarium of *Sjfs800* siRNA1–treated worms were observed using confocal laser scanning microscopy. The vitellarium was well developed in female worms that were transfected with scrambled siRNA. By contrast, transfection with *Sjfs800* siRNA1 suppressed the development and maturation of the vitellarium, with fewer mature vitelline cells found in the *Sjfs800* siRNA1–treated females than in the controls (Fig 5).

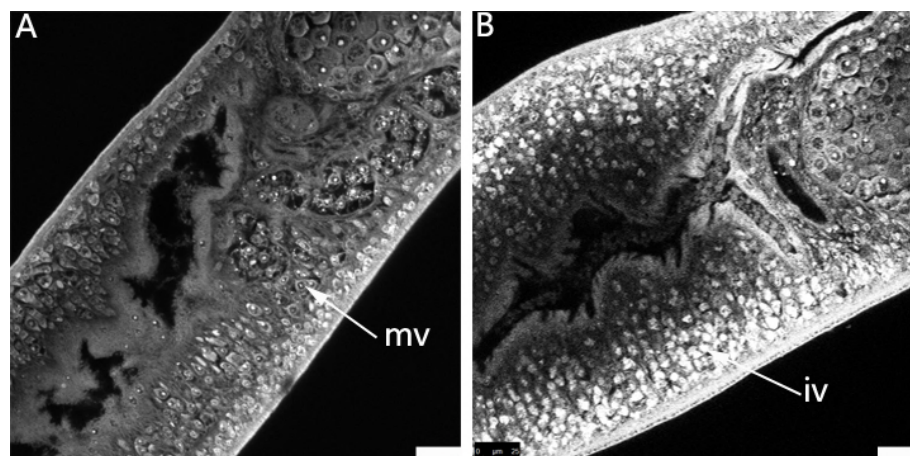


Fig 5. Confocal Scanning Laser Microscopy Images of the Vitellarium in *S. japonicum* Females After Transfection With *Sjfs800* siRNA1. A, Vitellarium from worms transfected with scrambled siRNA. Arrow points to mature vitelline cells (mv); B, Vitellarium from a trematode transfected with *Sjfs800* siRNA1. Arrow indicates immature vitelline cells (iv). Scale bars, 25 μm.

Discussion

Paired adult female schistosomes produce a large number of eggs, which are primarily responsible for the schistosomiasis disease pathology and are critical for

dissemination of the disease. The reproductive system of female schistosomes has been widely studied at the molecular level, and several studies have described transcriptomes that are differentially expressed before and after pairing in female schistosomes [16, 20-27]. For example, *SmFst* was recently identified as a regulatory molecule in the transforming growth factor β pathway that is pairing dependently transcribed in the male gonad, likely facilitating processes leading to male competence [21]. Lu et al. described pairing-induced processes within the gonads, including stem cell-associated and neural functions, by analyzing gonad-specific and pairing-dependent transcriptomes [22]. In 2017, Wang et al. mapped the dynamic transcriptome changes in male and female *S. japonicum* throughout the sexual developmental process from pairing to maturation to identify biogenic amines and insect-like hormones that regulate reproduction development in *S. japonicum* [16].

In the present study, gene microarray analysis was used to screen for differently expressed genes before and after pairing of the *S. japonicum* female. We found the transcript profiles in 16-dpi females were similar to those at 18 dpi. However, substantial changes in gene expression were observed in 24-dpi females, indicating that virgin female schistosomes undergo marked changes in gene expression before they complete maturation. Genes involved in reproduction, such as those associated with the cell cycle, egg formation, and protein synthesis, were substantially upregulated in the adult female trematodes. This result is consistent with that of others and on the basis of gender-related gene expression patterns reflects that the female, rather than the male, governs egg production [16, 20-27].

The results of our gene microarray analyses showed that the top 30 genes with respect to transcript abundance in 24-dpi females included representatives with proven roles in vitellarium development and egg production, including *eggshell precursor protein*, superoxide dismutase (*SOD*), and *Sjfs800*. The vitellarium, which occupies the posterior two-thirds of the female schistosome body, produces vitellocytes. Vitellocytes supply nutrition to the developing zygote and constituents essential to egg shell construction. Mature vitellocytes join with fertilized oocytes in the ootype, which is where mature eggs are formed [28]. Thus, the development of the vitellarium plays essential roles in the production of schistosome eggs. In the present study, we aimed to determine the role of some of the genes related to vitellarium development in the production of schistosome eggs.

SOD is an antioxidant that removes reactive oxygen species in an organism by converting the superoxide radical to molecular oxygen and hydrogen peroxide. *SOD* is in the cytoplasm and mitochondria and is also extracellular and is conserved across various species [29]. In the present study, *SOD* in 24-dpi *S. japonicum* females was located primarily in the ovary and vitellarium. Our transcriptome analysis indicated that *SOD* was expressed almost exclusively (>2000-fold enriched) and at high levels in 24-dpi (rather than in 16-dpi or 18-dpi) adult female trematodes, which may be under significant oxidative stress because of vitellarium development and egg production.

The schistosome eggshell is a hardened and tanned structure made from cross-linked proteins. Eggshell formation starts in the ootype. Contractions of the

ootype cause the vitelline cells to release their granules, which contain eggshell precursor proteins. The eggshell is shaped by the ootype and strengthened through tyrosinase activity that causes cross-linking of the released eggshell precursor proteins [30]. We found that the *eggshell precursor protein* gene was far more highly expressed in 24-dpi adult female trematodes than in 16-dpi or 18-dpi females. Transcripts of the *eggshell precursor protein* gene were expressed within the ovary and vitellarium, consistent with the findings of a previous study showing similar gene expression [31]. We also observed *eggshell precursor protein* signals in the ootype. Thus, the findings of the present study suggest that eggshell precursor protein maybe play an important role in helping the vitellarium promote eggshell formation.

Smfs800 gene was first found and identified by Reis et al., who also used in situ hybridization to determine that *Smfs800* mRNA was expressed only in female vitelline cells [32], suggesting that *Smfs800* may play role in egg development. However, little is known about the functions of *fs800* in vitellarium development and egg production. In the present study, *Sjfs800* mRNA was located in mature vitelline cells. Some developmental defects, especially a reduced number of mature vitelline cells in the vitellarium, were observed after *Sjfs800* gene knockdown by siRNA, and the pairing rate and oviposition rate were also significantly decreased. Therefore, we concluded that *fs800* is vital for vitelline cell development and maturation and that maturation of the vitellarium is required for *S. japonicum* females to produce eggs. The number male-female pairings was reduced by approximately 70% after *Sjfs800* siRNA transfection. The results of some studies have indicated that *Sjfs800* may be a

molecule downstream of *S. japonicum* Nanos1 or Abl tyrosine kinase activity [33-35].

In conclusion, our study showed substantial differences in the expression levels of some genes in the *S. japonicum* female before and after male-female pairing, including genes related to vitellarium development, which can affect pairing, sexual maturation, and egg production. These results provide a deeper understanding of the reproductive biology of schistosomes and may lead to the development of novel approaches for the prevention and treatment of schistosomiasis.

References

1. Gryseels B, Polman K, Clerinx J, Kestens L. Human schistosomiasis. *Lancet* 2006;368(9541):1106-18.
2. Weerakoon KG, Gobert GN, Cai P, McManus DP. Advances in the Diagnosis of Human Schistosomiasis. *Clin Microbiol Rev* 2015;28(4):939-67.
3. Colley DG, Bustinduy AL, Secor WE, King CH. Human schistosomiasis. *Lancet* 2014;383(9936):2253-64.
4. King CH, Dangerfield-Cha M. The unacknowledged impact of chronic schistosomiasis. *Chronic Illn* 2008;4(1):65-79.
5. Kasinathan RS, Greenberg RM. Pharmacology and potential physiological significance of schistosome multidrug resistance transporters. *Exp Parasitol* 2012;132(1):2-6.
6. Doenhoff MJ, Pica-Mattoccia L. Praziquantel for the treatment of schistosomiasis: its use for control in areas with endemic disease and prospects for drug resistance.

- 492 Expert Rev Anti Infect Ther 2006;4(2):199-210.
- 493 7. Melman SD, Steinauer ML, Cunningham C, Kubatko LS, Mwangi IN, Wynn NB,
494 et al. Reduced susceptibility to praziquantel among naturally occurring Kenyan
495 isolates of *Schistosoma mansoni*. PLoS Negl Trop Dis 2009;3(8):e504.
- 496 8. Wilson MS, Mentink-Kane MM, Pesce JT, Ramalingam TR, Thompson R, Wynn
497 TA. Immunopathology of schistosomiasis. Immunol Cell Biol. 2007;85(2):148–
498 54.
- 499 9. Wang S, Spear RC. Exploring the impact of infection-induced immunity on the
500 transmission of *Schistosoma japonicum* in hilly and mountainous environments
501 in China. Acta Trop 2014;133:8-14.
- 502 10. King CH, Sutherland LJ, Bertsch D. Systematic Review and Meta-analysis of the
503 Impact of Chemical-Based Mollusciciding for Control of *Schistosoma mansoni*
504 and *S. haematobium* Transmission. PLoS Negl Trop Dis 2015;9(12):e0004290.
- 505 11. LoVerde PT, Andrade LF, Oliveira G. Signal transduction regulates schistosome
506 reproductive biology. Curr Opin Microbiol 2009;12(4):422-8.
- 507 12. Beckmann S, Buro C, Dissous C, Hirzmann J, Grevelding CG. The Syk kinase
508 SmTK4 of *Schistosoma mansoni* is involved in the regulation of spermatogenesis
509 and oogenesis. PLoS Pathog 2010;6(2):e1000769.
- 510 13. Buro C, Oliveira KC, Lu Z, Leutner S, Beckmann S, Dissous C, et al.
511 Transcriptome analyses of inhibitor-treated schistosome females provide
512 evidence for cooperating Src-kinase and TGFbeta receptor pathways controlling
513 mitosis and eggshell formation. PLoS Pathog 2013;9(6):e1003448.

- 514 14. Vanderstraete M, Gouignard N, Cailliau K, Morel M, Hahnel S, Leutner S, *et*
515 *al.* Venus kinase receptors control reproduction in the platyhelminth parasite
516 *Schistosoma mansoni*. PLoS Pathog 2014;10(5):e1004138.
- 517 15. Ding H, Liu F, Zhu L, Wu F, Liu Q, He S, *et al.* Tyrosine kinase 4 is involved in
518 the reproduction of the platyhelminth parasite *Schistosoma japonicum*. Parasit
519 Vectors 2017;10(1):498.
- 520 16. Wang J, Yu Y, Shen H, Qing T, Zheng Y, Li Q, *et al.* Dynamic transcriptomes
521 identify biogenic amines and insect-like hormonal regulation for mediating
522 reproduction in *Schistosoma japonicum*. Nat Commun 2017;8:14693.
- 523 17. Cogswell AA, Collins JJ 3rd, Newmark PA, Williams DL. Whole mount in situ
524 hybridization methodology for *Schistosoma mansoni*. *Mol Biochem Parasitol*.
525 2011;178(1-2):46-50.
- 526 18. Zhang, W, Zhang, P, Liu, M, Ren, Shen, J. Worm morphology of *Schistosoma*
527 *japonicum* using confocal laser scanning microscopy. *Journal of Helminthology*
528 2012; 86(3):317-322.
- 529 19. Akram A. Da'dara and Patrick J. Skelly. Gene Suppression in Schistosomes
530 Using RNAi. *Parasite Genomics Protocols*, Methods in Molecular Biology, vol.
531 1201.
- 532 20. Moertel L, McManus DP, Piva TJ, Young L, McInnes RL, Gobert GN.
533 Oligonucleotide microarray analysis of strain- and gender-associated gene
534 expression in the human blood fluke, *Schistosoma japonicum*. Mol Cell Probes
535 2006;20(5):280-9.

- 536 21. Leutner S, Oliveira KC, Rotter B, Beckmann S, Buro C, Hahnel S, *et al.*
537 Combinatory microarray and SuperSAGE analyses identify pairing-dependently
538 transcribed genes in *Schistosoma mansoni* males, including follistatin. PLoS
539 Negl Trop Dis 2013;7(11):e2532.
- 540 22. Lu Z, Sessler F, Holroyd N, Hahnel S, Quack T, Berriman M, *et al.* Schistosome
541 sex matters: a deep view into gonad-specific and pairing-dependent
542 transcriptomes reveals a complex gender interplay. Sci Rep 2016;6:31150.
- 543 23. Cai P, Liu S, Piao X, Hou N, You H, McManus DP, *et al.* A next-generation
544 microarray further reveals stage-enriched gene expression pattern in the blood
545 fluke *Schistosoma japonicum*. Parasit Vectors 2017;10(1):19.
- 546 24. Waisberg M, Lobo FP, Cerqueira GC, Passos LK, Carvalho OS, Franco GR, *et*
547 *al.* Microarray analysis of gene expression induced by sexual contact in
548 *Schistosoma mansoni*. BMC Genomics 2007;8:181.
- 549 25. Sun J, Wang SW, Li C, Hu W, Ren YJ, Wang JQ. Transcriptome profilings of
550 female *Schistosoma japonicum* reveal significant differential expression of genes
551 after pairing. Parasitol Res 2014;113(3):881-92.
- 552 26. Dillon GP, Feltwell T, Skelton JP, Ashton PD, Coulson PS, Quail MA, *et al.*
553 Microarray analysis identifies genes preferentially expressed in the lung
554 schistosomulum of *Schistosoma mansoni*. Int J Parasitol 2006;36(1):1-8.
- 555 27. Williams DL, Sayed AA, Bernier J, Birkeland SR, Cipriano MJ, Papa AR, *et al.*
556 Profiling *Schistosoma mansoni* development using serial analysis of gene
557 expression (SAGE). Exp Parasitol 2007;117(3):246-58.

- 558 28. Wang J, Collins JR. Identification of new markers for the *Schistosoma mansoni*
559 vitelline lineage. *Int J Parasitol* 2016;46(7):405-10.
- 560 29. Warner HR. Superoxide dismutase, aging, and degenerative disease. *Free Radic*
561 *Biol Med* 1994;17(3):249-58.
- 562 30. DeWalick S, Tielens AG, van Hellemond JJ. *Schistosoma mansoni*: the egg,
563 biosynthesis of the shell and interaction with the host. *Exp Parasitol*
564 2012;132(1):7-13.
- 565 31. Cogswell AA, Collins JR, Newmark PA, Williams DL. Whole mount in situ
566 hybridization methodology for *Schistosoma mansoni*. *Mol Biochem Parasitol*
567 2011;178(1-2):46-50.
- 568 32. Reis MG, Kuhns J, Blanton R, Davis AH. Localization and pattern of expression
569 of a female specific mRNA in *Schistosoma mansoni*. *Mol Biochem Parasitol*
570 1989;32(2-3):113-9.
- 571 33. Liu Q, Zhu L, Liu F, Hua M, Ding H, He S, et al. Function of Nanos1 gene in the
572 development of reproductive organs of *Schistosoma japonicum*. *Parasitol Res*
573 2017;116(5):1505-1513.
- 574 34. Buro C, Beckmann S, Oliveira KC, Dissous C, Cailliau K, Marhofer RJ, *et al.*
575 Imatinib treatment causes substantial transcriptional changes in adult
576 *Schistosoma mansoni* in vitro exhibiting pleiotropic effects. *PLoS Negl Trop Dis*
577 2014;8(6):e2923.
- 578 35. Buro C, Oliveira KC, Lu Z, Leutner S, Beckmann S, Dissous C, *et*
579 *al.* Transcriptome analyses of inhibitor-treated schistosome females provide

580 evidence for cooperating Src-kinase and TGFbeta receptor pathways controlling
581 mitosis and eggshell formation. PLoS Pathog 2013;9(6):e1003448.