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Identification of a new panel of reference genes to study pairing-dependent gene expression in *Schistosoma mansoni*

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ABSTRACT

Facilitated by the *Schistosoma mansoni* genome project, multiple transcriptomic studies were performed over the last decade to elucidate gene expression patterns among different developmental stages of the complex schistosome life cycle. While these analyses enable the identification of candidate genes with key functions in schistosome biology, a diverse molecular tool set is needed that allows comprehensive functional characterization at the single gene level. This includes the availability of reliable reference genes to confirm changes in the transcription of genes of interest over different biological samples and experimental conditions. In particular, the investigation of one key aspect of schistosome biology, the pairing-dependent gene expression in females and males, requires knowledge on reference genes that are expressed independently of both pairing and of in vitro culture effects. Therefore, the present study focused on the identification of quantitative reverse transcription (qRT)-PCR reference genes suitable for the investigation of pairing-dependent gene expression in the *S. mansoni* male. The "pipeline" we present here is based on qRT-PCR analyses of high biological replication combined with three different statistical analysis tools, BestKeeper, geNorm, and NormFinder. Our approach resulted in a statistically robust ranking of 15 selected reference genes with respect to their transcription stability between pairing-unexperienced and -experienced males. We further tested the top seven candidate genes for their transcription stability during in vitro culture of adult *S. mansoni*. Of these, the two most suitable reference genes were used to investigate the influence of the pairing contact on the transcription of genes of interest, comprising a tyrosine decarboxylase gene Sm*tdc1*, an ebony ortholog Sm*ebony*, and the follistatin ortholog Sm*fst* in *S. mansoni* males. Performing pairing, separation and re-pairing experiments with adult *S. mansoni* in vitro, our results indicate for the first time that pairing can act as a molecular on/off-switch of specific genes to strictly control their expression in schistosome males.

1. Introduction

Caused by blood-dwelling trematodes of the genus *Schistosoma*, schistosomiasis is an infectious disease with tremendous impact on global health and socio-economic development. Endemic in 76 tropical and subtropical countries worldwide, over 230 million people are infected with at least one of the three major parasite species, *Schistosoma mansoni*, *Schistosoma japonicum* or *Schistosoma haematobium* (Colley et al., 2014).

Schistosomes exhibit a complex life cycle including two free-living larval and two parasitic stages, which infest mollusks as intermediate hosts and mammals as final hosts, respectively. In the case of *S. mansoni*, adult worms live as couples in the mesenteric system of the final host. Pathogenesis of the disease is triggered by the eggs, which are produced by paired female worms. Almost 50% of the released eggs are transported via the blood system to different organs where they get stuck in the tissue and provoke inflammatory reactions. Organs affected by the eggs are mainly liver, gut, and spleen. Chronic infections can finally lead to severe symptoms such as hepatosplenomegaly (Gryseels et al., 2006; Olveda et al., 2014). In recent decades, the control of schistosomiasis relied mostly on mass drug administration of the anthelmintic drug praziquantel, and alternative treatment strategies are not yet available (Siqueira et al., 2017; Vale et al., 2017). In the light of constant reinfections and reports of reduced cure rates upon praziquantel treatment in endemic regions, this lack

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of alternative treatment strategies raises fear of the emergence of resistant parasite populations (Wang et al., 2012; Bergquist et al., 2017; Vale et al., 2017). To deal with this threat, a detailed understanding of the parasite's biology is necessary to identify key molecules that could serve as future drug targets.

Facilitated by the *S. mansoni* genome project (Berriman et al., 2009; Protasio et al., 2012), several transcriptomic studies were performed in the last decade to elucidate global patterns of gene expression in different life-cycle stages of this parasite. This included studies on eggs (Anderson et al., 2015), larval stages (Taft et al., 2009; Parker-Manuel et al., 2011; Protasio et al., 2013), and adult worms (Ojopi et al., 2007; Almeida et al., 2012; Leutner et al., 2013; Picard et al., 2016). Finally, initial tissue-specific transcriptome analyses were realized by laser-microdissection (Nawaratna et al., 2011, 2014), or an enzyme-based isolation method, of schistosome organs (Lu et al., 2016, 2017). Additionally, recent progress on stem cell labeling (Collins et al., 2013; Wang et al., 2013) and cell isolation techniques (Lu et al., 2015) will allow single-cell transcriptomic studies in the near future. While these extensive transcriptome data sets can be bioinformatically explored to gain comprehensive insight into the potential functions of candidate genes and gene families (Grevelding et al., 2018; Hahnel et al., 2018), subsequent functional studies have to follow at the single-gene level to confirm transcriptome-based transcript patterns and to substantiate the predicted role(s) of specific gene(s) for schistosome biology by e.g. RNA interference (RNAi).

The 'gold standard' for the validation of transcriptomic data is the quantitative reverse transcription (qRT)-PCR (Endrullat et al., 2016; Costa-Silva et al., 2017). While qRT-PCR analyses in principle allow the detection of absolute transcript levels, the most commonly used approach is the relative quantification by normalization of gene transcription between two or more conditions against suitable reference genes (Vandesompele et al., 2002; Hayes et al., 2011). This approach is highly favored by most studies because it is less sensitive to variations in sample handling and preparation, RNA quality, or cDNA synthesis, but it requires the availability of reliable reference genes as internal standards. To fulfill this requirement, reference genes need to be stably transcribed under all investigated conditions and should be less affected by external variation (Weyrich et al., 2010). Due to the great increase in scientific questions that focus on gene expression, the use of globally expressed 'housekeepers' as universal reference genes has been widely approved. However, there is increasing evidence that classical reference genes such as GAPDH or actin can vary greatly in their stage- and/or tissue-specific transcript levels (Yuan et al., 2014; Zhai et al., 2014; Zheng et al., 2014; Chapman and Waldenström, 2015). Consequently, the perspective has changed, and it is progressively accepted that few universal reference genes exist which have constant expression in all tissues and under all experimental conditions. Instead, suitable reference genes have to be determined for every new comparison (Guénin et al., 2009).

In the post-genomic era of schistosome research, there is high demand for the functional characterization of genes of interest (GOI). In this context, in vitro studies have been performed which among others allow the investigation of schistosome sex-specific and/or pairing-dependent processes affecting GOI expression (You et al., 2009; Beckmann et al., 2010; Ressurreição et al., 2011). The latter is of great biological interest due to the pairing-dependent sexual differentiation of the schistosome female that finally leads to its egg production capability (Kunz, 2001; Hoffmann, 2004). Thus, the male fulfills an essential role for female reproductive biology. Interestingly, initial evidence was obtained that not only female but also male gene expression underlies pairing-dependent influences. One example is the expression of neuronal genes which points to an involvement of neuronal processes in male–female interaction (Leutner et al., 2013; Lu et al., 2016). Against this background, our study aimed to identify reference genes that are suitable for the investigation of pairing-dependent transcript profiles in *S. mansoni* males under in vitro conditions. Furthermore, we road-tested these reference genes, examining the transcript patterns of exemplary neuronal genes by qRT-PCR that were predicted by previous microarray and RNA-seq analyses to be pairing-dependently transcribed in males (Leutner et al., 2013; Lu et al., 2016). Using three different statistical algorithms, our results revealed suitable reference genes, which are stably transcribed under standard in vitro culture conditions, and which allowed us to obtain conclusive evidence for a direct influence of pairing on the transcription of three selected neuronal genes in schistosome males. This has implications for schistosome biology, pointing to the existence of competence factors of males that may have relevance for the male–female interaction and its consequences with respect to the pairing-dependent sexual development of the schistosome female.

2. Material and methods

2.1. Ethics statement

Animal experiments using Syrian hamsters (*Mesocricetus auratus*) as model hosts were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (ETS No 123; revised Appendix A) and were approved by the Regional Council (Regierungspraesidium), Giessen, Germany (V54-19c 20/15c GI 18/10).

2.2. Parasite maintenance

Adult and larval schistosome stages originated from a Liberian isolate of *S. mansoni* which was maintained in snails (*Biomphalaria glabrata*) and Syrian hamsters (*Mesocricetus auratus*). Unisexual worm populations were generated by monomiracidial intermediate host infection (Grevelding, 1995). Adult worms were obtained by hepatoportal perfusion at 42–49 days p.i. After perfusion, worms were transferred to Petri dishes of 60 mm diameter size containing 5 ml of M199 medium (Sigma-Aldrich; supplemented with 10% Newborn Calf Serum (NCS), 1% HEPES [1 M] and 1% ABAM-solution [10,000 units of penicillin, 10 mg of streptomycin and 25 mg of amphotericin B per ml]) in groups of 20 couples per Petri dish until further usage. All worms were cultured in vitro at 37 °C and 5% CO_2.

2.3. In vitro culture experiments

The in vitro culture experiments with *S. mansoni* males and couples were performed for a maximum of 2 weeks to obtain males of different pairing states for subsequent qRT-PCR analyses. Pairing-experienced males (EM) originated from couples and were separated from their female partners directly before freeze storage for subsequent RNA extraction, while pairing-unexperienced males (UM) were obtained from unisexual infections of the final host. If needed, males with pairing experience were separated (SEM) from female partners directly after perfusion and either cultured unmated in vitro for the whole experiment or re-paired (REM) with a female after a separation period of 96 h. Samples for RNA isolation were taken at different time points during the experiment, i.e. at $t = 0$ h, $t = 96$ h after perfusion. For re-pairing, males were cultured in a ratio of 1:3 for 48 h together with females that were obtained from bisex (=mixed sex) infections and after re-pairing cultured for further 96 h. All worms were cultured in six-well

plates (Greiner Bio-One, Germany) in 5 ml of supplemented M199 medium at 37 °C and 5% CO_2, and medium was refreshed every other day.

2.4. RNA isolation and cDNA synthesis

Prior to RNA isolation, in vitro cultured worms were incubated in 100 µl of TriFast reagent, immediately frozen in liquid nitrogen and stored at −80 °C until further usage. Sample sizes ranged between 5–10 worms per culture condition, and only viable worms that showed normal moving behavior were frozen. Total RNA from *S. mansoni* males was extracted using PeqGOLD TriFast (Peqlab, Germany) following the manufacturer's protocol. In short, worms were mechanically homogenized with a plastic pestle before the TriFast reagent was added to a final volume of 500 µl. Precipitation of total RNA in 2-propanol was aided by addition of 35 µg of glycogen (RNase-Free PeqGOLD Glycogen, Peqlab), and precipitated RNA was resolved in 10 µl of RNAse-free diethylpyrocarbonate (DEPC)-treated water. RNA quality and quantity were checked by electropherogram analysis using the BioAnalyzer 2100 (Agilent Technologies, USA). In brief, 1 µl of resuspended RNA was loaded on an Agilent RNA 6000 Nano Chip according to the manufacturer's instructions and analyzed using the device setting "Eukaryote Total RNA Nano assay." Isolated RNA was stored at −80 °C until cDNA synthesis. Synthesis of cDNA was performed using the QuantiTect Reverse Transcription Kit (QIAGEN, Germany) comprising a genomic (g)DNA wipe out step and 500 ng of total RNA per reaction. To test the efficacy of the gDNA removal, the obtained cDNAs were used as template for a standard PCR with the primer pair 5'PDIvs: AAA TGA TGC CCC GAC TTA CC, and 3'PDIvs: TCA TCC CAA ACT GGA GCA AG. These primers prime to exons 5 and 6 of the *S. mansoni pdi* gene (Smp_056760) (Finken et al., 1994). Spanning a 387 bp intron region, amplification of cDNA and gDNA can be distinguished by size differences. Those cDNAs that showed no amplification of the gDNA product were diluted 1:20 in RNAse-free water before they were used in subsequent qRT-PCR analyses.

2.5. qRT-PCR experiments

All primers used for qRT-PCR experiments were designed for a melting temperature of 60 °C and an amplicon size of 140–200 bp (Supplementary Table S1), using the Primer3Plus software tool (Untergasser et al., 2012). If possible, primer pairs were located on different exons of a gene to distinguish (contaminating) gDNA from cDNA amplification by size. Prior to qRT-PCR, all primer pairs were tested under standard PCR conditions using the FirePol taq polymerase (Solis BioDyne, Estonia). PCR products were checked for specificity and occurrence of primer dimers on 2% agarose gels. Appropriate PCR products were gel extracted (PeqLab gel extraction Kit), cloned into pDrive plasmid (QIAGEN, PCR Cloning Kit) and Sanger sequenced (LGC Genomics, Germany) to verify accuracy of the amplification. All primers were commercially synthesized by Biolegio (Nijmegen, The Netherlands).

All qRT-PCR experiments were performed on a Rotorgene Q cycler (QIAGEN) using the 2× PerfeCTa SYBR Green SuperMix (Quantabio, USA) for the detection of synthesized DNA double strands in a final volume of 20 µl. Primers were used in a final concentration of 400 nM, and qRT-PCR conditions were as follows: initial denaturation step at 95 °C for 3 min, 45 cycles at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s. For each primer pair, melting point analyses were performed after each run to verify primer specificity and to exclude the generation of unspecific side-products, primer dimers, or the amplification of an additional gDNA product. Primer efficiencies were determined by a standard curve on diluted PCR product with 1:10 dilution steps (Dorak, 2008), and only primers with an efficiency of 90–100% were used for subsequent analyses. All qRT-PCRs were performed in three to six biological replicates with three technical replicates for each sample. To investigate expression stability of potential reference genes, PCR products were calculated by absolute quantification against a standard curve (Leutner et al., 2013). Pairing-dependent changes in gene transcription during in vitro cultivation was investigated by relative quantification of the GOI against selected candidate reference genes using the delta delta Ct method (Livak and Schmittgen, 2001).

2.6. Evaluation of expression stability of selected reference genes

The transcription stability of selected candidate reference genes was analyzed by three different software algorithms, NormFinder, BestKeeper, and geNorm, according to original publications (Vandesompele et al., 2002; Andersen et al., 2004; Pfaffl et al., 2004). For NormFinder analysis, the calculated concentrations of qRT-PCR amplification products were used as input data. NormFinder calculates a stability value (M) based on intra- and inter-group variations across the different sample groups. This means the lower the variances and the lower the stability value is, the more stably transcribed a reference gene is. In addition, the best combination of two reference genes is determined.

As a second approach, BestKeeper analysis was performed on raw Ct values. This algorithm assumes that stable reference genes should display similar transcription patterns, i.e. are highly correlated to each other. This is reflected by a high coefficient of correlation (r), whereby the most stably transcribed genes exhibit values closest to 1. The number of genes that can be analyzed simultaneously in this program is limited to 10. To be able to rank 15 different genes, three independent analyses were performed, each comprising a selection of 10 genes with each gene being included in two of the three analyses. The average r value from the different analyses was created for each gene as a basis for the final ranking of all 15 genes. The standard deviations of all genes were <1.

The geNorm algorithm was used as a third approach and is based on calculation of the pairwise variation of each gene with all other candidate reference genes using relative Ct values as input data. The average of these pairwise variations yields a stability value (M), which means a low M value points to stable transcription.

2.7. Statistical analysis

Data on the influence of pairing on transcription of candidate genes in *S. mansoni* males are given as mean ± S.E.M. Statistical analysis was performed using paired *t*-tests. All *P* values <0.05 were considered significant.

3. Results

3.1. Identification of reference genes for the study of pairing-dependent gene expression in S. mansoni males

To identify genes suitable for the study of pairing-dependent gene expression in *S. mansoni* males, we preselected a total 15 candidate genes. As a basis for selection, we took advantage of previously performed transcriptomic studies to choose a set of genes that was already shown to be stably transcribed among different parasite stages. None of the selected genes has been studied before in detail for its biological function in trematodes, but their GeneDB annotation (Berriman et al., 2009; Protasio et al., 2012) and manual BLAST (Basic Local Alignment Search Tool of the National Center

for Biotechnology Information) analyses in our laboratory revealed homology to orthologs from different model organisms, suggesting roles in conserved cellular processes such as cytoskeleton arrangement, mRNA translation, mitochondrial organization, mRNA splicing, and the regulation of cell growth (Table 1). The first five candidate genes included Sm*wdr1* (Smp_073940), encoding an actin interacting protein, Sm*eif4g2a* (Smp_008900), encoding a eukaryotic translation initiation factor 4 gamma, Sm*letm1* (Smp_065110), encoding a LETM1 and EF hand domain containing protein 1, Sm*cwf19l1* (Smp_152510), encoding a CWF19 like protein 1, and Sm*ptpa* (Smp_166290), encoding a serine/threonine protein phosphatase 2A. These five genes were selected because they were previously shown to be among the most stably transcribed genes in adult *S. mansoni* and their gonads, independent of the pairing status (Lu et al., 2016) (Supplementary Table S2). Due to their transcription stability between UM and EM, the candidate gene pool was expanded by including four genes from a previous transcriptomic study (Leutner et al., 2013). These four genes encoded an ubiquinol cytochrome c reductase (Sm*uqcr*, Smp_061870), the exocyst complex component 7 (Sm*exoc7*, Smp_136580), an ortholog of the solute carrier transporter family 44 (Sm*slc44a2*, Smp_160740), and a putative cytochrome P450 reductase gene (Sm*porb*, Smp_030760). All four genes were shown not to be regulated in their transcript levels between EM and UM, neither by microarray nor SuperSAGE experiments (Leutner et al., 2013). Finally, the set was completed by six further candidates, which were either postulated to be suitable reference genes based on their constitutive transcription among different *S. mansoni* life stages as shown by microarray experiments (Fitzpatrick et al., 2009), or because they were orthologs of classic "housekeeping" genes and used for normalization of gene transcription in other systems (Kozera and Rapacz, 2013) (Table 1). This group includes Sm*map3k9* (Smp_176580), a mitogen activated protein kinase kinase kinase gene, Sm*nip7* (Smp_021760), an ortholog of the 60 s ribosome subunit biogenesis protein nip7, and Sm*psmb7* (Smp_073410), encoding the proteasome subunit beta type 7. As classical "housekeepers", two cytoskeleton-related genes, the actin gamma 1 coding gene Sm*actg1* (Smp_161930) and the tubulin beta 2B chain gene Sm*tubb4b* (Smp_030730) as well as the glyceraldehyde 3 phosphate dehydrogenase (GAPDH) gene Sm*gapdh* (Smp_056970) were chosen.

In a first step, the 15 selected candidate genes were evaluated for their transcription stability between *S. mansoni* UM and EM by qRT-PCR. To this end, transcript levels in six biological replicates per condition were determined by absolute quantification against a standard curve. Transcription stability was analyzed using three different algorithms which use as input data raw Ct values (BestKeeper), relative Ct values (GeNorm) and calculated concentrations of amplification products (NormFinder), respectively. A good reference gene is characterized by an M value below 1 in heterogeneous cell or tissue sample sets (Vandesompele et al., 2002). Based on this and our results, all of the tested genes showed acceptable expression stabilities between UM and EM, and thus were ranked as candidate reference genes. However, they differed slightly in their expression stability in a direct comparison. NormFinder ranked candidate reference genes from Sm*porb* as the less stably transcribed gene, to Sm*ptpa*, as the most stable gene between UM and EM (Fig. 1). The seven most stably transcribed genes were ranked referring to the M-values as follows (rank 7 to rank 1): Sm*letm1* > Sm*map3k9* > Sm*exoc7* > Sm*psmb7* > Sm*gapdh* > Sm*cwf19l1* > Sm*ptpa*. The two other algorithms, BestKeeper and geNorm showed the same overall tendencies (Supplementary Fig. S1), with Sm*gapdh*, Sm*cwf19l1*, and Sm*ptpa* as the three most stably transcribed genes. Based on these results, we selected seven genes shown to be most stably transcribed between UM and EM by at least two of three validation algorithms (Table 2).

Table 1
Selection of candidate reference genes to study gene expression in *Schistosoma mansoni* males.

Gene name	GeneID	Homology (NCBI BLAST)	Protein function
Sm*wdr1*	Smp_073940	WD repeat domain 1 [*Mus musculus*] (7e−154)	Orthologs of this gene are involved in regulation of actin polymerization
Sm*eif4g2a*	Smp_008900	Eukaryotic translation initiation factor 4, gamma 2a [*Danio rerio*] (8e−31)	This protein is one of three subunits of the eukaryotic translation initiation factor 4F involved in the specific recognition of the 5′cap structure of mRNA during translation
Sm*letm1*	Smp_065110	LETM1 and EF-hand domain-containing protein 1, mitochondrial [*Danio rerio*] (1e−101)	Members of this membrane protein family play a role in the potassium and hydrogen ion exchange of the mitochondrion
Sm*cwf19l1*	Smp_152510	CWF19-like protein 1 [*Mus musculus*] (3e−131)	This gene encodes a conserved protein with a potential function in pre-mRNA splicing
Sm*ptpa*	Smp_166290	Serine/threonine-protein phosphatase 2A activator isoform b [*Homo sapiens*] (1e−87)	This gene encodes a regulatory B subunit of the heterotrimeric protein phosphatase 2 A, a ubiquitously expressed enzyme with phosphatase activity in many eukaryotic cells
Sm*uqcrfs1*	Smp_061870	Ubiquinol cytochrome c reductase, Rieske [*Danio rerio*] (3e−79)	Component of the ubiquinol-cytochrome c reductase complex within the mitochondrion
Sm*exoc7*	Smp_136580	Exocyst complex component 7 [*Danio rerio*] (3e−38)	Component of the exocyst complex supporting interaction of exocytic vesicles with fusion sites on the plasma membrane
Sm*slc44a2*	Smp_160740	Solute carrier family 44, member 2 [*Mus musculus*] (1e−128)	Members of this membrane transporter family are among others involved in the transport of choline into acetylcholine-synthesizing neurons
Sm*porb*	Smp_030760	P450 (cytochrome) oxidoreductase b [*Danio rerio*] (4e−161)	This gene encodes an endoplasmic reticulum-associated oxidoreductase which is required for the electron transfer from NADPH to cytochrome P450 proteins
Sm*map3k9*	Smp_176580	Mitogen-activated protein kinase kinase kinase 9 [*Homo sapiens*] (8e−71)	This gene product has serine/threonine kinase activity and shows similarity to human MAPKKK, a member of the Mitogen-activated protein kinase (MAPK) signaling pathway
Sm*nip7*	Smp_021760	Nucleolar pre-rRNA processing protein [*Mus musculus*] (1e−68)	Ortholog of NIP7, which is involved in rRNA processing and ribosome assembly
Sm*psmb7*	Smp_073410	Proteasome subunit beta type 7 [*Mus musculus*] (5e−122)	Important component of the cellular protein degradation complex
Sm*actg1*	Smp_161930	Actin, gamma, cytoplasmic 1 [*Mus musculus*] (5e−120)	The Actin gamma 1 isoform is a cytoplasmic actin expressed in non-muscle cells
Sm*tubb4a*	Smp_030730	Tubulin beta 4B class IVb [*Homo sapiens*] (0.0)	Tubulins are the major component of microtubules
Sm*gapdh*	Smp_056970	Glyceraldehyde-3-phosphate dehydrogenase [*Homo sapiens*] (2e−178)	GAPDH catalyzes the sixth step of the glycolysis by converting D-glyceraldehyde 3-phosphate to 3-phospho-D-glyceroyl phosphate

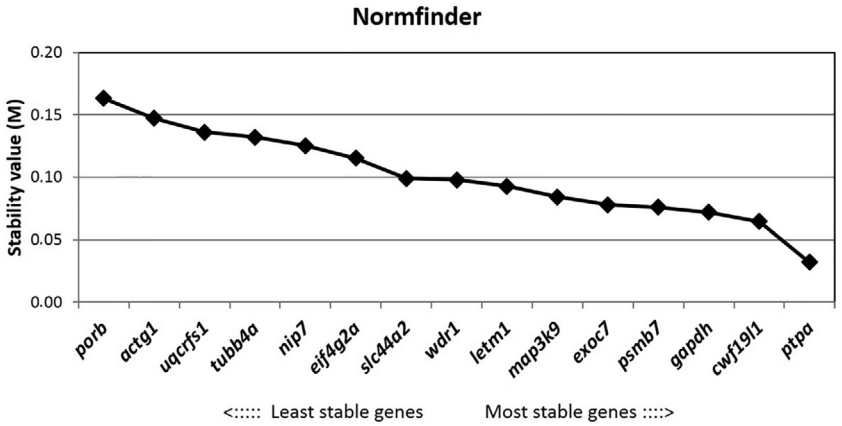

Fig. 1. Expression stability of candidate reference genes between *Schistosoma mansoni* pairing-unexperienced males and pairing-experienced males. The transcript levels of 15 candidate reference genes in unexperienced males and experienced males were determined by quantitative reverse transcription (qRT)-PCR, and expression stability between both conditions was analyzed using the NormFinder algorithm. Candidate genes were ranked according to their stability values (M). A lower M value indicates less variance and thus a more stable expression between unexperienced males and experienced males (n = 6 biological replicates).

Table 2
Expression stability of candidate reference genes between *Schistosoma mansoni* pairing-unexperienced males and pairing-experienced males (EM). Ranking of the most stably expressed candidate reference genes between UM and EM, determined by three different analyses: NormFinder, BestKeeper and geNorm. For each analysis, the seven most stably expressed genes are shown. Candidate reference genes selected for further analyses are written in bold.

Ranking	NormFinder		BestKeeper		geNorm	
	Reference gene	Stability value (M)	Reference gene	Coeff. of correlation ®	Reference gene	Expression stability (M)
1	**Sm*ptpa***	0.032	**Sm*ptpa***	0.965	**Sm*ptpa***	0.174
2	**Sm*cwf19l1***	0.065	**Sm*cwf19l1***	0.941	**Sm*cwf19l1***	0.189
3	**Sm*gapdh***	0.072	**Sm*gapdh***	0.933	**Sm*gapdh***	0.198
4	**Sm*psmb7***	0.076	Sm*wdr1*	0.931	**Sm*psmb7***	0.201
5	**Sm*exoc7***	0.078	Sm*uqcrfs1*	0.897	**Sm*letm1***	0.209
6	**Sm*map3k9***	0.084	Sm*tubb4a*	0.885	**Sm*exoc7***	0.213
7	**Sm*letm1***	0.093	**Sm*map3k9***	0.823	Sm*wdr1*	0.213

3.2. Stability of reference genes during in vitro cultivation of adult S. mansoni males

To allow valid gene expression analyses of in vitro cultured worms, a reference gene is needed that is stably expressed during in vitro culture conditions. To this end, the most stably transcribed reference genes obtained by our analyses were tested for their transcription stability during the in vitro cultivation of *S. mansoni* males over different time points. All three algorithms require a minimum number of three genes, while at least five genes has been recommended for NormFinder (Andersen et al., 2004). Therefore, we chose a cut-off of the top seven candidate genes to ensure adequate statistical significance for the subsequent analyses.

UM and EM were cultured for up to 264 h (11 days). Samples for qRT-PCR analyses were taken at time points t = 0 h, t = 96 h, and t = 264 h. A combined NormFinder analysis of all male samples from all culture time points showed that the most stably transcribed reference gene candidate was Sm*letm1* while the best combination of two genes was Sm*letm1* and Sm*psmb7* (stability value of combination: 0.04). The ranking of stability values of all seven candidate genes is shown in Fig. 2. These two top candidate reference genes were also the two most stably transcribed genes

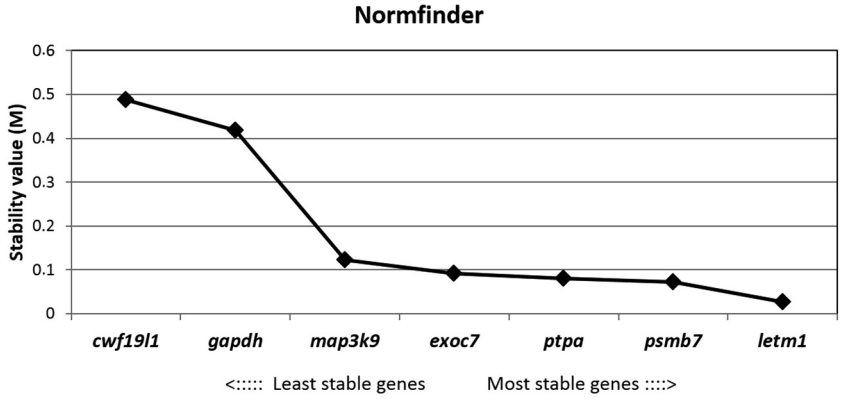

Fig. 2. Expression stability of reference genes during in vitro cultivation of *Schistosoma mansoni* males. The transcript levels of seven candidate reference genes in pairing-unexperienced males and pairing-experienced males over 11 days in vitro were determined by quantitative reverse transcription (qRT)-PCR (sampling time points t = 0 h, t = 96 h, and t = 264 h), and expression stability between both conditions was analyzed using the NormFinder algorithm. Candidate genes were ranked by their stability value (M). A lower M value indicates less variance and thus a more stable expression between unexperienced males and experienced males during in vitro cultivation (n = 3 biological replicates).

according to the BestKeeper analysis, and among the top three genes using geNorm (Supplementary Fig. S2). All three algorithms suggested that the most stably transcribed gene was Sm*letm1*, while the least stably one was Sm*cwf19l1*. The results obtained from NormFinder, geNorm and BestKeeper analyses are summarized in Table 3.

3.3. Validation of pairing-dependent gene transcription by candidate reference genes

The two candidate reference genes showing the most stable transcription in males with different pairing states and during in vitro culture, Sm*letm1* and *Smpsmb7*, were road-tested and used to quantify the influence of the pairing status on the relative transcription of different GOI in *S. mansoni* males. We focused on two neuronal genes with presumed pairing-dependent expression based on our previous microarray and RNA-seq studies. UM and EM (paired) were cultured for 11 days in vitro, and samples for qRT-PCR analysis were taken at time points, $t = 0$ h, $t = 96$ h, and $t = 264$ h. Additionally, some EM were separated from the female directly after perfusion and were cultured as SEM in parallel to UM and EM. After 96 h, half of the SEM were co-cultured with females for 48 h, and REM were kept in culture until $t = 264$ h (Fig. 3A). The four resulting conditions of this in vitro culture experiment, UM, EM, SEM and REM, allowed the investigation of a pairing influence on gene transcription in *S. mansoni* males.

Table 3
Expression stability of candidate reference genes between *Schistosoma mansoni* pairing-unexperienced males and pairing-experienced males during in vitro culture. Ranking of the most stably expressed candidate reference genes between UM and EM, determined by three different analyses: NormFinder, BestKeeper and geNorm. For each analysis, the seven most stably expressed genes are shown. Candidate reference genes selected for gene expression analyses are written in bold.

Ranking	NormFinder		BestKeeper		geNorm	
	Reference gene	Stability value (M)	Reference gene	Coeff. of correlation (r)	Reference gene	Expression stability (M)
1	**Sm*letm1***	0.027	**Sm*letm1***	0.992	**Sm*letm1***	0.314
2	**Sm*psmb7***	0.072	**Sm*psmb7***	0.984	Sm*map3k9*	0.314
3	Sm*ptpa*	0.081	Sm*map3k9*	0.977	**Sm*psmb7***	0.377
4	Sm*exoc7*	0.091	Sm*gapdh*	0.977	Sm*ptpa*	0.414
5	Sm*map3k9*	0.123	Sm*ptpa*	0.966	Sm*exoc7*	0.457
6	Sm*gapdh*	0.419	Sm*exoc7*	0.958	Sm*gapdh*	0.585
7	Sm*cwf19l1*	0.487	Sm*cwf19l1*	0.690	Sm*cwf19l1*	0.721

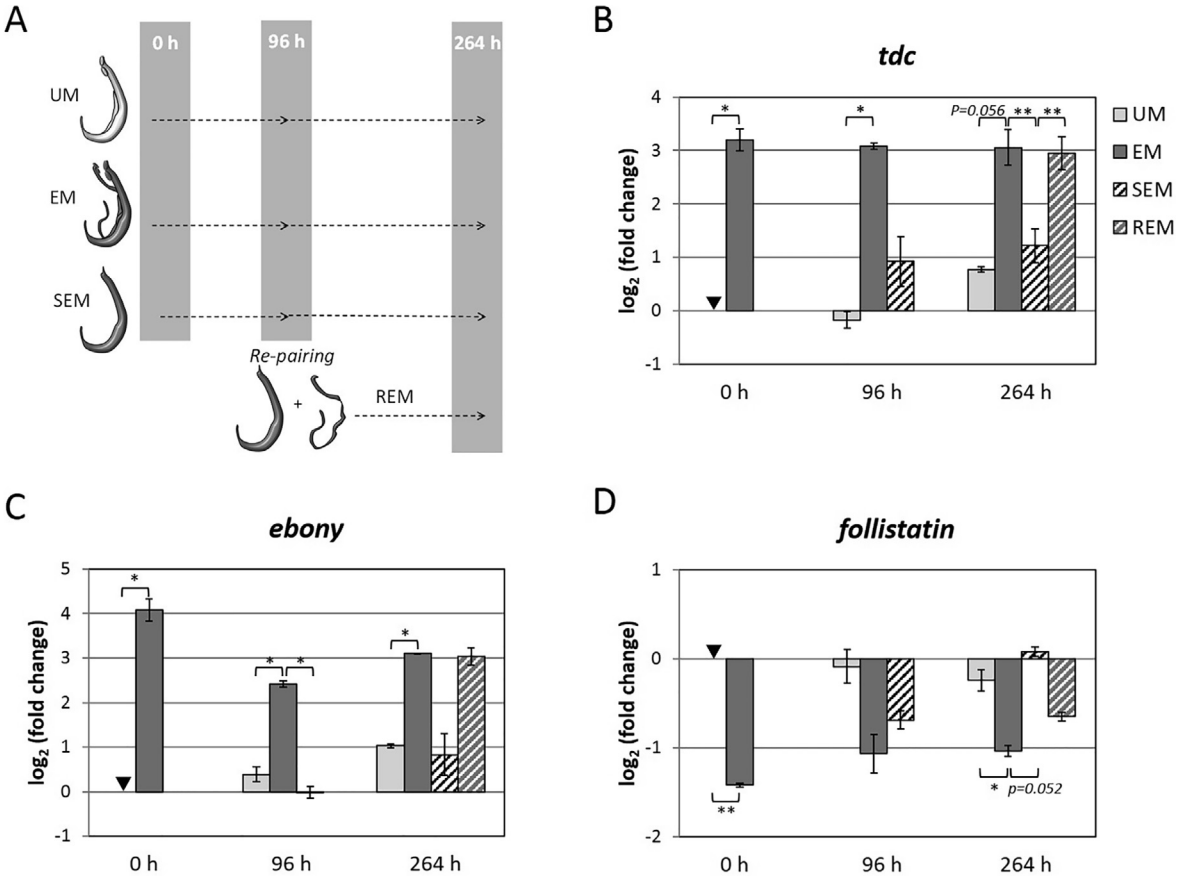

Fig. 3. Influence of pairing on expression of candidate genes in *Schistosoma mansoni* males. *Schistosoma mansoni* male samples, including pairing-experienced males, pairing-unexperienced males, separated pairing-experienced males and re-paired males, were collected at different time points (0 h, 96 h, 264 h) of two independent in vitro culture experiments (A). Three different genes related to neuronal (B, C) and signaling (D) processes in schistosomes, Sm*tdc* (B), Sm*ebony* (C) and Sm*fst* (D), were investigated for their pairing-dependent transcription in *S. mansoni* males by quantitative reverse transcription (qRT)-PCR Relative transcript levels among samples were calculated using the geometric mean of two candidate reference genes, Sm*lemt1* and Sm*psmb7*, for normalization. Significant differences by t-test are indicated: $^{*}P < 0.05$; $^{**}P < 0.01$.

The first GOI, Sm*tdc* (Smp_135230), shares homology to dopa decarboxylases and tyrosine decarboxylases (Ribeiro et al., 2012), both conserved key enzymes in neurotransmitter metabolism. Dopa decarboxylases catalyze the decarboxylation of L-3,4-dihydroxyphenylalanine (DOPA) to dopamine, L-5-hydroxytryptophan to serotonin, and L-tryptophan to tryptamine (De Luca et al., 2003), while tyrosine decarboxylases are involved in the production of invertebrate-specific phenolamines such as tyramine and octopamine (Cole et al., 2005; Ribeiro et al., 2012). Sm*tdc* showed significantly higher transcript levels in EM compared with UM by microarray, SuperSAGE and RNA-seq (Leutner et al., 2013; Lu et al., 2016). This finding was confirmed by our qRT-PCR analysis on UM and EM samples at $t = 0$ h, with transcript levels significantly up-regulated during pairing contact (fold change = 3.2. This difference between UM and EM was consistent during the whole in vitro culture experiment, while separation from the female led to a drastic decline in Sm*tdc* transcript level in males after 96 h. This decline in SEM was reversed by re-pairing, and REM showed equal Sm*tdc* transcript levels compared with EM after 264 h (Fig. 3B).

The GeneDB entry Smp_158480 represents a gene potentially encoding an *ebony* ortholog, which was first discovered in pigmentation mutants of the fruit fly *Drosophila* (Jacobs, 1961; Massey and Wittkopp, 2016). More recent studies in *Drosophila* also revealed its expression in the nervous system, and its function in neurotransmitter inactivation (Richardt et al., 2003; Hartwig et al., 2014). Transcription profiling by qRT-PCR resulted in a similar pairing-influenced tendency as with Sm*tdc*. In agreement with previous transcriptomic data (Leutner et al., 2013; Lu et al., 2016), we found a higher expression of Sm*ebony* in EM compared with UM by qRT-PCR at $t = 0$ h (fold change = 4.1). During in vitro culture, transcript levels generally declined in EM samples, but differences between UM and EM remained significant. As observed for Sm*tdc*, separation led to a reduction of transcription in SEM to the level observed in UM, while re-pairing reversed this effect completely in REM at time point $t = 264$ h (Fig. 3C).

Further, we investigated the pairing-dependent transcript profile of Sm*fst* (Smp_123300), a follistatin ortholog of *S. mansoni*. Previous microarray, SuperSAGE, and qRT-PCR data had already shown that Sm*fst* is differentially regulated in *S. mansoni* males. However, in contrast to Sm*tdc* and Sm*ebony,* the tendency of transcription was opposite with higher transcript levels in UM (Leutner et al., 2013). By analyzing UM and EM samples at $t = 0$ h, we obtained results which corresponded to our previous data (fold change = −1.4). Furthermore, after 264 h of in vitro culture the difference in transcript levels between both conditions remained significant. At this time point, SEM and REM samples were comparable with UM and EM levels, respectively, demonstrating here for the first time, to our knowledge, that separating and re-pairing with the female has a direct influence on Sm*fst* transcript levels in the male (Fig. 3D).

4. Discussion

Over the last decade, a large variety of transcriptomic studies was performed in *S. mansoni* (Ojopi et al., 2007; Nawaratna et al., 2011, 2014; Almeida et al., 2012; Leutner et al., 2013; Anderson et al., 2015; Lu et al., 2016, 2017; Picard et al., 2016), which allowed insights on gene expression patterns in different developmental stages and tissues of the parasite, enabling the identification of genes with potential key functions in schistosome biology. To narrow down the biological roles of those GOI, a diverse molecular tool set is needed which supports comprehensive functional characterizations at the single gene level. This includes the availability of reliable reference genes to confirm changes in the transcription of GOI over different biological samples and experimental conditions. To identify suitable reference genes that could support the investigation of pairing-dependent processes in the male in vitro, we selected 12 candidate genes from independent transcriptomic studies (Fitzpatrick et al., 2009, Leutner et al., 2013, Lu et al., 2016) but also three orthologs of classical "housekeeping" genes. All candidate genes selected from transcriptomic studies were chosen because they showed stable transcript levels among the different stages and/or tissues of the parasite. Although none of the candidate genes was previously characterized in detail, they show homology to orthologs from different model organisms, suggesting conserved roles in ubiquitous cellular processes throughout the Eumetazoa (Table 1). For the validation of candidate genes, we used three well established analysis tools that follow different strategies to determine transcription stability. This combination of the unique strengths of all three algorithms has proven its value in several independent studies (Hildyard and Wells, 2014; Zhang et al., 2014; Sarker et al. 2018). NormFinder (Andersen et al., 2004) applies a mathematical model to evaluate intragroup variation (here, biological replicates of either UM or EM) and intergroup variation (comparison between UM and EM samples) across different sample groups for all candidate genes individually, to identify genes that exhibit the highest transcription stability among all samples. Instead, BestKeeper (Pfaffl et al., 2004) and geNorm (Vandesompele et al., 2002) are both based on pairwise gene comparison. BestKeeper calculates the geometric mean over the whole dataset provided for analysis to generate an artificial normalization factor. Transcription levels of all candidate genes will be compared with this normalization factor to identify genes with the least variation. geNorm uses a similar approach. Each candidate gene is compared with the geometric mean of the data and the gene with the highest variation is excluded. This analysis is repeated until the two genes with the least variation are remaining. The pairwise comparison approach makes BestKeeper and geNorm tolerant against variation in RNA- or cDNA quality, but it is extremely sensitive to the initial preselection of candidate genes used as input. Groups of candidate genes that show similar expression patterns might get a high-ranking score even if they were systematically regulated between samples. To address this potential pitfall, we selected candidate genes with diverse biological functions. Compared with the two other tools, NormFinder performs no direct gene-gene comparison and is, therefore, less affected by correlated expression of subgroups of candidate genes.

Remarkably, in our analysis all three algorithms ranked Sm*gapdh*, Sm*cwf19l1* and Sm*ptpa* as the three most stably transcribed genes between UM and EM, which supports their suitability to serve as reference genes to study pairing-dependent gene expression in *S. mansoni* males. All three genes have distinct cellular functions that are not regulated by pairing. While Sm*gapdh* encodes a key enzyme of glycolysis (Ercolani et al., 1988), the gene product of Sm*cwf19l1* is potentially involved in pre-mRNA splicing (Ohi et al., 2002). On the other hand, Sm*ptpa* encodes a regulatory B subunit of the heterotrimeric protein phosphatase 2A, a ubiquitously expressed enzyme with phosphatase activity in many eukaryotes including schistosomes (Azam et al., 2007; Zhao et al., 2018). With respect to the ranking of the top seven candidates, NormFinder and geNorm showed more overlap than each of the algorithms with BestKeeper. In total, the same six candidate genes were ranked in both analyses among the top seven while BestKeeper still shares four and five genes of its top seven candidates with NormFinder and geNorm, respectively. This large overlap provided confidence to select the seven genes that were shown to be most stably transcribed between UM and EM by at least two of three validation algorithms for further analysis (Table 2). Interestingly, classical "housekeepers" such as actin (Sm*actg1*) or tubulin (Sm*tubb4a*) showed transcription stabilities that were among the

lowest of the 15 candidate genes addressed in our study. These findings are in accordance with multiple studies of different systems and confirm the necessity to individually validate the reliability of reference genes for each experimental setup (Yuan et al., 2014; Zhai et al., 2014; Zheng et al., 2014; Chapman and Waldenström, 2015). We made similar observations when we tested our top seven candidate genes for their transcription stability between pairing-unexperienced females (UF) and pairing-experienced females (EF) in an independent experiment (Supplementary Fig. S3). Here, the NormFinder analysis led to a different ranking than in males, and identified Sm*psmb*7, the proteasome subunit beta type 7, as the most stably transcribed candidate gene.

In vitro cultivation can be a crucial step for the investigation of gene function in larval and adult schistosomes because currently available protocols are not able to completely mimic natural conditions associated with the host environment. As a consequence, important developmental processes of the parasite cannot be followed in vitro. Moreover, some biological aspects including the sexual reproduction of adult worms are only traceable over a limited period of time until egg production declines. This observation is accompanied by a regression of the female's reproductive organs while paired with the male, suggesting that essential factors for egg production are absent in vitro (Galanti et al., 2012; Huang et al., 2012; Pearce and Huang, 2015). However, due to the lack of detailed gene expression studies, it remains unclear how much the transcriptome is changing in both sexes during long-term in vitro culture. Thus, stable in vitro culture conditions are urgently needed, not only because in vitro culture of adult schistosomes is a central part of current medium-throughput drug-screening approaches (Long et al., 2016; Guidi et al., 2017; Weeks et al., 2018), but also for subsequent validation of drug targets and functional studies such as RNAi. The knowledge on reference genes with stable expression under in vitro culture conditions represents an essential step towards this goal. Therefore, we tested the seven most stably expressed candidate genes between UM and EM for expression stability during 11 days of in vitro culture. Compared with our initial analysis, in this context Sm*letm1* and Sm*psmb7* were identified as the most suitable reference genes, indicating that the in vitro culture conditions applied here had no critical influence on the transcript levels of the selected candidate genes.

The close pairing contact between adult *S. mansoni* changes global gene expression in the female drastically by inducing maturation processes in its reproductive organs (Kunz, 2001; Neves et al., 2005; Beckmann et al., 2010; Lu et al., 2016). While these changes can be tracked microscopically, pairing-induced processes in the male are not accompanied by extensive morphological changes and are therefore less understood (Fitzpatrick and Hoffmann, 2006; Picard et al., 2016). Nevertheless, previous transcriptomic studies also revealed pairing-dependent regulation of genes in schistosome males. Among these are genes which, according to their annotation, are associated with neuronal processes. This included neurotransmitters, neuropeptides as well as G-protein coupled receptors (GPCRs) and indicated the involvement of the nervous system during male–female interaction (Wang et al., 2017; Hahnel et al., 2018). To investigate the differential regulation of two GOI with predicted neuronal function (Sm*tdc* and Sm*ebony*) and Sm*fst*, a potential regulator of TGF-beta signaling under the influence of pairing (Leutner et al., 2013), we performed pairing/separation/re-pairing experiments with *S. mansoni* females and males in vitro. In a first step, we were able to verify pairing-dependent transcription of all three GOI previously detected by independent transcriptomic approaches (Leutner et al., 2013; Lu et al., 2016), confirming the suitability of the selected reference genes. Both Sm*tdc* and Sm*ebony*, which may play potential roles in neurotransmitter metabolism, are up-regulated in males after pairing contact. This strongly supports the hypothesis that neuronal processes might be altered in EM (Leutner et al., 2013; Lu et al., 2016; Hahnel et al., 2018). Furthermore, we investigated for the first time how separation and re-pairing influences the transcription of these genes in *S. mansoni* males. Our qRT-PCR results demonstrate that the expression of both genes is switched on and off depending on the actual pairing status. Suggested by their homology, both genes might be associated with dopamine or octopamine signaling. Remarkably, these neurotransmitters play important roles in reproduction of invertebrate model organisms. In *Caenorhabditis elegans*, dopaminergic cells are involved in male sperm ejaculation (LeBoeuf et al., 2014), while octopamine regulates mating behavior in *Drosophila* males (Andrews et al., 2014). On the other hand, Sm*fst*, a *S. mansoni* follistatin ortholog, is up-regulated in UM. Follistatins are known to be negative regulators of TGF-beta signaling pathways. They bind TGF-beta receptor ligands such as BMP (Balemans and Van Hul, 2002), and thus negatively influence TGF-induced signal transduction. Since the TGF-beta signaling is postulated to be involved in reproductive processes in schistosomes (LoVerde et al., 2007; Buro et al., 2013), Sm*fst* might prevent the activation of this pathway prior to pairing in UM (Leutner et al., 2013). As for the other GOI, Sm*fst* expression seems to be strictly regulated by contact with the female. These findings have implications for schistosome biology, pointing to the existence of pairing-influenced competence factors in males that may have functional relevance for the male–female interaction and its consequences for the pairing-dependent sexual development of the schistosome female, a topic for future studies.

As a guideline for the selection of appropriate reference genes for future gene expression studies in the schistosome field, we recommend the following steps: (i) If appropriate for the specific research question, the preselection of candidate reference genes should be based on existing transcription profile data such as the RNA-seq dataset by Lu et al. (2016) which provides the first information on the stability of gene expression in different sexes or pairing states of schistosomes. (ii) Select multiple candidate reference genes and rank them according to their transcription stability in the desired experimental setting (e.g. in vitro culture) by using multiple analysis algorithms. The most stable genes would appear among the top candidates in more than just one analysis. (iii) For the most accurate normalization of qRT-PCR data, a geometric averaging of at least two reference genes should be considered (Vandesompele et al., 2002). (iv) For each new experimental setup (e.g. change in in vitro culture conditions, change in stage or sex of worms), the selected reference genes should be newly evaluated.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.01.006.

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