# Characterization of genes with a putative key role in the parasitic lifestyle of the nematode *Strongyloides ratti*

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(*Received 5 March 2012; accepted 17 March 2012; first published online 2 May 2012*)

SUMMARY

Parasitic nematodes are significant pathogens of humans and other animals. The molecular and genetic basis of animal parasitism is not yet fully understood. *Strongyloides* spp. are a genus of gastrointestinal nematodes of which species infect approximately 100–200 million people worldwide. *S. ratti* is a natural parasite of the rat, and a useful and amenable laboratory model. Previous EST and microarray analyses of the *S. ratti* life cycle have identified genes whose expression was specific, or biased, to the parasitic adult stage, suggesting that they may play a key role in parasitism in this species. Here we have further investigated the expression of these genes (by RT-PCR) throughout the *S. ratti* life-cycle. We produced recombinant proteins *in vitro* for a subset of these genes, which were used in Western blot analyses to investigate the distribution of the gene products among different stages of the *S. ratti* life cycle. We tested the efficacy of these recombinant proteins as anti-*S. ratti* vaccines. One of the proteins was detected in the excretory/secretory products of the parasitic stages.

Key words: Strongyloides, protein expression, gene expression, vaccination.

## INTRODUCTION

Infection is normal: most species of plants and animals, including humans, are subject to parasitism at some stage during their life. Approximately a quarter of the world's human population are infected with gastrointestinal nematodes, with the infection concentrated in the poor in tropical, developing countries. *Strongyloides* spp. is a genus of gastrointestinal nematodes which infect a wide range of hosts. There are two species that infect humans, *S. stercoralis* and *S. fuelleborni*. It is estimated that 100–200 million people are infected with *S. stercoralis* worldwide (Albonico *et al*. 1999). *S. ratti* is a natural parasite of rats and can be used as a model for other *Strongyloides* spp. infections (Fisher and Viney, 1998).

Infection occurs when the infective larvae of *S. ratti* penetrate the skin of the host and migrate to the gut where they embed themselves in the gut mucosa and moult (*via* an L4 stage) into parasitic females. Parasitic females reproduce by mitotic parthenogenesis and produce eggs which (as a mixture of eggs and newly hatched L1s) are passed out of the host in faeces (Viney, 1994). These eggs are genetically male or female. Larvae develop into free-living adults (*via* 4 larval stages). Female larvae can develop directly *via* direct or, so-called, homogonic development into infective third-stage larvae (iL3s). Alternatively, female larvae can moult into free-living females (*via* indirect or, heterogenic, development); male larvae only moult into free-living males (Viney and Lok, 2007). Adult free-living males and females reproduce by sexual reproduction, and the progeny moult into infective larvae that are ready to infect new hosts (Viney *et al*. 1993).

A central challenge in parasitic nematode biology is to understand the molecular and genetic basis by which parasitic nematodes have adopted a parasitic lifestyle (Thompson *et al*. 2008). An approach to this is to identify genes and gene products that are expressed uniquely in the parasitic stage. The underlying concept is that genes and gene products that are expressed only in the parasitic stage must have a specific role in that life-cycle stage, and hence the parasitic lifestyle. The life-cycle of *Strongyloides* is particularly powerful in such an analysis, because it has both a parasitic and a free-living adult female stage, which are genetically identical. Thus, this life-cycle provides the potential to identify genes and gene products that are expressed in parasitic females but not in free-living females (Thompson *et al*. 2009). This comparison is in contrast to most parasitic nematodes for which, typically, adult stages are parasitic, but larval stages are free-living; therefore, the 'developmental stage' (i.e. adult *vs* larval) is confounded with 'lifestyle' (parasitic *vs* free-living) in any comparative analysis (Viney and Cable, 2011).

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*Parasitology* (2012), **139**, 1317–1328. 
doi:10.1017/S0031182012000637

For parasitic nematodes, the principal features of the within-host environment are those provided by aspects of host physiology (temperature, pH, gaseous balance *etc*.) as well as the host immune response. Many aspects of the within-host environment (perhaps especially that it is more stable and predictable than a free-living environment; Gardner *et al*. 2006) may present very favourable niches (Viney and Cable, 2011). However, the host immune response can actively cause harm to parasites, and parasites seek to protect themselves from this harm. Helminth parasites use strategies of host mimicry, host immunomodulation and the generation of counter-effector molecules to survive with the host (Blackburn and Selkirk, 1992; Maizels *et al*. 1993, 2004; Viney and Cable, 2011).

Previously for *S. ratti*, 14761 ESTs were obtained which were grouped into 4152 clusters, each representing a putative gene (Thompson *et al*. 2005). The relative abundance of ESTs within each cluster in different *S. ratti* life-cycle stages was used as a measure of the relative level of gene expression (Thompson *et al*. 2006); this latter assumption has some empirical support (Alkharouf *et al*. 2006). EST abundance data were used to identify genes whose expression was putatively specific, or biased, to the parasitic female stage (Thompson *et al*. 2008). Further, this approach sought genes that, by bioinformatic analyses, appeared to be unique to parasitic species, rather than common with free-living species (Thompson *et al*. 2008). This initial approach was used to identify a series of genes whose further characterization we present here. For these genes we have undertaken a detailed life-cycle-stage expression analysis and then used these data (together with additional sequence data) to select a subset of genes for *in vitro* protein expression, which allowed *S. ratti* life cycle stage-specific Western blot analyses of these proteins, together with vaccination experiments.

## MATERIALS AND METHODS

### *Rationale*

We had previously identified 7 genes: SR00007 (GenBank ID: JQ437394), SR00034 (GenBank ID: JQ437395), SR00035, SR00449 (GenBank ID: JQ437396), SR00984 (GenBank ID: JQ437397), SR01000 (GenBank ID: JQ437398), SR01014 (GenBank ID: JQ437399) that, by analysis of life-cycle stage-specific EST abundance, suggested that their expression was specific, or biased, to the parasitic female stage of *S. ratti* (Thompson *et al*. 2005). Further, previous bioinformatic analyses of these genes were consistent with the idea that these genes may have novel functions in *S. ratti* (Thompson *et al*. 2005, 2008). We therefore sought to further investigate and characterize these genes in the parasitic life of *S. ratti*.

### *Parasites*

The *S. ratti* isofemale line ED321 Heterogonic was used throughout, and maintained in female Wistar rats as previously described (Viney, 1996). Free-living stages were obtained from feces of infected animals. L1s were isolated from fresh faeces using a Baermann funnel held at 25 °C; L2s were obtained by culturing faeces at 19 °C for 24 h, followed by Baermann funnel extraction, as previously described (Thompson *et al*. 2005). L4s were obtained by culturing faeces at 19 °C for 48 h, after which the worms in the water surrounding the faecal mass were removed. Infective third-stage larvae (iL3s) were isolated from faecal cultures grown at 19 °C for 3 days, at which time the iL3s had crawled away from the faecal mass into the water, from which they were harvested. To collect free-living males and females, faecal cultures were grown for 3 days at 19 °C and worms in the water surrounding the faecal mass were removed (Thompson *et al*. 2005). Free-living stages were purified by density centrifugation using sucrose (Thompson *et al*. 2005), and free-living adult males and adult females were separated into sex-specific pools by microscopic observation (Thompson *et al*. 2009). Parasitic females were harvested from the intestine of sacrificed animals at 6 days post-infection (p.i.) and cleaned using a Percoll gradient, as previously described (Thompson *et al*. 2005). All worm material was snap-frozen in an equal volume of TRI-Reagent with liquid nitrogen and then stored at −70 °C.

### *Nucleic acid preparation*

To prepare RNA or DNA from these samples, the material was defrosted into TRI-Reagent (Sigma), mechanically disrupted using an electronic homogenizer (VWR) and RNA or DNA extracted following the TRI-Reagent protocol. cDNA was prepared from RNA using the Quantitech reverse transcription kit (Qiagen).

### *Reverse transcriptase PCR (RT-PCR)*

Semi-quantitative RT-PCR was performed on 5 $\mu$g of total RNA that was reverse transcribed using Superscript II (Qiagen) following the manufacturer's instructions. Four replicate preparations of RNA were used to prepare cDNA; two replicate RT-PCR reactions were performed on each cDNA preparation, therefore giving a total of 8 amplicons. The primers used are shown in Table 1. The control gene was *act-3* (Crook and Viney, 2005). PCR amplification for each gene was sampled in 3 cycle increments over 14–35 cycles and the highest cycle number from the exponential phase was used to calculate expression, a method developed for, and previously validated with, *S. ratti* (Crook and Viney, 2005). In these analyses, the relative quantity of cDNA

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Table 1. Primers used in RT-PCR and RACE

| Gene | Primer | Sequence |
|---|---|---|
| SR00007 | RTF[1] | AAAATAACAACCAATTTATT |
| | RTR | AAGTTATGAATAATTTATTG |
| | 5GSP1[2] | TTGCATGGTTTTCTTGGAGA |
| | 5GSP2 | TTCTTTTCGATTCAGCAGCA |
| | 5GSP3 | TGCTCCCATGATTTGATATGA |
| | 3GSP1 | TCATATCAAATCATGGGAGCA |
| | 3GSP2 | ATAGAGGGGCATGGAGAAGC |
| SR00035 | RTF | GATGATATCTTTTCTTTTGCTAT |
| | RTR | AACAGACTGAAACATATGAGAAT |
| SR00034 | RTF | TGATACTGGTATTGGTATGACTA |
| | RTR | ACAAGTTTTGATAAAGTATCTGG |
| SR00449 | RTF | TGATGAAGTATCTAACAACGTC |
| | RTR | CAAATCATTAGCGAATTTAATA |
| | 5GSP1 | TCGTGGGTTTTTCTTCCTTG |
| | 5GSP2 | CACCCATAGGACGTCTGTGTT |
| | 5GSP3 | TCGCTTCCACCTTGAAAAAC |
| | 5GSP4 | ACTTCGGTGACGTAGCTCGT |
| | 3GSP1 | GGAAGCGAGGGCTACTTAGG |
| | 3GSP2 | GATGACTGGAGCGAAGAGGA |
| SR00984 | RTF | CATGTCCAGAGGTAGTAAATAAC |
| | RTR | GAAATTAGTCCCATAAAATAAAA |
| | 5GSP1 | TCAATTGGGACAGTCTTGACA |
| | 5GSP2 | ACGGACGAAACTTCTTTGGA |
| | 5GSP3 | CCTCTGGACATGGTTCCATAA |
| | 3GSP1 | ATGGAACCATGTCCAGAGGT |
| | 3GSP2 | CGGAACCATCCAAAGAAGTT |
| | 3GSP3 | CTAAGGACGGTGTCCTCACAG |
| SR01000 | RTF | ACTGACAATACTTTACCACTACC |
| | RTR | AACATTTACTGTTCCAGTACTTC |
| SR01014 | RTF | GCTATTTATTATTCGCTATGTTT |
| | RTR | ATATTTGTCACTCTTCTCTTCAT |

[1] RTF and RTR, forward and reverse primers, respectively, for RT-PCR. [2] 5GSP and 3GSP, primers used for 5′ and 3′ RACE, respectively.

between the different life-cycle stages is sought, which obviates the need to measure absolute quantities. The relative expression of genes between the different *S. ratti* life-cycle stages was calculated as the ratio of the quantity of gene amplicon/control *act-3* amplicon. The data presented are the mean (±1 S.D.) of the 8 replicate PCR ratios; failed PCRs were excluded from these analyses.

### *RACE*

To extend the sequence information available from the relevant ESTs (Thompson *et al*. 2005), both 5′ and 3′ RACE were performed using the primers listed in Table 1. RACE was performed using a 3′ and 5′ RACE kit (Invitrogen) following the manufacturer's instructions. PCR products resulting from this were cloned into pCR2.1-TOPO (Invitrogen) and commercially sequenced. Further sequence information was also obtained by resequencing existing ESTs.

### *Bioinformatic analysis*

A selection of bioinformatic analyses was performed using widely available software. BLASTP was used to compare the predicted protein sequence to known proteins in the GenBank non-redundant protein database in August 2011 (http://blast.ncbi.nlm.nih.gov). Gene sequences were further annotated with the use of Motif scan (Pagni *et al*. 2007) and SMART (Letunic *et al*. 2009). SignalP 3.0 (Bendtsen *et al*. 2004) was used to predict the presence of signal peptides present for each of the genes studied. With the full length *S. ratti* genome nearing completion it was also possible to identify the contigs (September 2011) on which these genes occurred, which was done using BLASTN (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/strongyloides).

### *Recombinant protein expression*

The full open reading frame (ORF) of SR00449 (nucleotides 87 to 2120) was expressed by cloning it into the pcDNA3.1/V5-His vector (Invitrogen) which was used to transfect HEK 293 cells (Invitrogen) using the Lipofectamine 2000 method (Invitrogen). A shorter length portion (nucleotides 1741-2035) of SR00449, incorporating the sequence with significant homology to CPG-3 (see below), was

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codon optimized for bacterial expression (MWG Operon) and expressed using the pET28$\alpha$(+) vector in BL21 *Escherichia coli* (NEB). For SR00007, in an approach analogous to that used with the shorter SR00449 fragment, nucleotides 96 to 1581 (encompassing the CPG-3 domain) were first cloned into the pET28$\alpha$(+) vector before subsequent expression in BL21 *Escherichia coli* (NEB). The entire ORF of SR00984 (nucleotides 68 to 547) was expressed in bacteria. To do this the sequence was codon optimized for bacterial expression (MWG Operon) and expressed as for the shorter version of SR00449. For bacterial expression, expression of the recombinant proteins was induced by IPTG (to a final concentration of 0·5 mM) and purified using a His-Trap column (GE Healthcare) following the manufacturer's protocol. Proteins with a molecular mass greater than 12 kDa were concentrated using a 12 000 MWCO vivaspin filter (Sartorius).

### *Immunization, antisera and ELISA*

Recombinant proteins SR00007, SR00449 and SR00984 were used to immunize female Wistar rats. Animals were immunized with 100 $\mu$g of purified protein (for SR00449 this was a mixture of 50 $\mu$g of each of the full length and shorter version) in 50 $\mu$l of PBS with an equal volume of TiterMax Gold adjuvant (Sigma). Control immunizations were the same, but used 50 $\mu$l of PBS in place of the recombinant protein. Animals were immunized 3 times, with each immunization 14 days apart (i.e. days 1, 15, 27). Animals were killed on day 41 and exsanguinated by cardiac puncture, the blood left to clot at room temperature for 1 h, after which it was centrifuged at 14 000 ***g*** at 4 °C for 5 min, the serum removed, aliquoted and stored at −20 °C. The titre of these antisera was measured by ELISA, as described by Wilkes *et al*. 2007, but using a polyclonal goat anti-rat IgG HRP conjugate (Nordic) antiserum for detection. In an analogous experiment to that described above, *S. ratti* hyperimmune serum (Wilkes *et al*. 2007), and serum samples from animals with a primary *S. ratti* infection (6, 12 and 18 days p.i.) were used to identify whether an immune response to these proteins occurs naturally during the course of infection.

A second ELISA was performed which sought to detect SR00449 in *S. ratti* excretory/secretory (ES) products. To collect ES products free-living males and free-living females were obtained (as above) and then held in PBS at 19 °C for 24 h; for parasitic females, they were obtained (as above) and held in PBS at 25 °C for 24 h. Each sample was then centrifuged at 2000 ***g*** for 2 min at 4 °C and the ES supernatant removed; the pellet (which contained the worms) was re-suspended in PBS and snap-frozen in liquid nitrogen before being thawed and then sonicated (MSE) 3 × 30 s on ice, after which the samples were centrifuged at 12 000 ***g*** for 5 min at 4 °C, and the supernatant retained. Protein concentrations of the ES and sonicated worm samples were determined using Bradford's reagent (Sigma). ELISA plates were coated with 50 $\mu$g of these protein samples for 24 h and an ELISA conducted, as described above.

### *Western blot*

Protein extracts of different *S. ratti* life-cycles stages were prepared by sonication (as above), after which the samples were centrifuged at 12 000 ***g*** for 10 min at 4 °C, the supernatant removed, and stored in aliquots at −20 °C. Control protein samples of mixed-stage *C. elegans*, a 1 cm length of rat gut tissue, and distilled water, were prepared in the same manner. The protein concentration of all samples was determined as described above and then 100 $\mu$g of each sample was separated on 15% (w/v) SDS-PAGE gels and transferred to a nitrocellulose membrane (Amersham) using standard methods. Nitrocellulose membranes were then probed with 1:5000 dilutions of the relevant rat antisera. The Western blots were visualized using the ECL detection kit (Amersham) following the manufacturer's protocol. A goat anti-rat IgG HRP antiserum (Nordic) was used for detection.

### S. ratti *vaccination and challenge*

We investigated whether vaccination of rats with these recombinant proteins provided protection against a subsequent *S. ratti* challenge infection. In the first experiment, animals were immunized, as above, with the SR00007 recombinant protein ($n = 8$), or for the control, PBS only ($n = 8$), on days 1, 15 and 27. Blood samples were taken by tail venipuncture on day 41 and the anti-SR00007 titres determined by ELISA (as above). Immunized and control animals were challenged with 1000 iL3s on day 45. To determine the effect of immunization, faeces were collected (days 51, 55, 59, 62 and 66) and cultured for 3 days at 19 °C, after which the total number of worms in those cultures was determined, as previously described (Paterson and Viney, 2002).

In the second experiment, animals were immunized, as described above, in a total of 4 groups: (i) SR00449 this was a mixture of 50 $\mu$g each of the full length and shorter version ($n = 4$); (ii) 100 $\mu$g of the SR00984 recombinant protein ($n = 4$); (iii) a mixture group consisting of 25 $\mu$g SR00007, 25 $\mu$g SR00449 (full length), 25 $\mu$g SR00449 (shorter version) and 25 $\mu$g SR00984 ($n = 4$); (iv) for the negative control, PBS only ($n = 4$). All protein was mixed with an equal volume of adjuvant and used for immunization on days 1, 15 and 27. Blood sampling and subsequent ELISA was performed as described above. A positive

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Table 2. Seven *Strongyloides ratti* genes and their four most significant BLASTP alignments

| Gene | Alignment | Species | Accession no. | Score | Probability |
|---|---|---|---|---|---|
| SR00007 | Chondroitin proteoglycan 3 | *Ascaris suum* | ADY47757.1 | 104 | $1e^{-23}$ |
| (75349)+ | Chondroitin proteoglycan 3 | *A. suum* | ADY49493.1 | 96·7 | $5e^{-21}$ |
| | Chondroitin proteoglycan 3 | *C. briggsae* | XP_002629883.1 | 97·8 | $2e^{-20}$ |
| | Chondroitin proteoglycan 3 | *A. suum* | ADY47377.1 | 93·6 | $6e^{-20}$ |
| SR00449 | Chondroitin proteoglycan 3 | *A. suum* | ADY47757.1 | 120 | $1e^{-28}$ |
| (74969) | Hypothetical protein | *Loa loa* | XP_003138540.1 | 116 | $5e^{-28}$ |
| | Chondroitin proteoglycan 3 | *A. suum* | ADY48277.1 | 116 | $1e^{-27}$ |
| | Chondroitin proteoglycan 3 | *A. suum* | ADY47584.1 | 107 | $2e^{-23}$ |
| SR00984 | Small HSP | *Trichinella pseudospiralis* | ABJ55915.1 | 105 | $7e^{-27}$ |
| (75470) | Small HSP | *T. spiralis* | ABJ55914.1 | 105 | $1e^{-26}$ |
| | HSP-17b | *C. elegans* | NP_001023958.1 | 103 | $3e^{-26}$ |
| | HSP-17a | *C. elegans* | NP_001023957.1 | 103 | $3e^{-26}$ |
| SR01014 | Small HSP | *A. suum* | ADY49707.1 | 115 | $2e^{-31}$ |
| (75451) | HSP-17 | *C. brenneri* | EGT60481.1 | 102 | $1e^{-25}$ |
| | HSP-17b | *C. elegans* | NP_001023958.1 | 101 | $2e^{-25}$ |
| | HSP-17a | *C. elegans* | NP_001023957.1 | 101 | $2e^{-25}$ |
| SR00034 | Endoplasmin precursor | *Brugia malayi* | XP_001899398.1 | 1058 | 0·0 |
| (74959) | Endoplasmin | *A. suum* | ADY41733.1 | 1055 | 0·0 |
| | Endoplasmin | *A. suum* | ADY41505.1 | 1047 | 0·0 |
| | Hypothetical protein | *C. remanei* | XP_003107918.1 | 1038 | 0·0 |
| SR01000 | Sortilin-related receptor | *A. suum* | ADY42121.1 | 390 | $9e^{-127}$ |
| (75319) | Low density lipoprotein receptor | *L. loa* | XP_003147428.1 | 383 | $1e^{-123}$ |
| | Low density lipoprotein receptor | *B. malayi* | XP_001898946.1 | 377 | $1e^{-121}$ |
| | EGG-1 | *C. elegans* | XP_002636254.1 | 322 | $2e^{-100}$ |
| SR00035 | No significant alignment | – | – | – | – |

+ Denotes *S. ratti* genome assembly contig number (September 2011).

control consisted of animals that had been given a previous *S. ratti* infection ($n=4$). To do this, animals were infected subcutaneously with 500 iL3s on day 29. Animals were twice given an *S. ratti* curative dose of 176 mg Thiabendazole in 200 $\mu$l of sunflower oil on days 41 and 42, as previously described (Paterson and Viney, 2002). Confirmation that this treatment cleared the infection was done by faecal examination 3 days post-Thiabendazole administration. Immunized and control animals were challenged with 500 iL3s on day 45, faeces collected (days 51, 53, 55, 58, 61, 64 and 67) and, as above, the total number of worms in those cultures determined.

### *Data analysis*

To determine whether the expression of these genes differed across the life cycle of *S. ratti*, data were analysed by one-way ANOVA with *post-hoc* Tukey HSD tests. RT-PCR data are shown as the mean ratio between the gene and control *act-3* gene fluorescence (±1 S.E.).

The ELISA OD data are values recorded at 490 nm above a background reading of PBS-only wells. Data reported here are the mean (±1 S.D).

To investigate the effect of the immunization treatments on the challenge *S. ratti* infection, we used generalized linear models (with a normal error structure) to analyse the total *S. ratti* reproductive output of the rats. Total reproductive output was calculated by summing the total number of worms present in faecal cultures on sample days, with interpolation for non-sample days (Crook and Viney, 2005). The mean total worm count (±1 S.E.) for each treatment group is presented here.

## RESULTS

### *Bioinformatic analyses*

RACE analyses and further EST sequencing identified more sequence data for these 7 genes, which facilitated bioinformatic analyses of each gene, whose putative identification is shown in Table 2.

The sequence data for genes SR00007, SR00034, SR00449, SR00984, SR01000 and SR01014 are in GenBank as JQ437394, JQ437395, JQ437396, JQ437397, JQ437398, JQ437399 respectively. The sequence data for SR00035 is too short for GenBank submission.

The genes SR00007 and SR00449 both had significant alignments to chondroitin proteoglycan 3 (CPG-3) of *Ascaris suum* ($1e^{-23}$ and $1e^{-28}$ respectively). More specifically, these alignments were localized towards the C-terminal end of the computationally translated protein sequence (Fig. 1). Outside of this region there is no significant alignment of SR00007 and SR00449 to other genes. Within the current *S. ratti* genome assembly, SR00007 and

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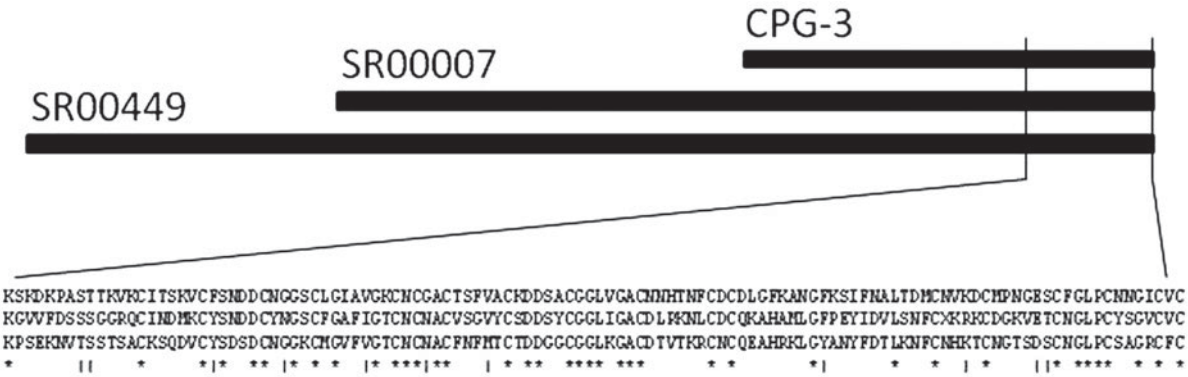


Fig. 1. A schematic depiction of the alignment of the complete sequences of *Ascaris suum* CPG-3, SR00007 and SR00449, with the amino acid alignment of the specified region shown below with common identity shown by *.

SR00449 occur on different contigs (Table 2), which suggests that they are different loci. SignalP predicted a signal peptide present in the SR00449 sequence.

The genes SR00984 and SR01014 also shared significant alignments to the alpha crystalline HSP superfamily of proteins from *Trichinella pseudospiralis* and *A. suum*, respectively (Table 2). Interestingly, these 2 *S. ratti* genes had significant alignments to the 2 isoforms (i.e. a and b) of the *C. elegans* HSP-17 (Table 2). Within the current *S. ratti* genome assembly, SR00984 and SR01014 occur on different contigs also suggesting that they are different loci.

The gene SR00034 had significant alignments to many proteins belonging to the HSP-90 family, as identified through domain analysis. The most significant alignment was to an endoplasmin precursor of *Brugia malayi* (Table 2). SMART analysis identified a histadine kinase-like ATPase domain (typically found in the HSP-90 superfamily of proteins) towards the N-terminal end of SR00034. Despite these alignments and domain analyses, it is unlikely that SR00034 is an HSP-90 coding gene of *S. ratti*, because SR00034 does not have the N-terminal MEEVD sequence motif that is conserved among HSP-90 molecules (Devaney *et al.* 2005).

The gene SR01000 had significant alignment to a Sortillin-related receptor of *A. suum* (Table 2) and to low density lipoprotein receptors of *Loa loa*, *B. malayi* and *C. elegans*. Domain analysis showed that these alignments have an almost identical domain structure (i.e. transmembrane and low-density lipoprotein receptors) to that of SR01000 (Fig. 2).

There was no significant alignment of the available sequence for the gene SR00035 (Table 2). RACE analysis did not discover additional sequence of this gene.

### *Gene expression*

The expression of the genes SR00007 and SR00449 was significantly greater in the parasitic female stage

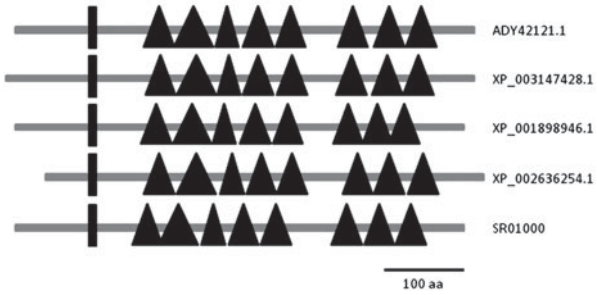


Fig. 2. SMART domain analysis of SR01000 and its most significant BLAST alignments (Table 2). (▮) Transmembrane region, (▲) low-density lipoprotein receptor (LDLr).

compared with other life-cycle stages (Fig. 3A, B). For SR00007, expression varied significantly between life-cycle stages ($F_{1,22}=31{\cdot}3$, $P=0{\cdot}001$), and expression in parasitic females was significantly different to all other stages ($P=0{\cdot}001$). For SR00449, expression varied significantly between life-cycle stages ($F_{1,24}=24{\cdot}3$, $P=0{\cdot}001$) and expression in parasitic females was significantly different to L1, L2, L4 and iL3 stages ($P=0{\cdot}001$), but not to expression in free-living males and females ($P=0{\cdot}072$ and $P=0{\cdot}149$, respectively).

The expression of SR00984 was also greatest in the parasitic female stage (Fig. 3C). (Expression varied significantly between life-cycle stages, $F_{1,24}=115{\cdot}4$, $P=0{\cdot}001$; expression in parasitic females was significantly different to all other stages, $P=0{\cdot}001$). The expression of SR01014 was significantly greater in the parasitic female, free-living female and L4 larval stage compared with other life-cycle stages (Fig. 3D). (Expression varied significantly between life-cycle stages, $F_{1,26}=9{\cdot}164$, $P=0{\cdot}001$; expression in parasitic females was significantly different to expression in L1, L2 and iL3 stages, $P=0{\cdot}001$, and free-living males, $P=0{\cdot}041$, but not significantly different from that of L4s and free-living females).

There was no significant difference in the expression of SR00034 among the *S. ratti* life-cycle stages (Fig. 3E). The expression of SR01000 was

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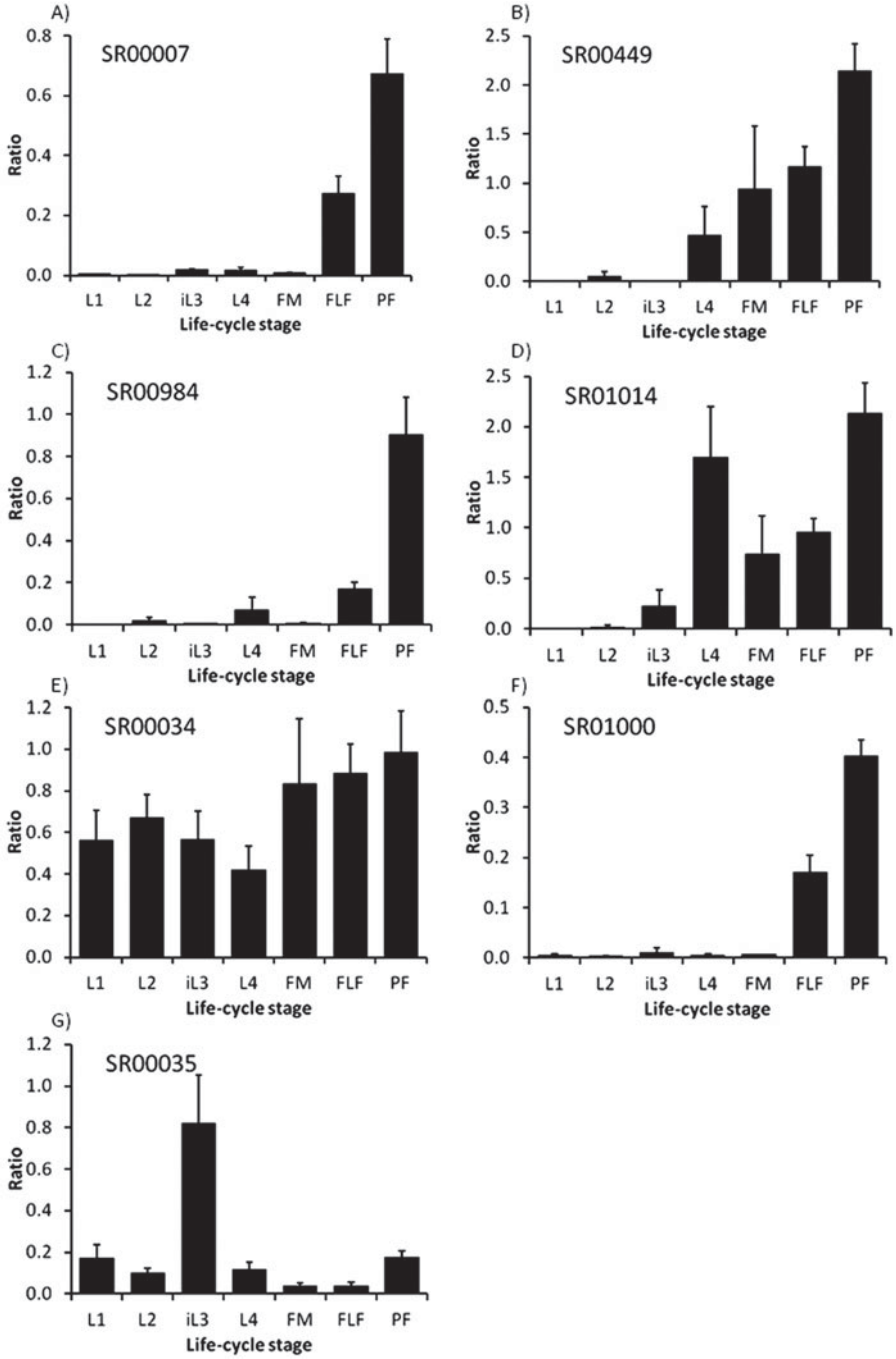


Fig. 3. Gene expression between different life-cycle stages for (A) SR00007, (B) SR00449, (C) SR00984, (D) SR01014, (E) SR00034, (F) SR01000 and (G) SR00035. Expression is shown as a ratio of the gene's expression compared with the control *act-3* gene. Error bars are ±1 S.E. L1 L2 iL3 FM, free-living male; FF, free-living female; PF, parasitic female.

greatest in the parasitic female stage (Fig. 3F). Expression varied significantly between life-cycle stages, $F_{1,26}=76{\cdot}009$, $P=0{\cdot}001$; expression in parasitic females was significantly different to all other stages, $P=0{\cdot}001$.

The expression of SR00035 was significantly different across the different life-cycle stages ($F_{1,74}=8{\cdot}890$, $P=0{\cdot}001$), with greatest expression in the iL3 stage (Fig. 3G); expression of SR00035 in the parasitic female stage was not significantly different from other life-cycle stages.

These RT-PCR data have, therefore, identified genes whose expression was, overall, greatest in the parasitic female stage. These data were used to select a subset of genes, namely SR00007, SR00449 and SR00984, for *in vitro* expression and further analyses.

### *Protein expression*

The antisera raised to SR00007, SR00449 (both full and shorter length), and SR00984 had titres of

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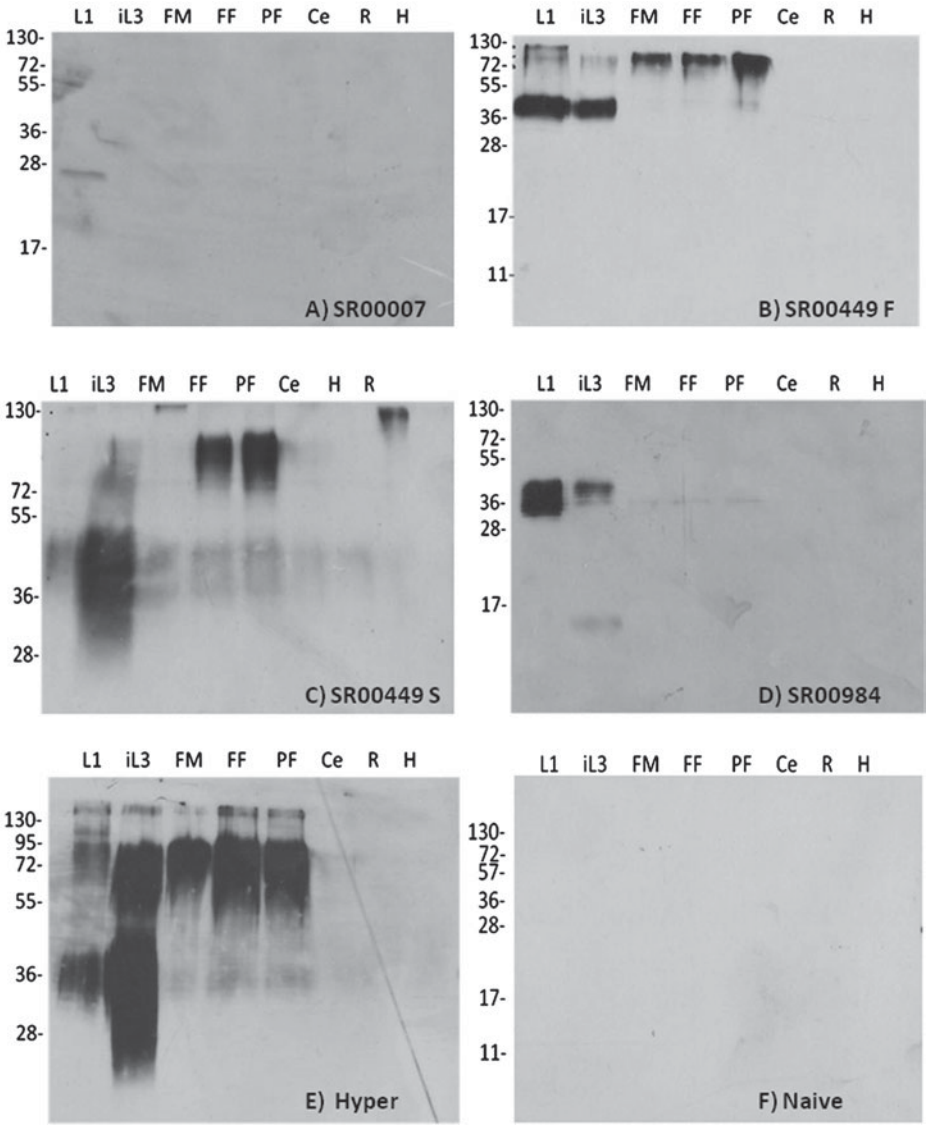


Fig. 4. Western blots probed with antisera to (A) SR00007, (B) SR00449 (full length), (C) SR00449 (shorter version), (D) SR00984 and (E) hyperimmune serum (F) naïve rat serum. L1, first-stage larvae; iL3s, infective third-stage larvae; FM, free-living male; FF, free-living female; PF, parasitic female; Ce, *C. elegans* mixed stage; R, rat gut; H, water.

1:64 000, 1:256 000, and 1:64 000, respectively. In Western blot analysis the anti-SR00007 serum recognized only a protein of approximately 28 kDa in L1 stages (Fig. 4A). Naive serum did not detect any bands, showing the specificity of these anti-SR00007 serum results (as well as for other sera used, below). This is in notable contrast to the RT-PCR data, where the greatest expression was in the parasitic female stage (Fig. 3A). The parasitic female stage will, of course, contain *in utero* eggs within which there are developing L1s. It is therefore possible that the parasitic female *in utero* eggs contain mRNA for SR00007, but that this is only translated in hatched L1s.

The anti-SR00449 serum (generated to the full length SR00449 version) detected a major band of approximately 72 kDa in parasitic females, and of the same size (but less intensely) in the free-living male and female stages (Fig. 4B). This is consistent with the RT-PCR expression data for this gene. However, the anti-SR00449 serum also detected bands of approximately 40 kDa in the first and infective third-stage larvae. There is a suggestion that this band is also present in the parasitic female stage, consistent with the *in utero* presence of L1s. The second anti-SR00449 serum (generated to the shorter length version) identified bands in the free-living and parasitic females (but not free-living males) which may be the equivalent of the 72 kDa band (above); this antiserum also recognized protein that may be equivalent to the 40 kDa band (above), principally in the iL3 stage, but in all stages (and with evidence in control lanes) (Fig. 4C).

The anti-SR00984 serum detected bands of approximately 36 kDa predominantly in the L1 and, to a lesser extent, in the iL3 stage, although these bands were also, just, detectable in all life-cycle stages tested (Fig. 4D). These results are not

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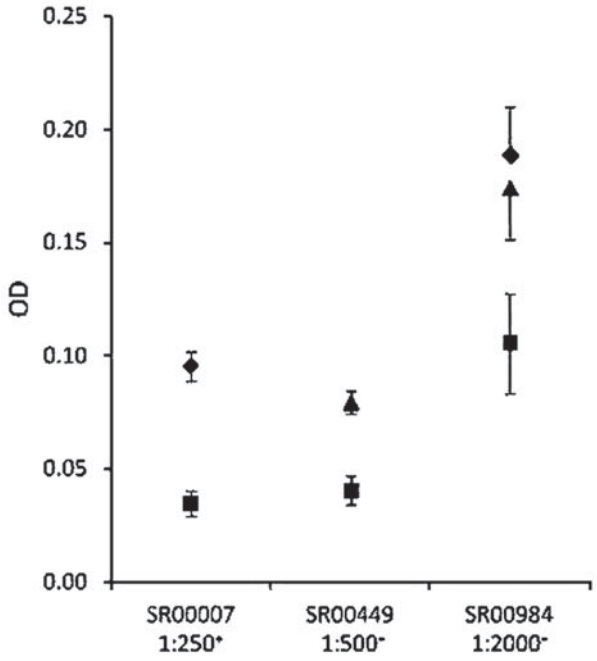


Fig. 5. The optical density (OD) determined by ELISA of 6 days p.i. serum (♦) or hyperimmune serum (▲), at the titres shown by +, compared with serum from naïve animals (■) against SR00007, SR00449 and SR00984. Error bars are ±1 S.E.

consistent with the RT-PCR data (where greatest expression was in the parasitic females) although, analogous to the above, the L1 stages will be within *in utero* eggs.

Hyperimmune serum (Wilkes *et al*. 2007) identified many different proteins particularly in the iL3 sample ranging from approximately 20 kDa to greater than 130 kDa (Fig. 4E). There appears to be some overlap in the proteins recognized by this hyperimmune serum and those recognized by the anti-SR00449 serum (Fig. 4C).

### *Excretory/secretory products and infection serum*

A 1:800 dilution of anti-SR00449 (full-length) serum resulted in an OD of 0·245 (±0·03) and 0·153 (±0·02) against parasitic female ES and sonicated material, respectively (for free-living males and females, and *C. elegans* and PBS controls OD < 0·08). This suggests that SR00449 is present in *S. ratti* parasitic female ES and is consistent with the computational predication of a signal peptide (above).

We investigated whether sera taken from rats during a *S. ratti* infection, or *S. ratti* hyperimmune serum, recognized SR00007, SR00449 (shorter and full-length version) and SR00984. This found that the 6-day p.i. *S. ratti* infection serum recognized SR00007 and SR00984 (at titres of 1:250 and 1:2000, respectively) and that the hyperimmune serum recognized SR00449 and SR00984 (at titres of 1:500 and 1:2000, respectively) (Fig. 5). (The 12 or 18 day p.i. *S. ratti* sera did not recognize any of these proteins.)

### S. ratti *vaccination and challenge*

Immunized animals had high antigen-specific titres (1:64 000, 1:128 000, 1:64 000 and 1:128 000 for immunization with SR00007, SR00449, SR00984 and the 3-protein mixture, respectively). Overall there was no significant effect of these vaccinations ($F_{1,7}=0{\cdot}52$, $P=0{\cdot}52$; $F_{1,7}=0{\cdot}97$, $P=0{\cdot}40$; $F_{1,7}=3{\cdot}25$, $P=0{\cdot}17$ for SR00984, SR00449 and the 3-protein mixture, respectively) on the total reproductive output of the *S. ratti* challenge infection, compared with the negative control (PBS) immunization (Fig. 6). In the first experiment (Fig. 6A) the total reproductive output (±1 S.E.) for SR00007 (50 078 ± 9420) was greater than for the PBS control group (37 061 ± 8394). In the second experiment (Fig. 6B) immunization with SR00984, SR00449 and the 3-protein mixture reduced the mean total reproductive output by 18·4%, 11·6% and 26·1%, respectively, compared with the PBS control group.

## DISCUSSION

Genomic-scale gene discovery has intensified the need to understand the function of genes. Parasites evolved from free-living ancestors which has required that parasites acquired altered physiological processes and mechanisms that we now see in parasitic lifestyles. These parasitism-specific functions are encoded in parasite genomes and one of parasitology's tasks is to discover these genes and understand this function. Therefore, the purpose of this study was to further characterize genes for which previous data had suggested the hypothesis that they played a key role in the parasitic lifestyle of *S. ratti* (Thompson *et al*. 2008). We analysed the expression of 7 such genes in detail by RT-PCR. For 5 genes (SR00007, SR00449, SR00984, SR01014 and SR01000) their expression was, indeed, significantly greater in the parasitic female stage, compared with all other life-cycle stages tested, suggesting that the function of these genes is important in parasitism. Among the remaining 2 genes, SR00035 had significantly greater expression in the iL3 stage (i.e. the pre-parasitic stage); SR00034 was expressed equally across life-cycle stages. Overall, these data show that EST-abundance is a strongly indicative, but not completely accurate, measure of gene expression.

### *SR00007 and SR00449*

Bioinformatic analyses of complete sequence data for these genes showed that they shared significant alignments to the *A. suum cpg*-3 gene, although

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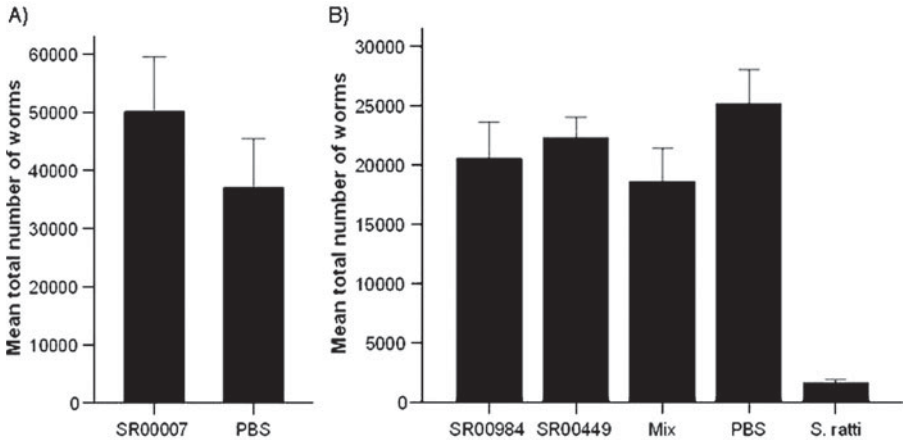


Fig. 6. The total reproductive output (±1 S.E.) following a *Strongyloides ratti* challenge infection of rats previously vaccinated with (A) SR00007 and (B) SR00984, SR00449 or a 3-protein mixture (Mix), compared with the negative control of PBS and the positive control of a previous *S. ratti* infection.

the 2 *S. ratti* genes appear to be different genes. In *C. elegans*, expression of 5 *cpg* genes (1, 2, 3, 5 and 6) are enriched in the germ line (Olson *et al.* 2006). *C. elegans cpg* genes have been found (by RNAi analysis) to have a role in oocytes and fertilized eggs *in utero*; progeny of such RNAi-treated worms were either not viable or had poor gonad formation (Mizuguchi *et al.* 2003). RNAi of *C. elegans cpg-1* and *cpg-2* together (but not individually) resulted in multi-nucleated single-celled embryos (Olson *et al.* 2006). Together, these results suggest that in *C. elegans* these genes play a role in oocyte formation, fertilization and early embryo development. By analogy, these 2 *S. ratti* genes (SR00007 and SR00449) could therefore play a role in its oogenesis. This would be consistent with their expression in parasitic females. Further, for SR000449 (and to a lesser extent SR00007) there was greater expression in the free-living female stages, consistent with the idea of these genes playing a role in oogenesis, or some other aspect of female gametogenesis or reproduction. If so, however, this is clearly not a parasitism-specific trait.

Analysis of the presence of SR00007 and SR000449 proteins through the *S. ratti* life cycle added further detail as to how these genes/gene products may be controlled in the life cycle. Specifically, for SR00007, its protein product was not detected in parasitic females, but it was detected in L1s (the progeny of parasitic females; eggs containing embryos and L1s are present in parasitic females *in utero*). This suggests the idea that in parasitic females the SR00007 gene is transcribed (either in the parasitic female or in her *in utero* eggs/larvae), but that translation to protein occurs in freshly hatched L1s. The storage of maternal mRNA in mouse oocytes, by P-bodies in subcortical cellular regions, has been observed in analogous processes to those that underlie the relationship of transcription and translation of this gene in *S. ratti* (Pepling, 2010). The situation for SR00449 is different. Specifically, there is correspondence between SR00449 mRNA and protein (*ca.* 72 kDa molecule) in the parasitic female (and free-living females and free-living males). However, in L1s (and iL3s) a molecule of a different size (*ca.* 40 kDa) is strongly recognized. One possibility here is that differential splicing or post-translational modification occurs for this gene between the adult and larval stages. Further, SR00449 protein was recognized in *S. ratti* parasitic female ES. It is interesting that these clearly related molecules differ in that one appears to be secreted/excreted and one does not.

### *SR00984 and SR01014*

Bioinformatic analyses of the complete sequence for these genes showed that they shared alignments to the small heat shock proteins (smHSPs) (Russnak *et al.* 1983), although the 2 *S. ratti* genes appear to be different genes. Both SR00984 and SR01014 have a significant alignment to the 2 isoforms of HSP-17 of *C. elegans*. In *T. spiralis* HSP-17 is expressed in the body wall muscle, hypodermis and other tissues; comparison of expression among life-cycle stages showed that it was expressed most in muscle-stage larvae; its expression could also be induced by heat shock (Wu *et al.* 2007). In *S. ratti* SR00984 has recently been found to be present in parasitic female ES (Soblik *et al.* 2011). In *C. elegans* similar HSPs are expressed in different tissues and under a wide variety of conditions (Ding and Candido, 2000). More widely, these smHSPs can have chaperone functions and capture unfolding proteins, creating a stable complex, preventing irreversible damage (Nakamoto and Vigh, 2007). Our findings with *S. ratti* have some analogies with the *T. spiralis* observations, especially the different expression of this molecule among the life-cycle stages.

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There was a non-correspondence between expression of the SR00984 mRNA and protein among life-cycle stages. Analogously to the SR00007 data, despite the detection of SR00984 mRNA in the parasitic female stage, protein was only detected in the L1 stage, again suggesting the idea that in parasitic females this gene is transcribed (either in the parasitic female or in her *in utero* eggs/larvae), but that translation to protein occurs in freshly hatched L1s. We did not analyse the presence of SR01014 protein in the *S. ratti* life cycle, but this would be interesting to do.

### *SR01000*

Bioinformatic analysis of the complete sequence of this gene showed that it had alignment to a Sortillin-related receptor and that it shares patterns of low-density lipoprotein receptor (LDLr) motifs with other genes. LDLrs have been found to play an important role in mammalian cholesterol metabolism (Yamamoto *et al*. 1984). LDLrs are usually localized on the surface of epithelial cells and bind to low-density lipoproteins, which are then transported into the cell (Yochem and Greenwald, 1993).

In *C. elegans* EGG-1 is thought to be part of the molecular mechanism involved in egg–sperm interaction (Kadandale *et al*. 2005), such that EGG-1 and EGG-2 are required for fertilization in hermaphrodites. The *egg-1* and *egg-2* genes show partial redundancy because RNAi knockout of *egg-1* was partially recovered by *egg-2*. The role of these genes in *C. elegans* and *S. ratti* must differ, because *S. ratti* parasitic females are parthenogenetic and therefore there is no egg–sperm interaction. However, these findings in *C. elegans* and the observed expression of SR01000 in parasitic and free-living females is consistent with this gene playing some molecular role in reproduction in this species.

### *SR00034*

Bioinformatic analysis of the complete SR00034 gene sequence identified numerous significant alignments. Many of the identified proteins were found to be members of the HSP-90 family of proteins. It is unlikely, however, that SR00034 is the *S. ratti* HSP-90 protein because the amino acid sequence lacks the highly conserved N-terminal MEEVD sequence motif (Devaney *et al*. 2005). Possible roles proposed for SR00034 are therefore in the re-folding of denatured or misfolded protein, as has been identified for many other proteins of the HSP-90 family (Picard, 2002).

### *SR00035*

Bioinformatic analyses failed to identify any significant alignment to this gene. The iL3-specific expression of this gene suggests that it may play a role in the initiation of parasitism, but this is unknowable without further information about the identity of the gene.

### *Immune response and vaccination*

Sera from animals infected with *S. ratti* recognize SR00007, SR00449 and SR00984. This, together with the discovery of the parasitic female-specific or -biased expression of these genes suggested that these molecules may be able to induce an immune response that affects an *S. ratti* infection. Experimentally the data were suggestive of such an effect, but this was not statistically supported although further immunization experiments with larger sample sizes may give further experimental power to identify significant effects.

In conclusion, this study has examined and further characterized the genes and gene products that putatively play a key role in parasitism. The *S. ratti* genome project is discovering all of the genes of this species, so the next challenge is to understand their role in parasitism. Stable transgenesis of *S. ratti* (and *S. stercoralis*) has been reported (Li *et al*. 2011; Lok, 2011). While still experimental, this important step in parasitic nematode transgenesis has great potential for investigating gene function in these species.

## ACKNOWLEDGEMENTS

We would like to acknowledge the help of Louise Hughes and Simon Carley-Smith in the production of the RT-PCR data.

## FINANCIAL SUPPORT

This work was supported by a BBSRC Studentship.

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