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Suppressive subtractive hybridization analysis of *Rhipicephalus* (*Boophilus*) *microplus* larval and adult transcript expression during attachment and feeding

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ABSTRACT

Ticks, as blood-feeding ectoparasites, affect their hosts both directly and as vectors of viral, bacterial and protozoal diseases. The tick's mode of feeding means it must maintain intimate contact with the host in the face of host defensive responses for a prolonged time. The parasite–host interactions are characterized by the host response and parasite counter-response which result in a highly complex biological system that is barely understood. We conducted transcriptomic analyses utilizing suppressive subtractive hybridization (SSH) to identify transcripts associated with host attachment and feeding of larval, adult female and adult male ticks. Five SSH libraries resulted in 511 clones (assembled into 36 contigs and 90 singletons) from differentially expressed transcripts isolated from unattached frustrated larvae (95), feeding larvae (159), unattached frustrated adult female ticks (68), feeding adult female ticks (95) and male adult ticks (94 clones). Unattached 'frustrated' ticks were held in fabric bags affixed to cattle for up to 24 h to identify genes up-regulated prior to host penetration. Sequence analysis was based on BLAST, Panther, KOG and domain (CDD) analyses to assign functional groups for proteins including: cuticle proteins, enzymes (ATPases), ligand binding (histamine binding), molecular chaperone (prefoldin), nucleic acid binding (ribosomal proteins), putative salivary proteins, serine proteases, stress response (heat shock, glycine rich) and transporters. An additional 63% of all contigs and singletons were novel *R. microplus* transcripts or predicted proteins of unknown function. Expression was confirmed using quantitative real time PCR analysis of selected transcripts. This is the first comprehensive analysis of the *R. microplus* transcriptome from multiple stages of ticks and assists to elucidate the molecular events during tick attachment and development.

1. Introduction

The cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, is one of the most economically important ticks affecting the global cattle population (McCosker, 1979). Currently, *R. microplus* and its associated pathogens which can be transmitted to cattle can lead to severe agricultural losses

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in milk and beef production and restrict the movement of livestock. The most affected regions of the world are tropical and sub-tropical countries including northern Australia, Mexico, South America and South Africa, with threats to USA cattle populations at southern borders with Mexico (George et al., 2002). Treatment with acaricide is the primary means used to control cattle tick infestations, however resistance to acaricide families is rapidly developing (Li et al., 2003; Miller et al., 1999, 2005). Currently resources for tick research are increasing, with the availability of a global EST library for *R. microplus* (Wang et al., 2007) and the current *Ixodes scapularis* genome sequencing project (Hill and Wikel, 2005). These resources will provide the basis for increased cattle tick research activities, thereby increasing our knowledge of the biology of ticks and host parasitism.

Molecular events associated with attachment and feeding have been studied in a number of hard and soft tick species (Mans et al., 2008; Mulenga et al., 2007a; Ribeiro et al., 2006). The main basis of these studies has been to elucidate the parasite: host relationship by improved understanding of tick salivary gland components and how the tick's secretion of these components is able to manipulate the host's defense systems. This understanding of the processes which ticks use to adapt to the blood-feeding environment can greatly facilitate the discovery of new tick control methods. Limited study of the *R. microplus* tick–host interaction has been reported using approaches applied to other tick species including cDNA library analysis of salivary gland ESTs in *Argas monolakensis* (Mans et al., 2008), *Amblyomma cajennense* (Batista et al., 2008), *I. scapularis* (Ribeiro et al., 2006), *Ixodes ricinis* (Chmelar et al., 2008), *Ixodes pacificus* (Francischetti et al., 2005) *Dermacentor andersoni* (Alarcon-Chaidez et al., 2007); *Ornithodoros parkeri* (Francischetti et al., 2008b); differential display analysis of male tick salivary glands (*Amblyomma americanum*, *D. andersoni*) (Bior et al., 2002; Anyomi et al., 2006); and proteomic analyses of female tick sialome of *A. monolakensis* (Mans et al., 2008) and *O. coriaceus* (Francischetti et al., 2008a). One study has utilized suppressive subtractive hybridization to isolate transcripts expressed by *Amblyomma* female ticks which are up-regulated during 'host finding' or pre-attachment (Mulenga et al., 2007a). Although *R. microplus* morphological studies have been undertaken (Nunes et al., 2006b; Saito et al., 2005), there are no reports which investigate the molecular basis of feeding or attachment of *R. microplus* ticks and furthermore, no studies to date have attempted to isolate differentially expressed sequences from tick larval stages.

This study utilizes a suppressive subtractive hybridization technique to isolate 511 sequences up-regulated by feeding larvae, adult female and adult male ticks and by adult female and larval ticks responding to host stimuli.

2. Materials and methods

2.1. Ticks and animal sampling

On Day 1, a tick naïve Hereford female (~9 months age) was infested with 1.5 g (~30,000) N strain larvae (Stewart et al., 1982) using a tick collar which remained on the animal while kept in a moat pen (DPI&F Animal Ethics approval SA2006/03/96). On Day 2, approximately 1000 larvae were placed into a 4 cm^2 mesh bag and attached to the neck of animal for 24 h in order for the larvae to 'sense' host stimuli while also in the presence of other attached ticks. These 'frustrated' larvae were subsequently frozen in liquid nitrogen or collected into RNAlater RNA Stabilization Reagent (Ambion Inc., TX, USA) prior to frozen storage and extraction, respectively. At 24 h (Day 3) approximately 100 attached larvae (feeding larvae) were collected and stored in RNAlater for RNA extraction. At Day 10, 100 nymphal ticks were collected. Similarly, at Day 17 adult females were carefully collected and placed into mesh bag attached to the neck for 24 h prior to harvesting and RNA extraction ('frustrated' females). On Day 17, 40 adult male ticks (collected from the underside of feeding females) and 50 semi-engorged adult female ticks were collected and stored in RNAlater for subsequent RNA extraction. Skin and blood were collected from the Hereford to provide host material for subsequent experiments. For skin tissue removal, 5 ml lignocaine 2% was injected subcutaneously to form a line block in the middle of the left side of the neck with a 25-G needle. Immediately adjacent and distal to one side, a 5 mm biopsy punch was used to take one full thickness skin biopsy. The skin was sprayed with disinfectant containing fly repellent. Blood was collected from the tail vein. Tissue was collected from the animal on Days 3, 10 and 17. At Day 21, engorged adult ticks were collected. On Day 22 the Hereford was treated with Ivermectin to eliminate remaining ticks at the conclusion of tick collection.

2.2. Total RNA extraction

RNA was prepared from the whole larvae (frustrated and feeding), nymph, adult male (feeding/mating stages) and adult female (frustrated and feeding) ticks collected as described above. The ticks were ground in liquid nitrogen using a sterile mortar and pestle prior to processing using the Qiagen Rneasy kit (QIAGEN CA, USA). RNA was also prepared from 4000 unattached 'resting larvae' and gut, salivary gland and ovary tissue dissected from 20 adult female ticks (semi-engorged at 17 days post-infestation). Samples were used for suppressive subtractive hybridization (SSH) and/or qRT-PCR analysis as described below.

2.3. Suppressive subtractive hybridization

SSH was undertaken using the Clontech PCR-Select™ cDNA Subtraction Kit using cDNA prepared using the Super SMART™ PCR cDNA Synthesis Kit as per manufacturer's instructions (Clontech, CA, USA). In order to isolate specific up-regulated sequences in the following 'tester' samples, cDNA from a mix of up to three 'driver' cDNA sequences were included in each subtraction experiment mixed 1:1 with the tester as follows (also summarized in Table 1): (1) frustrated larvae: mix of control larvae (unfed), feeding larvae and skin biopsy 1:1:1 proportions of driver cDNA (Day 3); (2) feeding larvae: mix of control larvae, frustrated larvae and skin biopsy 1:1:1 (Day 3); (3) male ticks: mix of

Table 1
Summary of each SSH library including the total number of clones and corresponding contigs and singletons following assembly.

Library (tester sample)	Drivers	No. of clones	No. of contigs	No. of singletons
'Frustrated' larvae (L3)	Unfed larvae, feeding larvae, bovine skin biopsy	95	7	23
Feeding larvae (L2)	Unfed larvae, frustrated larvae, bovine skin biopsy	159	4	3
Adult males (M1)	Unfed larvae, feeding females, bovine skin biopsy	94	9	19
'Frustrated' adult females (F2)	Feeding females, bovine skin biopsy	68	5	27
Feeding adult females (F1)	Unfed larvae, male ticks, bovine skin biopsy	95	11	18
Totals		511	36	90
Summary		511 clones	126 contigs and singletons	

control larvae, feeding adult females and skin biopsy 1:1:1 (Day 17); (4) frustrated females: mix of control feeding adult female ticks and skin biopsy 2:1 (Day 17); (5) feeding females: mix of control larvae, male adult ticks and skin biopsy 1:1:1 (Day 17).

2.4. Cloning and sequencing

The differentially amplified transcripts were sub-cloned into TOPO TA-cloning vectors (ONE SHOT chemically competent cells) following the manufacturer's instructions (Invitrogen Corp., CA, USA). Individual *E. coli* colonies were grown in 5 ml LB ampicillin broths for 18 h prior to plasmid extraction from 4 ml using the QIAprep Spin miniprep kit (QIAGEN, MD, USA) and glycerol storage of the remaining 1 ml of broth culture (−80 °C). Direct sequencing of plasmid inserts was undertaken using the BigDye Vers 3.1 technology (Applied Biosystems, CA, USA) and analyzed on the Applied Biosystems 3130xl Genetic Analyser at the Griffith University DNA Sequencing Facility (School of Biomolecular and Biomedical Science, Griffith University, Qld, Australia). Sequencing reactions were prepared using M13 and T7 primers in 96 well plate format according to the manufacturer's instructions (Applied Biosystems, CA, USA). Sequences were visualized, edited and aligned using Sequencher Vers 4.5 (Gene Codes Corporation, MI, USA) to remove all vector and adaptor sequences prior to further analysis.

2.5. Analysis and sequence function prediction

Vector pCR®2.1-TOPO® and SSH adaptor sequence (Clontech, CA, USA) were clipped from library sequences using cross-match (Ewing et al., 1998). Individual libraries were assembled using CAP3 (Huang and Madan, 1999). Clones within a library were aligned to construct tentative contigs. Differentially expressed sequences were screened against the following databases on the CCG (Centre for Comparative Genomics: http://ccg.murdoch.edu.au/) Grendel HPC system (Hunter et al., 2005): NCBI protein (non-redundant and patent) (National Centre for Biotechnology Information: http://www.ncbi.nlm.nih.gov), String v7 database built on Unicellular (COG) and Eukaryotic Clusters (KOG) of Orthologous Groups (von Mering et al., 2007), Clusters of Orthologous Groups of proteins COG (Tatusov et al., 2003), tigr_bmigi.062608 (The Gene Index Project http://compbio.dfci.harvard.edu/tgi/) (Quackenbush et al., 2000) and NCBI Conserved Domain database (CDD) (Goonesekere and Lee, 2008). All alignments were conducted using the BLAST program suite (Altschul et al., 1990) except for the NCBI Conserved Domain data where RPSBLAST was used (Goonesekere and Lee, 2008). The alignment results were then summarized using BIOPERL (Stajich et al., 2002) scripts based on alignment percent identity (PID), query coverage and an expected value thresholds, >25%, >75% and <1e-05 respectively. For categories not found in the COG database Panther (Protein ANalysis THrough Evolutionary Relationships http://www.pantherdb.org/panther/goToPanther.jsp) categories were assigned manually.

2.6. qRT-PCR analysis

Primers were designed using emboss version 6.0.1 eprimer3 (Rice et al., 2000) set using the following parameters: -minsize 22, -osize 24, -maxsize 27, -mintm 55, -maxtm 65, -maxpolyx 4, -gcclamp 2, -productsize 100, -mingc 35, -maxgc 65. Primer sets were then screened against bovine nucleotide sequence using Blastn (Altschul et al., 1990) with an expected value 100. Primer alignments were then screened using a custom Bioperl (Stajich et al., 2002) script for matches forward and reverse to ensure these sets would not amplify bovine sequences. Primer sequences, PCR product and annealing temperatures for all targets are listed in Table 2. cDNA was synthesized using Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen Corp., CA, USA) and duplicate qPCRs (10 ng per reaction) undertaken using the SensiMix dT kit (Quantace Ltd., Watford, UK) in the Corbett RotorGene 3000 (QIAGEN/Corbett, Sydney, Australia) using the following profile: 95 °C 10 min, 45 cycles of 95 °C 15 s, 55 or 60 °C 30 s (see Table 2 for optimal temperatures per assay), 72 °C 30 s, followed by a melt analysis 72–90 °C 30 s on the first step, 5 s holds for subsequent steps, according to manufacturer's instructions for SYBR green detection. All assays were first optimised on a cDNA pool consisting of whole adult female, adult male and larval cDNAs prior to screening samples prepared from all stages including extracts prepared specifically from female gut, salivary gland and ovaries. Assays with the observed consistent amplification of duplicates on a standard curve ($R^2 > 0.95$) giving efficiency values of 2.0 (within 15%) were considered acceptable for normalisation and expression analysis. The expression profiles (average of two reactions) were normalised against the *R. microplus* actin gene (Nijhof et al., 2007) using the Mean Normalised Expression method (Muller et al., 2002).

Table 2
Primer sequences for qRT-PCR validation of libraries.

Library and contig/clone	Protein identity/GenBank accessions	Primers (5′-3′) f: forward; r: reverse	Annealing, °C
Frustrated larvae			
Clone 55	Glycine rich	f TTCGAAGGTTCGCTTTATCC r GTGGTTATGGCGGCTATGG	55
Clone 15.5	Putative salivary secreted	[a]GenBank accession to be updated	
Contig 2	Hypothetical proteobacterial	f CCTGTTTCCCATCGACTACG r ACCTACACGCCGAAAGTCC	60
Clone 21	Hypothetical Drosophila	f CCACTGCTGACGTCACTCC r CTGCGGTGAACCTAACATCC	60
Clone 17	Unknown	f TAGGACTGCCACAATCATCG r TTCGTCTAAAATGGGACTGC	60
Feeding larvae			
Contig 1	Cuticle	f GCGACTGCATTATTTCTATTTCCT r TCGAAGTTAGAAGGTTCACAACAG	60
Contig 1A	ATPase	f TGATTCTCATCGGTCTAAACTCAG r GACCTCGATGTTGGATTAGGATAC	60
Contig 2	*A. hebraeum* mitochondrial genes	f TGATTCTCATCGGTCTAAACTCAG r GACCTCGATGTTGGATTAGGATAC	60
Adult male ticks			
Clone 63	*R. appendiculatus* immunodominant saliva	f CGTCGGTCTTGTGAACTTCG r CCAGTACCTCAGCCATACCC	60
Clone 99	RNA polymerase	f TTCAGCTCTAGACGCAATCG r TCTCCGTGTTTTCAACATGC	60
Clone 10	Trypsin-like serine protease	[a]GenBank accession to be updated	
Contig 2	Unknown	f AGTCTTCATTTTCCGCAACG r CCATAAATGCGTCAGACACG	60
Contig 5	Unknown	f CCTGAGGACACCTCTCATCC r CTGCGATGACCGCTAATACC	60
Frustrated adult female ticks			
Clone 54	Trypsin-like serine protease	[a]GenBank accession to be updated	
Contig 3	Cuticle	f CGTTGAGGGTCACATCAGC r CCGAGGTACTGCCAACTACG	55
Clone 68	Unknown	f GATCTACGTTTCCTTCAATCATAGG r GCAACAATTTGATGATACAGTTCG	60
Clone 78	Unknown	[a]GenBank accession to be updated	
Feeding adult female ticks			
Contig 9	Female specific tick histamine binding protein-1	[a]GenBank accession to be updated	
Clone 64	Hypothetical culex	f ACCAGGTGTGACTGCTCTCC r GGGTATAGGGGCGAAAGACC	55
Contig 6	Unknown	f ACGGCACCCAAACTAACG r TTTCTGAACCAGCGGATACC	55
Clone 59	Unknown	f ATTTCGCCTTCGAAGATTGC r CAAGCTTCCTGCTCTGTCG	55
Clone 75	Unknown	[a]GenBank accession to be updated	

[a] Sequences for these primers can be obtained from the corresponding author following patent registration. See Table 4 describing GenBank accessions for the remainder of the clones listed in this table.

3. Results

3.1. Subtraction library summary

A total of 511 clones were isolated from the 5 libraries as described below and a summary of the number of clones, contigs and singletons per library is presented in Table 1. The 511 clones resulted in 36 consensus contigs and 90 singletons, 63% of which were novel *R. microplus* transcripts or similar to hypothetical proteins in other species with no known function. Table 3 describes the protein hits for each of the contigs and singletons using Panther, KOG and CDD analysis into specific categories. Two hypothetical protein hits, *Thermobia domestica* (insect) and *Brugia malaya* (nematode), present in most libraries were confirmed using Blastn to be homologous

Table 3
Summary of predicted protein hits (Blast-X and CDD analysis) in each library based on Panther and specific categories.

Molecular function classification (Panther)	Total contigs	Tick stage enriched–no. of clones per contig					Description of protein hits[a]		
		Frustrated larvae	Feeding larvae	Male	Frustrated female	Feeding female	Tick species homologue[a]	e-Value	Non-tick or domain homology
Cuticle protein	2		2				*Ixodes ricinis* cuticle protein 10.9	1e-32	
					2		*I. ricinis* cuticle protein 10.9	4e-10	
Enzyme	3	1						9e-42	*Nasonia vitripennis* ubiquitin-conjugating enzyme rad 6 [predicted]-Ubiquitin protein ligase
			52				*Amblyomma americanum* ATPase FoF1 subunit 6	6e-8	
						2	*Rhipicephalus sanguineus* ATP-synthase F0 subunit 6	7e-14	
Glycine rich protein	2	3					*Argas monolakensis* GGY domain protein	3e-7	
		1						2e-9	Domain pfam07172–glycine rich protein family
Immunodominant protein	1			1			*R. appendiculatus* 20/24 kDa immunodominant saliva protein	3e-13	
Ligand binding	1					3	*R. appendiculatus* female-specific histamine binding protein-1	3e-56	
Molecular chaperone	2				1			1e-31	*Ornithorhynchus anatinus* similar to T-complex protein 1 subunit epsilon
				1				6e-25	*Nematostella vectensis* predicted protein [domain cd00632, Prefoldin]
Nucleic acid binding	10	1					*I. scapularis* ribosomal protein L21	4e-78	
		1						2e-54	*Limulus polyphemus* elongation factor-2
						5		3e-35	*Lycosa singoriensis* translation elongation factor-2
				1	1			4e-8 1e-6	Domain PRK11192–ATP dependent RNA helicase
				1				9e-6	Domain pfam08208–RNA polymerase A34
					1		*Ornithodoros parkeri* 40S ribosomal protein S3	8e-77	
					1		*O. parkeri* ribosomal protein L19	4e-7	
						2	*Haemaphysalis qinghaiensis* ribosomal protein L23	2e-22	
						1	*I. scapularis* ribosomal protein S17	4e-47	
Putative salivary protein	3	1					*I. scapularis* putative salivary secreted protein	3e-41	
		1					*I. scapularis* putative salivary protein	1e-20	
						2		1e-9	*Culicoides sonorensis* putative salivary protein
Thyropin precursor	1	1					*O. moubata* putative thyropin precursor	1e-13	
Serine protease	2			1				3e-27	*Drosophila pseudoobscura* GA17401-PA predicted [domain cd00190-secreted trypsin-like serine protease]
					1			6e-24	Domain cd00190-secreted trypsin-like serine protease

Stress response	3					1		1e-37	*Opistophthalmus carinatus* heat shock protein 90
						3		1e-34	*Lilium longiflorum* putative senescence associated protein
					6			4e-35	*Cupressus sempervirens* putative senescence associated protein
Toxin	1			2				0.024	Domain pfam07740, Toxin 12, Spider potassium channel inhibitory toxin
Transferase	1				1			1e-22	*Tetraodon nigroviridis* hypothetical [domain pfam04101, glycosyltransferase]
Transporter	1			1				1e-50	Domain pfam04752, Ca^{2+}/H^{+} cation transport-like protein
DNA mitochondrial and rRNA sequences	11	1	105[a]	59[a]	17[a]	49[a]	[[a]*A. hebraeum* mitochondrial NADH dehydrogenase subunit rDNA, AY059171]	<1e-5	[a]*Thermobia domestica* hypothetical protein
	3	35			14	3	[[a]*R. haemaphysaloides* 18S rDNA, DQ839552]	<3e-22	[a]*Brugia malayi* hypothetical protein
Hypothetical proteins	8	1						7e-10	*Drosophila erecta*
		25						2e-24	*V. vinifera*
		3						2e-16	Uncultured beta proteobacterium
					1		*H. qinghaiensis*	2e-4	
					3			2e-17	*Rattus norvegicus*
						1	*R. haemaphysaloides*	3e-8	
						1		3e-12	*Culex pipiens quinquefasciatus*
						1		2e-8	*Tribolium castaneum*
Unknowns	70								
Singletons		13	0	13	19	13			
Contigs/no. clones		3/6	0	6/15	0	3/8			
Total clones $n = 511$	126	95	159	94	68	95			

[a] Although hypothetical protein hits are described as *T. domestica* GenBank CAM36311 and *B. malaya* XP_001895031, the DNA sequences are highly homologous to tick mitochondria (*A. hebraeum*) and ribosomal genes (*R. haemaphysaloides* 18S rDNA) at e values of <7e-87 and 0 (100% homology), respectively. For the *A. hebraeum* sequences: feeding larval library consisted of 105 clones which were assembled into 2 contigs and 3 singletons; adult male library consisted of 2 contigs; frustrated adult female library consisted of 1 contig and 1 singleton; and the feeding adult female library consisted of a single contig.

Table 4
List of singleton and contig clones with corresponding GenBank accessions (this study).

Library	Contig or clone no.	dbEST Id	User Id	Genbank accession
Feeding female	Clone 59	62562036	F1-2-A_Clone_59	GE650060
	Clone 64	62562035	F1-3-A_Clone_64	GE650059
	Contig 6	64498247	F1-2-A_Clone_54	GO253184
		64498248	F1-2-A_Clone_50	GO253185
Feeding larvae	Contig 1	62562129	L2-2-A_clone_11	GE650153
		62562080	L2-2-A_Clone_59	GE650104
	Contig 1A	62562127	L2-3-A_Clone_41	GE650151
		62562128	L2-3-A_Clone_10	GE650152
		62562057	L2-3-A_Clone_11	GE650081
		62562116	L2-3-A_Clone_12	GE650140
		62562076	L2-3-A_Clone_13	GE650100
		62562079	L2-3-A_Clone_14	GE650103
		62562049	L2-3-A_Clone_15	GE650073
		62562077	L2-3-A_Clone_18	GE650101
		62562063	L2-3-A_Clone_2	GE650087
		62562083	L2-3-A_Clone_20	GE650107
		62562081	L2-3-A_Clone_21	GE650105
		62562058	L2-3-A_Clone_25	GE650082
		62562096	L2-3-A_Clone_26	GE650120
		62562055	L2-3-A_Clone_27	GE650079
		62562109	L2-3-A_Clone_29	GE650133
		62562106	L2-3-A_Clone_36	GE650130
		62562059	L2-3-A_Clone_37	GE650083
		62562065	L2-3-A_Clone_38	GE650089
		62562105	L2-3-A_Clone_39	GE650129
		62562132	L2-3-A_Clone_4	GE650156
		62562068	L2-3-A_Clone_40	GE650092
		62562112	L2-3-A_Clone_43	GE650136
		62562066	L2-3-A_Clone_46	GE650090
		62562056	L2-3-A_Clone_5	GE650080
		62562126	L2-3-A_Clone_50	GE650150
		62562073	L2-3-A_Clone_53	GE650097
		62562053	L2-3-A_Clone_57	GE650077
		62562061	L2-3-A_Clone_58	GE650085
		62562100	L2-3-A_Clone_59	GE650124
		62562060	L2-3-A_Clone_60	GE650084
		62562117	L2-3-A_Clone_62	GE650141
		62562084	L2-3-A_Clone_65	GE650108
		62562102	L2-3-A_Clone_66	GE650126
		62562101	L2-3-A_Clone_67	GE650125
		62562099	L2-3-A_Clone_7	GE650123
		62562047	L2-3-A_Clone_71	GE650071
		62562115	L2-3-A_Clone_76	GE650139
		62562110	L2-3-A_Clone_77	GE650134
Feeding larvae	Contig 1A	62562097	L2-3-A_Clone_78	GE650121
		62562054	L2-3-A_Clone_79	GE650078
		62562113	L2-3-A_Clone_81	GE650137
		62562074	L2-3-A_Clone_82	GE650098
		62562120	L2-3-A_Clone_85	GE650144
		62562121	L2-3-A_Clone_86	GE650145
		62562067	L2-3-A_Clone_87	GE650091
		62562130	L2-3-A_Clone_89	GE650154
		62562118	L2-3-A_Clone_9	GE650142
		62562104	L2-3-A_Clone_91	GE650128
		62562088	L2-3-A_Clone_93	GE650112
		62562093	L2-3-A_Clone_94	GE650117
		62562069	L2-3-A_Clone_95	GE650093
		62562125	L2-3-A_Clone_96	GE650149
	Contig 2	62562070	L2-3-A_clone_1	GE650094
		62562103	L2-3-A_Clone_100	GE650127
		62562071	L2-3-A_Clone_17	GE650095
		62562108	L2-3-A_Clone_19	GE650132
		62562092	L2-3-A_Clone_22	GE650116
		62562135	L2-3-A_Clone_23	GE650159
		62562124	L2-3-A_Clone_24	GE650148
		62562050	L2-3-A_Clone_28	GE650074
		62562094	L2-3-A_Clone_3	GE650118
		62562082	L2-3-A_Clone_31	GE650106

Table 4 (*Continued*)

Library	Contig or clone no.	dbEST Id	User Id	Genbank accession
		62562062	L2-3-A_Clone_32	GE650086
		62562095	L2-3-A_Clone_33	GE650119
		62562087	L2-3-A_Clone_42	GE650111
		62562086	L2-3-A_Clone_45	GE650110
		62562072	L2-3-A_Clone_47	GE650096
		62562051	L2-3-A_Clone_48	GE650075
		62562075	L2-3-A_Clone_49	GE650099
		62562123	L2-3-A_Clone_52	GE650147
		62562090	L2-3-A_Clone_54	GE650114
		62562107	L2-3-A_Clone_55	GE650131
		62562098	L2-3-A_Clone_56	GE650122
		62562122	L2-3-A_Clone_61	GE650146
		62562134	L2-3-A_Clone_63	GE650158
		62562078	L2-3-A_Clone_64	GE650102
		62562111	L2-3-A_Clone_68	GE650135
		62562048	L2-3-A_Clone_70	GE650072
		62562089	L2-3-A_Clone_72	GE650113
		62562137	L2-3-A_Clone_73	GE650161
		62562052	L2-3-A_Clone_75	GE650076
		62562119	L2-3-A_Clone_83	GE650143
		62562064	L2-3-A_Clone_84	GE650088
		62562133	L2-3-A_Clone_88	GE650157
		62562131	L2-3-A_Clone_90	GE650155
		62562114	L2-3-A_Clone_92	GE650138
Feeding larvae	Contig 2	62562085	L2-3-A_Clone_98	GE650109
		62562136	L2-3-A_Clone_99	GE650160
Frustrated female	Clone 68	62562042	F2-3-A_Clone_68.1	GE650066
	Contig 3	62562043	F2-3-A_Clone_67.2	GE650067
		62562045	F2-3-A_Clone_77	GE650069
Frustrated larvae	Clone 17	62562138	L3-3-A_Clone_17	GE650162
	Clone 17	62562138	L3-3-A_Clone_17	GE650162
	Clone 21	62562140	L3-3-C_Clone_21	GE650164
	Clone 55	62562139	L3-3-A_Clone_55	GE650163
	Contig 2	62562144	L3-3-A_Clone_13	GE650168
		62562143	L3-3-A_Clone_14	GE650167
		62562142	L3-3-A_Clone_19	GE650166
Male	Clone 63	62562145	M1-2-A_Clone_63	GE650169
	Clone 99	62562157	M1-2-A_Clone_99	GE650181
	Contig 2	62562154	M1-2-A_Clone_26	GE650178
		62562151	M1-2-A_Clone_33	GE650175
	Contig 5	64498250	M1-2-A_Clone_89	GO253187
		64498251	M1-2-A_Clone_58.3	GO253188
		64498249	M1-2-A_Clone_49	GO253186
		64498252	M1-2-A_Clone_48.4	GO253189

with the *Amblyomma hebraeum* mitochondrial region (containing NADH dehydrogenase subunit 1/ND1 gene, partial cds-tRNA-Leu gene-16S ribosomal RNA gene, partial sequence; and ND1 gene, partial sequence), and *Rhipicephalus haemaphysaloides* 18S rDNA, respectively. These DNA clone/contigs hits were retained in the analysis for each library described below. Quantitative real time PCR (qRT-PCR) analysis was used to both confirm tester differential expression and to demonstrate the expression of selected transcripts across multiple stages and female tissues (described for each tester/library below). Table 4 summarises corresponding GenBank accessions for clones and contigs screened in this study.

3.2. Frustrated larvae library

A total of 95 clones were characterized from the 'frustrated' larvae tester with functional proteins identified as a ubiquitin-conjugating enzyme, glycine rich proteins, ribosomal proteins, thyropin precursor, and three hypothetical proteins (insect, plant and bacterial) as summarized in Table 3 and Fig. 1. A further 19 clones (13 singletons, 3 contigs) did not return any known protein hits or known domains. The majority of transcripts were represented by 25 clones (single contig) similar to a plant (*Vitis vinifera*) hypothetical protein and 35 clones (single contig) homologous with *R. haemaphysaloides* 18S rDNA sequence. qRT-PCR analysis (Fig. 2) confirmed up-regulation of the specific sequences including the glycine rich protein at 5× higher expression in frustrated larvae and other stages (nymph 24×, frustrated adult females 2.5×, feeding adult females 1.55×, salivary gland 1.29×) compared to the pooled control. A putative salivary secreted protein up-regulated in larvae and nymphs was also detected at high levels in female salivary gland tissue (~5×). Two clones with hypothetical protein hits only

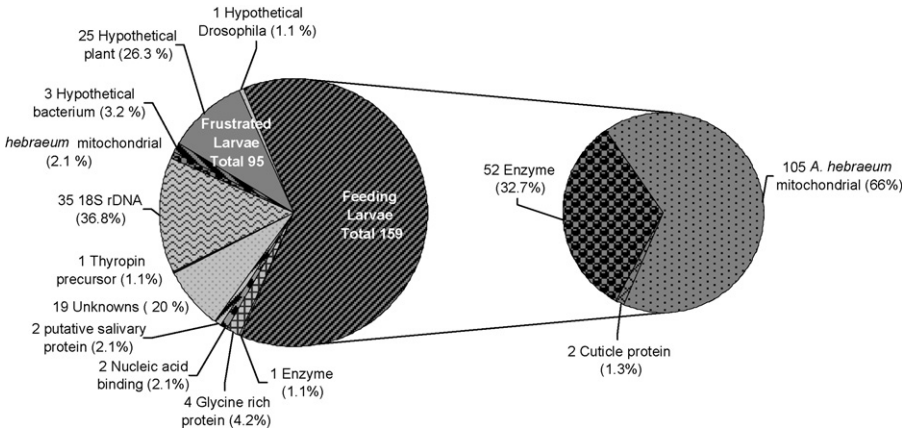

Fig. 1. Proportion of the number of transcripts up-regulated in frustrated and feeding larval tick stages (large pie chart) with detailed pie of feeding larvae in small pie chart.

(insect and bacterial) were confirmed by qRT-PCR as up-regulated in frustrated larvae, as well nymphs, adult males and female salivary gland tissue. A clone (clone 17) with no known protein hit or domain was shown to be highly expressed in the frustrated larval (18×) and nymphal stages (7×) with little or no expression detected in the other samples screened.

3.3. Feeding larvae library

A total of 159 clones were isolated from feeding larvae which represented cuticle proteins (2 clones, single contig), ATPase (52 clones, single contig), and *A. hebraeum* mitochondrial genes (105 clones: 2 contigs, 3 singletons), see Fig. 1. Despite repeated attempts to isolate novel sequences from this library, a large number of the clones isolated aligned with the DNA sequence from the *A. hebraeum* mitochondrial region which includes NADH dehydrogenase and 16S rDNA. The collection of feeding larvae (24 h post-infestation) was difficult thus the limited sample was not incorporated into qRT-PCR analysis as all materials were used in the preparation of the libraries. However, the mitochondrial sequence was highly expressed in nymphs (22×), with levels detected also in frustrated larvae, feeding adult female ticks and salivary gland tissue (Fig. 2). The cuticle protein sequence was highly expressed in nymph stages (129×) and feeding females (8×) but not in unfed or frustrated larval tick stages (feeding larval samples not available for screening). Under the qRT-PCR conditions used here (normalisation to actin) the ATPase was also shown to be highly expressed in unfed larvae (212×) and nymphs (25×), Fig. 2.

3.4. Adult male library

A total of 94 male tester clones were isolated and characterized including a *Rhipicephalus appendiculatus* immunodominant saliva protein, RNA polymerase, prefoldin (molecular chaperone), insect serine protease, toxin domain (2 clones, 1 contig), 28 unknown sequences (6 contigs and 13 singletons) and an abundance of *A. hebraeum* mitochondrial sequence clones ($n = 59$ which assembled into 2 contigs). Fig. 3 summarizes the composition of the adult male tick library and Fig. 4 demonstrates qRT-PCR analysis of selected transcripts. All of the qRT-PCR assays demonstrated male-specific expression (1–4× higher expression) with nil/poor expression in all other samples tested.

3.5. Frustrated adult females

Table 3 and Fig. 5 describe the 68 clones isolated from the 'frustrated' adult female tick library which includes hits with a cuticle protein (2 clones, single contig), molecular chaperone, 40S and L19 ribosomal proteins, serine protease, senescence associated protein (6 clones, single contig), transferase, 2 hypothetical proteins (*Haemophysalis qinghaiensis* and a rat sequence) and the *A. hebraeum* mitochondrial DNA sequence (17 clones assembling into 1 contig plus 1 singleton). An additional 30% of the transcripts were unknown with no Blast or CDD hits represented by 19 singletons. Although feeding adult female ticks were used in the driver mix, 3 of the 4 qRT-PCR assays used to validate the results from this library did not demonstrate higher expression in the 'frustrated' female tester compared with the feeding female tick sample (Fig. 6). The qRT-PCR assay based on unknown clone 68 showed a higher expression in frustrated adult female ticks as well as nymphs and ovary tissue compared with other targets. An assay based on unknown clone 78 showed a large increase in expression in 'frustrated' larvae and feeding adult females at 780 and 324× respectively. In addition, a sequence containing a trypsin-like serine protease domain was shown to be highly expressed in ovary tissue (4×) with nil expression detected in all other samples.

3.6. Feeding adult females

A total of 95 feeding female tester clones were isolated and characterized as an ATP synthase (2 clones, single contig), histamine binding protein (3 clones, single contig),

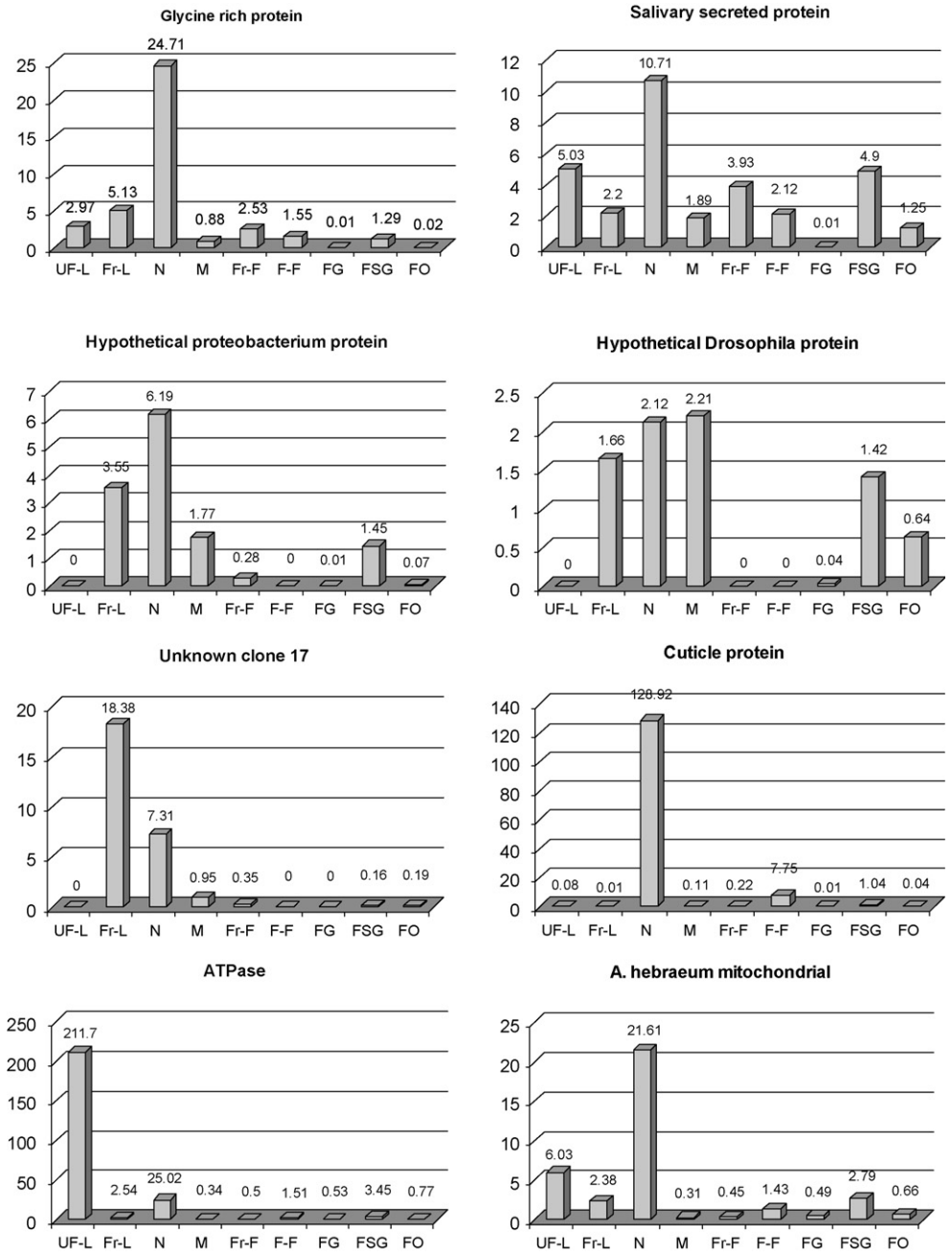

Fig. 2. qRT-PCR expression profiles (*y*-axis indicates average fold change of target gene compared to internal qRT-PCR control) of selected differentially expressed larval sequences screened across different stages and adult female tissues. Frustrated larvae library assays: glycine rich protein, salivary secreted protein, hypothetical proteobacterium protein, hypothetical *Drosophila* protein, and unknown clone 17. Feeding larvae library assays: cuticle protein, ATPase, and *A. hebraeum* mitochondrial genes. *Legend*: UF-L: unfed larvae, Fr-L: 'frustrated' larvae, N: nymphs, M: males, Fr-F: 'frustrated' females, F-F: feeding females, FG: female gut, FSG: female salivary gland, and FO: female ovary.

ribosomal L23 protein (2 clone, single contig), putative salivary protein (2 clones, single contig), heat shock protein, 3 hypothetical proteins (*R. haemaphysaloides*, mosquito, and beetle), 16 unknowns (3 contigs and 13 singletons) and 49 clones (single contig) homologous to the *A. hebraeum* mitochondrial DNA sequence. Results are summarized in Table 3 and Fig. 5. qRT-PCR results are presented in Fig. 6. Six qRT-PCR assays confirmed the differential expression of transcripts in feeding females with exceptionally high expression of the female specific histamine binding protein (FSHBP) in feeding (695×) and frustrated adult females (1388×). High expression of the FSHBP was also noted in nymph (27×) and slightly higher levels were amplified in female salivary gland tissue (2.4×). An unknown (contig 6) also demonstrated exceptionally high levels of expression during attachment

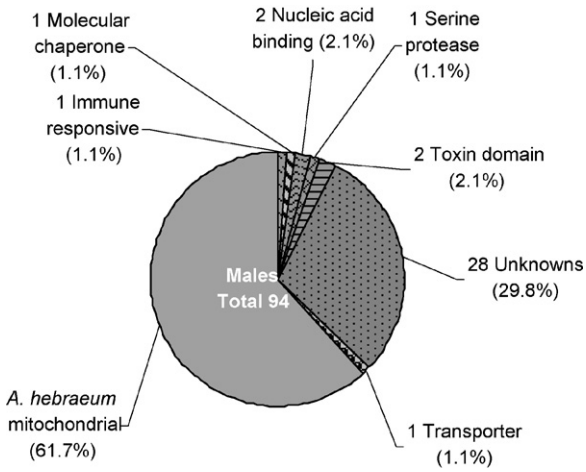

Fig. 3. Summary of transcripts up-regulated in adult male ticks.

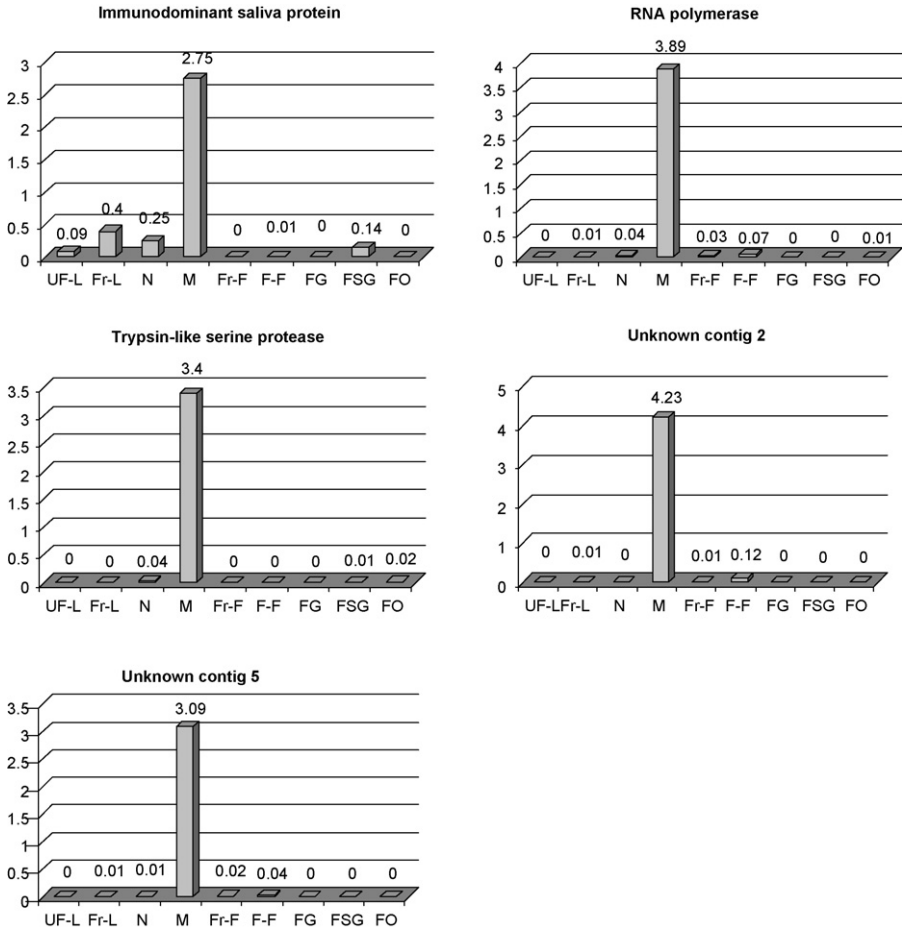

Fig. 4. qRT-PCR expression profiles (*y*-axis indicates average fold change of target gene compared to internal qRT-PCR control) of selected differentially expressed adult male tick sequences screened across different stages and adult female tissues. Assays: immunodominant saliva protein (*R. appendiculatus*), RNA polymerase, trypsin-like serine protease, *A. hebraeum* mitochondrial, and unknown contig 2. *Legend*: UF-L: unfed larvae, Fr-L: 'frustrated' larvae, N: nymphs, M: males, Fr-F: 'frustrated' females, F-F: feeding females, FG: female gut, FSG: female salivary gland, and FO: female ovary.

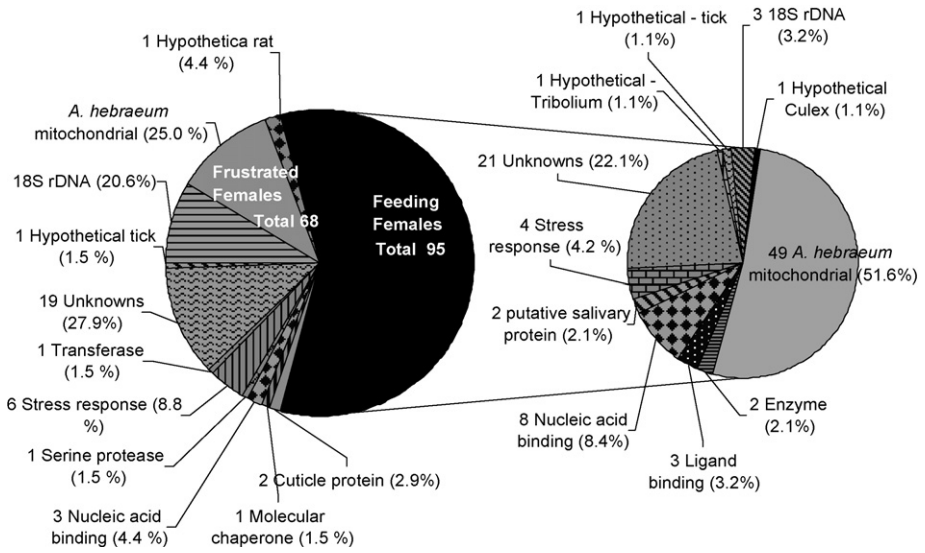

Fig. 5. Summary of transcripts up-regulated in frustrated and feeding adult female tick stages (large pie chart with details of frustrated female transcripts) with the smaller pie chart describing feeding female transcripts.

('frustrated') and feeding of adult female ticks, as well as nymphal stages and female gut tissue but not in adult male or larval stages (Fig. 6). qRT-PCR analysis of the hypothetical mosquito protein sequence was also shown to be highly expressed in the frustrated larval sample (8×) and feeding females (5×), with transcript levels also detected in frustrated females (2×), nymphs (1.4×) and female gut tissue (1.4×). Assays based on unknown clones 59 and 75 demonstrated high transcript levels in feeding and frustrated female tick samples.

4. Discussion

This study demonstrates the application of subtractive suppressive hybridization (SSH) analysis to identify differentially expressed transcript associated with *R. microplus* larval and adult tick attachment and feeding in response to host stimuli. Out of the five libraries studied, the feeding larval library did not yield a large variety of clones, however it is feasible that transcripts associated with cuticle production, the mitochondria (NADH dehydrogenase, 16S rRNA) and energy (ATPase) are most abundant in this larval stage. Although the *A. hebraeum* mitochondrial sequences were identified in most of the libraries in this study, gene expression analysis confirmed that library specific transcripts similar to the *A. hebraeum* mitochondrial sequence were in fact stage specific. In addition, approximately 16–28% of clones from all tester libraries represented novel sequences with no known protein or domain hits. An additional 8 sequences matched hypothetical proteins in other species, also with no known associated function. The difficulty of identifying tick sequences has been noted in other tick transcriptome studies (Mulenga et al., 2007a; Ribeiro et al., 2006). Not all transcripts were confirmed in qRT-PCR analyses with a few assays preferentially amplifying driver sequences (unfed larvae and feeding adult female ticks in frustrated larvae and adult female libraries respectively). This was mainly attributed to the use of a single gene for qRT-PCR normalisation as the generally the tester transcripts which could be identified showed evidence of relevant stage specificity. Insights into the potential function of specific sequences identified in the libraries will contribute to the elucidation of *R. microplus* attachment and feeding. This is the first study analyzing the expression of tick larvae in response to host stimuli ('frustrated' larvae) and the first analysis of *R. microplus* differentially expressed sequences isolated using SSH techniques.

Our study demonstrated the differential expression of a tick ATPase in 32% of the transcripts isolated from the feeding larval library and a slight up-regulation of this ATPase in the female salivary gland. We also demonstrated the up-regulation of an ATP synthase in female ticks, at higher levels than the larval ATPase (not shown). ATPase activity has been shown to be localized in the salivary glands of female *R. microplus* ticks during the feeding period (Nunes et al., 2006a) and an abundance of ATP synthase ESTs were identified in *I. ricinis* and *D. andersoni* female salivary gland studies (Alarcon-Chaidez et al., 2007; Chmelar et al., 2008), confirming ATPase activity in unfed females as well as feeding female stages as demonstrated in this study. The above studies (Alarcon-Chaidez et al., 2007; Chmelar et al., 2008; Nunes et al., 2006a) did not measure ATPase in larval stages but high levels of ATPases are linked to intensive secretory activity and thus logically could be expressed by larval stages (Sauer et al., 2000).

A number of housekeeping genes were identified in each library, which did appear to be stage specific. Each library (apart from feeding larvae) yielded tick specific ribosomal protein clones which appeared to be unique for each particular tick stage. Specific ribosomal proteins have been identified in male *A. americanum* and *D. andersoni* ticks, and *I. ricinis* female sialome analyses (Bior et al., 2002; Chmelar et al., 2008). In addition, elongation factor 2

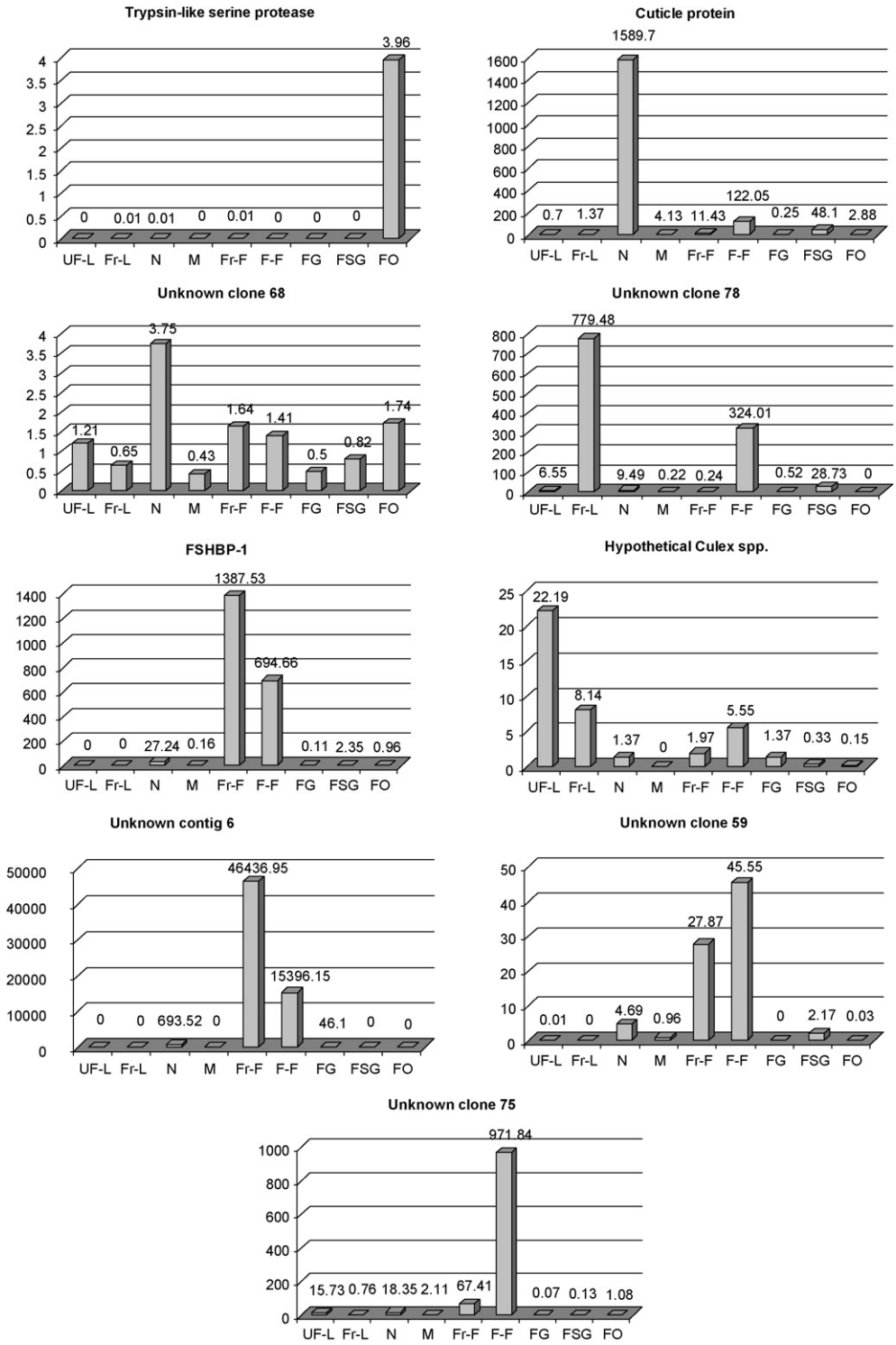

Fig. 6. qRT-PCR expression profiles (*y*-axis indicates average fold change of target gene compared to internal qRT-PCR control) of selected differentially expressed female sequences screened across different stages and adult female tissues. Frustrated female library assays: trypsin-like serine protease, cuticle protein, unknown clone 68, and unknown clone 78. Feeding female library assays: ATP synthase, female-specific histamine binding protein-1, senescence associated protein, hypothetical *Culex* spp. protein, unknown clone 59, and unknown clone 75. *Legend*: UF-L: unfed larvae, Fr-L: 'frustrated' larvae, N: nymphs, M: males, Fr-F: 'frustrated' females, F-F: feeding females, FG: female gut, FSG: female salivary gland, and FO: female ovary.

sequences associated with enhanced ribosomal function which were previously identified in *D. andersoni* female salivary gland EST analyses (Alarcon-Chaidez et al., 2007) were also isolated in the *R. microplus* feeding female and frustrated larval libraries in this study. The up-regulation of ribosomal RNA particularly of mitochondrial origin may be associated with the increased activity of the mitochondria during certain stages of tick development such as feeding. It has been demonstrated that growth and differentiation relies on the mitochondrial respiratory chain as the major source of ATP in nematode development (Tsang and Lemire, 2002) and that tick mitochondrial NADH dehydrogenase and lipids are produced in feeding females and salivary gland cells, respectively (Chmelar et al., 2008; Denardi et al., 2006). A large number of clones were isolated from feeding larvae that were associated with mitochondrial sequences (NADH dehydrogenase, 16S rRNA), thus correlating with the abundance of ATPase clones possibly associated with the rapid growth phase of feeding larvae. In contrast, the 'frustrated' larval library consisted of many more diverse transcripts including glycine rich and GGY proteins, salivary proteins, a putative cysteine protease inhibitor, ribosomal proteins and a number of diverse transcripts with no known prediction.

Cuticular protein transcripts were shown to be up-regulated during the stages of feeding larvae (the same clone up-regulated to a lesser degree in feeding females), whereas a cuticle protein clone isolated from the frustrated female library was up-regulated in nymph and female stages but not unfed and frustrated larvae (expression in feeding larval stages could not be confirmed). There are thus perhaps stage-specific cuticle proteins and indeed the structure of the cuticle has been shown to change in *I. ricinis* during feeding (Dillinger and Kesel, 2002), thus the concept that the protein could change during development is feasible. Curiously, recent tick female transcriptomic studies undertaken in *Ixodes*, *Dermacentor* or *Amblyomma* ticks did not yield cuticle protein transcripts (Alarcon-Chaidez et al., 2007; Chmelar et al., 2008; Mulenga et al., 2007a). Logically however, cuticle proteins were found to be most abundant in feeding stages but would not necessarily be found in salivary gland secretions.

The 'frustrated' larval library aimed to isolate transcripts differentially expressed by larvae while attempting to attach to the host. This is the first tick study that targets larval stages to study the molecular basis of attachment to the host. Glycine rich proteins were identified confirming results obtained in a proteomic analysis of unfed *R. microplus* tick larvae (Untalan et al., 2005). Other studies have demonstrated that GGY proteins are mostly associated with female salivary gland secretion, tick attachment (Francischetti et al., 2005; Zhou et al., 2006) and pathogen transmission (Macaluso et al., 2003; Nene et al., 2004). It is currently not certain what is the function of these proteins however they possess extracellular matrix (ECM) domains and have also been reported to be similar to the cement proteins utilized by ticks for host attachment and to also putatively have a role in platelet aggregation inhibition (Guilfoile and Packila, 2004; Ribeiro et al., 2006). Although both were up-regulated in frustrated larvae, one was highly expressed in unfed larvae and the other in nymphet stages as demonstrated by qRT-PCR analysis. Thus it is feasible, given the abundance in female stages also in the above reports, that there is a diverse family of GGY domain and glycine rich proteins perhaps associated with different stages and functions. This observation was recently confirmed in a comparative sialomic study of soft and hard ticks where it was demonstrated that *R. microplus* appears to harbour an abundance of Pro/Gly rich protein genes ($n = 27$) compared to other tick species: *Amblyomma* spp., *Ixodes* spp., *D. andersoni* and *R. appendiculatus* which comprised approximately 0–9 Pro/Gly rich genes only (Mans et al., 2008).

A cysteine proteinase inhibitor (cystatin) previously isolated from the *R. microplus* fat body (engorged females cDNA library) was thought to assist with yolk processing during embryogenesis (Lima et al., 2006). The putative cysteine proteinase inhibitor isolated from the frustrated larval library here is more similar to the soft tick *Ornithodoros moubata* thyropin precursor that has an identified thyroglobulin Type 1 domain associated with cysteine protease inhibitors. In studies using RNAi to inhibit the *A. americanum* cystatin, tick blood feeding was subsequently inhibited, thus indicating that cystatins are associated with feeding success and *O. coriaceus* sialomic studies have confirmed that cystatins are secreted by female salivary glands (Francischetti et al., 2008a; Karim et al., 2005). It is not certain what the function of the frustrated larval *R. microplus* cystatin isolated here however it is likely to be associated with early attachment and/or the initiation of the host feeding cascade.

Blood feeding is known to up-regulate proteases of various types (Sonenshine and Hynes, 2008). In this study, we report the isolation of two stage-specific putative trypsin-like serine proteases from male and female ovary tissue respectively. Expression of either protease was not evident in any other tick stages or organs tested. Bior et al. (2002) identified a cysteine protease in *Amblyomma* and *Dermacentor* male ticks and a male salivary gland study did not identify serine proteases in other tick species (Anyomi et al., 2006). In general, fewer male specific tick transcriptome studies have been undertaken and thus it is possible that male specific serine proteases are employed by male *R. microplus* ticks for feeding or other purposes. In addition, most female tick transcriptome studies are focused on salivary gland secretion and thus have not addressed ovary specific gene expression. This unique transcript arose from our 'whole' female tick cDNA samples which were used as testers in our SSH experiments. Approximately 34–39 ESTs with trypsin-like serine protease domains have been identified in the BmGI2 *R. microplus* EST database (Mans et al., 2008; Wang et al., 2007), indicating a potential diverse range of function and stage-specific activities. This abundance of these serine proteases has been confirmed in transcriptome studies of female salivary glands from both soft and hard tick species (Alarcon-Chaidez et al., 2007; Chmelar et al., 2008; Francischetti et al., 2008b; Mans et al., 2008). In addition, tick research literature has focused on the activity of serine protease inhibitors rather than the serine proteases possibly due to their high abundance and putative role in host thrombin inhibition (Batista et al., 2008; Mulenga

et al., 2007b). Further work to characterize these two exclusively expressed serine proteases will assist to define their activities in the male tick and the female ovary, respectively.

Tick histamine binding proteins (HBPs) are associated with Ixodid tick salivary glands and exhibit "histamine-blocking" activity (Chinery and Ayitej-Smith, 1977). Histamine-mediated cutaneous inflammation is one of the defence reactions host animals mount against blood-feeding ticks. Histamine is a principal mediator of inflammatory reactions and is released by the host in response to tissue damage such as tick feeding. Ixodid ticks feed on their host for extended period of time and thus HBP sequester host histamine at the wound site outcompeting host histamine receptors thereby overcoming the hosts' inflammatory responses and enabling successful feeding (Paesen et al., 1999). Logically and consistently with other tick species studies, female tick histamine binding protein was up-regulated in the adult female tick feeding library in this study. FSHBP knockdown led to a reduced histamine binding ability in *A. americanum* salivary gland and an aberrant tick feeding pattern or host response (Aljamali et al., 2003). Our adult female libraries also demonstrated the up-regulation of stress response proteins such as heat shock protein and 'senescence associated' proteins, putatively associated with adaptation to the host environment as previously demonstrated (Batista et al., 2008; Mulenga et al., 2007a).

Glycosyl transferase has been shown to be associated at the end of the hexosamine biosynthetic pathway facilitating the protein glycosylation of uridine diphosphate-*N*-acetylglucosamine which is also an active precursor of chitin (Huang et al., 2007). An enzyme up-regulated in our frustrated female library was demonstrated to have a glycosyl transferase domain. A recent study by Huang et al. (2007) demonstrated that this pathway is essential for *Haemaphysalis longicornis* stage development, up-regulated with blood feeding but decreasing with engorgement. Our RT assays demonstrated an up-regulation in salivary gland and ovary tissue. This is consistent with Huang et al. (2007) study where knockdown of glutamine:fructose-6-phosphate aminotransferase (an enzyme associated with the start of the hexosamine biosynthetic pathway) led to the inhibition of tick blood feeding and egg production.

Frustrated larval and feeding adult female tick libraries identified clones which matched previously isolated salivary secreted proteins from *Ixodes* ticks and insects (midges). The results were confirmed in nymph and female stages including salivary gland thus confirming salivary gland secretion. Their abundance however, has been demonstrated in female salivary gland secretions in many tick species including *D. andersoni*, *O. parkeri*, and *Ixodes* spp. (Alarcon-Chaidez et al., 2007; Chmelar et al., 2008; Francischetti et al., 2008b, 2005; Ribeiro et al., 2006). No specific function for these hits is known due to the lack of recognizable protein domains or motifs however a comprehensive study in *I. scapularis* has provided preliminary evidence for conservation of particular domains which are suggesting a role in anti-haemostasis (Francischetti et al., 2005; Ribeiro et al., 2006). Antibodies against these *I. scapularis* proteins demonstrate an inhibition of tick feeding and pathogen transmission providing further evidence of the importance of these salivary proteins (Narasimhan et al., 2007). A number of other hypothetical and unknown sequences were identified in our experiments. Expression analysis confirmed the specificity of some of these unknown sequences to various stages or organs. Until the completion of several tick genome sequences and the development of tick specific annotation resources, the putative functional identification of unknown sequences will continue to be limited.

The *R. microplus* male tick library was enriched using males collected from the under-side of semi-engorged female ticks. All male transcripts screened in RT-PCR assays demonstrated a strong stage specificity of the male sequences as compared to expression analysis of the larval and adult female libraries in this study. Apart from ribosomal proteins, no similarity of the *R. microplus* transcripts from our SSH experiments could be identified in male specific analyses of *Amblyomma* or *Dermacentor* male ticks. Apart from the putative male-specific serine protease described above, 2 clones with low homology to a spider toxin domain were identified. It is feasible that the putative toxin is associated with feeding and thus has anti-haemostatic activity (Yamazaki and Morita, 2007) or perhaps the toxin is involved in assisting the male tick to stabilize movement of the female during mating. Putative toxins have been identified in female salivary gland secretions, also with low sequence homology (Batista et al., 2008; Francischetti et al., 2005; Ribeiro et al., 2006). Apart from the paralysis tick, *Ixodes holocyclus*, with known toxin production, putative tick toxins identified in these studies have not been described or studied in detail. Other adult male specific sequences included homologues for an ATP dependent RNA helicase, prefoldin molecular chaperone, cation transport and a *R. appendiculatus* immunodominant protein. The adult male library also had the greatest abundance of novel sequences compared to the larval and female libraries.

5. Conclusion

In this study, we investigated the use of the SSH technique to identify novel transcripts differentially expressed in attaching ('frustrated') and feeding larvae and adult female ticks, and adult male *R. microplus* ticks. From a total of 511 clones, 126 contigs and clone sequences were analyzed using bioinformatics and gene expression. A high proportion of unknown novel and hypothetical sequences were identified at 63% of the total contigs and singletons. The study identified similarities between genes differentially expressed during attachment and feeding in larval, nymphal and adult female stages. The feeding larval library appeared to be mostly associated with growth and developmental housekeeping genes with the unattached ('frustrated') larvae producing transcripts also expressed by adult female tick stages (pre-attachment and feeding). Adult male gene expression resulting from the SSH clones demonstrated stage-specific expression of the screened transcripts, with the highest abundance of unknowns. Novel differentially expressed sequences iden-

tified have further elucidated stage-specific activities in *R. microplus* growth and development during host attachment and feeding. The SSH analysis undertaken here successfully identified stage-specific differentially expressed sequences; however a full gene expression study utilizing using microarray technologies would provide a more thorough understanding of the molecular basis of attachment and feeding of *R. microplus* tick stages.

Conflict of interest

The authors have declared that no competing interests exist.

Acknowledgments

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