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THE SALIVARY TRANSCRIPTOME OF *Anopheles gambiae* (DIPTERA: CULICIDAE) LARVAE: A MICROARRAY-BASED ANALYSIS

M. Neira Oviedo[*], **J.M.C. Ribeiro**[†], **A. Heyland**[‡], **L. VanEkeris**[*], **T. Moroz**[*], and **P.J. Linser**[*]

[*]The Whitney Laboratory for Marine Bioscience, University of Florida, St. Augustine, FL

[†]Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD

[‡]Department of Integrative Biology, University of Guelph, Guelph, ON, Canada.

Abstract

In spite of the many recent developments in the field of vector sialomics, the salivary glands of larval mosquitoes have been largely unexplored. We used whole-transcriptome microarray analysis to create a gene-expression profile of the salivary gland tissue of fourth-instar *Anopheles gambiae* larvae, and compare it to the gene-expression profile of a matching group of whole larvae. We identified a total of 221 probes with expression values that were (a) significantly enriched in the salivary glands, and (b) sufficiently annotated as to allow the prediction of the presence/absence of signal peptides in their corresponding gene products. Based on available annotation of the protein sequences associated with these probes, we propose that the main roles of larval salivary secretions include: (a) immune response, (b) mouthpart lubrication, (c) nutrient metabolism, and (d) xenobiotic detoxification. Other highlights of the study include the cloning of a transcript encoding a previously unknown salivary defensin (AgDef5), the confirmation of mucus secretion by the larval salivary glands, and the first report of salivary lipocalins in the Culicidae.

Keywords

Anopheles gambiae; salivary gland; Diptera; gene expression; salivary defensin; transcriptome; salivary lipocalin

1. Introduction

The study of the salivary secretions of arthropod vectors of disease (a field known as ‘vector sialomics’) has gained considerable momentum since Ribeiro (1987) first proposed that certain

Corresponding author: Dr. Marco Neira Oviedo, The Whitney Laboratory for Marine Bioscience, St. Augustine, FL 32080, USA. Tel: +1 904 461 4037; fax: +1 904 461 4052; e-mail: mneira@whitney.ufl.edu.

components of the salivary secretions of hematophagous arthropods play a key role in regulating the host's hemostatic and immune reactions. Currently, the list of vector species for which a published salivary transcriptome and/or proteome exists include *Anopheles gambiae* (Arca et al.,1999; Lanfrancotti et al.,2002; Arca et al.,2005, Calvo et al.,2006a), *An. funestus* (Calvo et al.,2007), *Aedes aegypti* (Ribeiro et al.,2007), *Ae. albopictus* (Arca et al.,2007), *Triatoma brasiliensis* (Santos et al.,2007), *Triatoma infestans* (Assumpcao et al.,2008) *Glossina morsitans* (Van Den Abbeele et al.,2007), *Culicoides nubeculosus* (Langner et al., 2007) and *Xenopsylla cheopis* (Andersen et al.,2007), among others. Within the mosquitoes (Diptera: Culicidae), most research efforts have been aimed at characterizing the salivary glands (SG) of adult stages, leaving the larval SG largely unexplored.

In mosquito larvae, the SG are two well developed structures located in the thorax, on each side of the digestive tract (fig. 1A). In *Anopheles* species, each gland is divided into two lobes (fig. 1B and 1C) and is connected to the digestive tract by a salivary duct that opens directly into the mouth cavity. A ring of imaginal cells, which will develop into the adult SG during pupation, is located in the most anterior part of each gland (Jensen and Jones, 1957;Christophers, 1960).

Information regarding the nature and purpose of the mosquitoes' larval salivary secretions is extremely scarce. In contrast with other Diptera, mosquito larvae do not need to secrete glue proteins to attach their pupae to a substrate (as *Drosophila* larvae do), nor do they bind particles together to form protective structures (as some *Chironomus* larvae do). In fact, the only published reference we are aware of regarding the function of the larval SG in mosquitoes, states that they have "no such special function to serve" (Christophers, 1960). It seems unlikely, however, that such well developed structures as the larval SG would have evolved in the Culicidae if they had no physiological role to fulfill. In other insect species, larval salivary secretions have been associated with digestion (Verma and Baylan, 1972; Agarwal, 1976; Verma et al, 1977), immunity (Turillazzi et al.,2004; Korayem et al.,2004, Candido-Silva et al.,2007; Liu, 2004), larval molting (Gelman et al, 1991; Zheng et al, 2003) and even social interaction (Cummings et al, 1999; Hunt, 1991).

In this work, we aimed at identifying the gene products most abundantly synthesized by the salivary glands of *An. gambiae* larvae, and therefore throw some light on the nature and function of their secretions. We performed whole-genome microarray analysis of gene expression, a technique that has been successfully used in the past to create transcriptional profiles of different tissues of *An. gambiae* larvae (Neira Oviedo et al.,2008) and adults (Marinotti et al., 2005, 2006; Warr et al.,2007). Based on available annotation of the selected gene products, we discuss the potential physiological roles of the larval salivary glands and their secretions.

2. Materials and methods

2.1. Mosquito rearing and dissection

An. gambiae (G3 strain) eggs were obtained from the Centers for Disease Control and Prevention (CDC; Atlanta, Georgia). Mosquito larvae were reared in distilled water at 28°C, 12h: 12h light: darkness cycle, and provided with ground Tetramin® flakes (Tetra Werke) as food.

For each biological replicate of the experiment, 50 fourth-instar larvae were anesthetized on ice and dissected in nuclease-free 70% ETOH. Their salivary glands were extracted and stored at −20°C in nuclease-free 70% ETOH until RNA extraction. Three biological replicates of the experiment were performed.

2.2. RNA sample preparation, processing and hybridization to Affymetrix GeneChip® Arrays

Total RNA was extracted using the RNAeasy-Mini Kit™ (Qiagen) following the manufacturer's recommendations. RNA processing and hybridization were performed at the University of Florida's microarray core facility. Briefly, RNA concentration was determined on a NanoDrop Spectrophotometer (NanoDrop Technologies) and sample quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies) as per the manufacturers guidelines. cDNA was synthesized from 5ug of total RNA using the One-Cycle Eukaryotic Target Labeling reagents (Affymetrix, Inc.). The cDNA was used as a template for *in vitro* transcription (IVT) in the presence of T7 RNA Polymerase and a biotinytlated nucleotide/ribonucleotide mix for cRNA amplification and biotin labeling using the IVT labeling kit (Affymetrix, Inc). The biotin-labeled cRNA was fragmented and hybridized for 16 hours to the Affymetrix GeneChip® *Plasmodium/Anopheles* Genome Array according to the manufactures' protocols.

The arrays were then washed and stained with streptavidin-phycoerythrin on an Affymetrix Fluidics Station 450, and scanned on a GeneChip Scanner 3000 (Affymetrix, Inc) using default values to generate signal intensities. Quality control of hybridized chips was performed following Affymetrix recommendations (Affymetrix, 2004a).

As of December 2008, the microarray design used in this study probes for ~11,200 *An. gambiae* transcripts, as annotated in the VectorBase database (http://www.vectorbase.org/Genome/MartView/).

2.3. Statistical analysis

The GeneSifter™ (VizX Labs) software package was used to perform all statistical analyses. Transcripts were considered as present in a group (whole larvae or SG) if they were scored as such in all three biological repeats by Affymetrix built-in detection call (Affymetrix, 2004a). Expression values were background-adjusted, normalized, and $\log_2$-transformed by applying the 'robust multiarray average' (RMA) algorithm (Irizarry et al.,2003). Using a t-test, we compared the average signal intensity of each transcript in the SG to that found in a matching group of whole 4th instar larvae processed identically as part of a recently published study by our laboratory (Neira Oviedo et al.,2008). False discovery rate was adjusted to 1% using a Benjamini & Hochberg correction (Benjamini and Hochberg, 1995). Transcripts showing an intensity value two-fold or higher ($P < 0.01$) in the SG as compared to whole larvae were considered to be significantly enriched in the SG and therefore selected for further analysis.

2.4. Bioinformatics

The predicted amino acid sequences of transcripts encoding a methionine within the first 50 residues were submitted to the SignalP server (Bendtsen et al.,2004) to identify potentially secreted gene products. Functional annotation of selected transcripts was obtained from the June 2007 version of AnoXcel (Ribeiro et al.,2004), a comprehensive annotation repository that compiles information from several relevant sources, including databases such as Gene Ontology (Asburner et al.,2001), SWISSPROT (Bairoch and Boeckmann, 1992), KOG (Tatusov et al.,2003), PFAM (Bateman et al.,2000), SMART (Letunic et al.,2002), among others. This database can be downloaded from http://exon.niaid.nih.gov/transcriptome/A_gambiae/AnoXcel-Nov-2007-Web.zip; the stand alone Affymetrix annotated data can be downloaded from http://exon.niaid.nih.gov/transcriptome/A_gambiae/AffyXcel-Nov-2007.tar.gz.

Based on the results of the aforementioned analyses, transcripts were classified as putative secreted, putative non-secreted, or unknown. Assignment to sub-categories within these groups was based on available functional annotation of the known or predicted gene products. All

microarray data discussed in this publication have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE9642. This data will also be available to the public through the expression data repository at VectorBase. (http://www.vectorbase.org).

Sequence alignments were performed using ClustalW (Thompson et al.,1994) available through the BioEdit software package (Hall, 1999) using default parameters. No manual adjustments of the alignment were necessary. Phylogenetic and molecular evolutionary analyses were conducted in the MEGA (version 4) software package (Kumar et al.,2004), using the maximum parsimony (MP) method and neighbor joining (NJ) algorithms. Specifically, we run NJ analysis with 100,000 bootstrap replicates and pair-wise comparisons. The putative annotation of disulfide bridges shown in figure 6a was performed using the crystal structure of sapecin from the flesh fly *Sarcophaga peregrina* (GenBank accession number P18313) as a model.

2.5. RT-PCR

Total RNA was extracted from dissected SG using the RNAeasy-Mini Kit™ (Qiagen) following the manufacturer's recommendations and using the kit's on-column DNAse treatment. cDNA synthesis was performed using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) using random hexamers as primers and following the manufacturer's recommendations.

Amplification reactions were assembled using 22μl PCR Supermix HiFidelity (Invitrogen), 1μl (≥100ng) cDNA, 1μl forward primer (10μM), and 1μl reverse primer (10μM). Primer sequences are listed in the supplementary materials, table 1. PCR conditions were: 94°C for 3 min; 35 cycles of [94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min]; 72°C for 3 min; hold at 4°C. PCR products were visualized on a 1% agarose/EtBr gel.

2.6. Quantitative real-time PCR

RNA extraction and cDNA construction were performed as described for RT-PCR analysis. Quantitative real-time PCR (qRT-PCR) reactions were prepared using the SYBR Green PCR Master Mix (Applied Biosystems), following the manufacturer's recommendations. Reactions were prepared in triplicate and run in an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). Relative abundance levels were calculated by the $\Delta\Delta C_T$ method (Applied Biosystems, 2004), using the ribosomal protein S7 transcript as endogenous control (Warr et al.,2007). All primer sequences used for qRT-PCR are listed in the supplementary materials, table 1.

2.7. An. gambiae defensin 5 (AgDef5) cDNA cloning and sequencing

An. gambiae defensin 5 PCR product was excised from the agarose gel and extracted using the MinElute gel extraction kit (Qiagen). Purified PCR product was cloned and used to transform TOP10® *Escherichia coli* cells using the TOPO TA Cloning® Kit for Sequencing (Invitrogen), following the manufacturer's instructions. Colonies producing a PCR product of the appropriate size were cultured overnight at 37°C in 5ml of LB medium containing 50μg/ml carbenicilin. Plasmid vectors were isolated from these bacterial cultures using the Qiaprep Spin Miniprep Kit (Qiagen), following the manufacturer's recommendations.

Sequencing was performed by using the ABI Prism® BigDye™ Terminator Cycle Sequencing Reaction (Applied Biosystems), following the manufacturer's guidelines. Output sequence files were edited using SeqMan software (DNASTAR).

2.8. Two-dimensional (2D) gel electrophoresis and protein identification by mass spectrometry (MS)

Approximately 50μg of sample proteins (200 pairs of larval SG) were used to perform 2D gel electrophoresis using ZOOM IPGRunner System (Invitrogen) under manufacturer's recommended running conditions. Protein identification of 2D gel-separated proteins was performed on reduced and alkylated trypsin-digested samples prepared by standard mass spectrometry protocols. Tryptic digests were analyzed by coupling the Nanomate (Advion BioSciences) – an automated chip-based nano-electrospray interface source – to a quadrupole time-of-flight mass spectrometer, QStarXL MS/MS System (Applied Biosystems/Sciex). Computer-controlled, data-dependent automated switching to MS/MS provided peptide sequence information. AnalystQS software (Applied Biosystems/Sciex) was used for data acquisition. Data processing and databank searching were performed with Mascot software (Matrix Science). The NR protein database from the NCBI, National Library of Medicine, NIH, was used for the search analysis.

2.9. Microscopy and histochemistry

For images showing the gross morphology of the larval alimentary canal and SG, early 4^{th} instar larvae were dissected in 70% ethanol and photographed using an Olympus SZX12 stereoscope (Olympus Life Sciences) connected to a Nikon DS-L1 digital camera (Nikon Corporation).

For histochemical staining, longitudinal paraffin sections of whole 4^{th} instar larvae were prepared as previously described (Smith et al.,2008). Rehydrated sections were incubated for 1 hr at 37°C with a 1/500 dilution of either FITC-conjugated *Erythrina cristagalli* lectin or FITC-conjugated wheat-germ agglutinin (Vector laboratories) in pre-incubation buffer (TBS, 1% bovine serum albumin, 1% normal goat serum, 0.1% TritonX100). Sections were then washed three times in TBS, mounted in 60% glycerol in TBS, and analyzed using a Leica LSCM SP2 laser scanning confocal microscope (Leica Microsystems).

3. Results and discussion

The gene expression platform used in this work (GeneChip® *Plasmodium/ Anopheles* array, Affymetrix Inc.) assigns both a qualitative value (present/absent call) and a quantitative 'expression level' value to each one of its probes. By comparing the expression profile of the fourth-instar larval SG (fig. 1) to that of whole larvae, we identified a total of 4,719 probes that were scored as 'present' in the SG, 747 of which were unique to this tissue (i.e. were scored as either 'absent' or 'undetermined' in one or more repeats of the whole larva sample) (supplementary materials, table 2). Among these, 221 probes (associated with 292 genes, encoding 318 transcripts) were selected for further analysis because they were (a) significantly ($P<0.01$) enriched at least twofold in the salivary glands, and (b) sufficiently annotated as to allow the prediction of the presence or absence of a signal peptide in their associated proteins.

The mismatch between the number of probes (221), transcripts (318) and genes (292) we list as significantly enriched in the larval SG can be explained by the constant evolution of our knowledge of the *An. gambiae* genome: the design of the Affymetrix microarray chip used in our study was based on the genomic information available in 2003 (Affymetrix, 2004b), and attempted to provide a probe for each transcript known at the time. However, many novel genes and transcripts have been identified since the commercial release of this design, and these can often be associated with existing probes. As a result, more than one transcript (sometimes from different genes) can be associated with several of the chip's probes.

The 221 probes selected for this study, as well as their corresponding transcripts and relevant functional annotation, are listed in the supplementary materials, table 2. Scatter and volcano plots of the transcriptomic comparison between larval SG and whole larvae are available in the supplementary materials, fig. 1.

In order to check for any potential contamination of our SG samples with tissues from other regions of the digestive tract, we compared the enrichment levels of the aforementioned 221 selected probes in the SG, gastric caeca, anterior midgut, posterior midgut and hindgut. For this purpose, we used whole-transcriptome expression profiles of each gut section previously generated by our laboratory (Neira Oviedo *et al.*, 2008) (accession number GSE7149 in NCBI's gene expression omnibus, available at http://www.ncbi.nlm.nih.gov/geo/). The clear enrichment that virtually all the selected probes showed in the SG (supplementary materials, figure 2) suggests that contamination of our tissue samples is rather unlikely.

Ten transcripts found to be significantly enriched in the SG by our microarray analysis were randomly selected to cover the entire spectrum of transcript enrichment, and used to perform qRT-PCR. A high degree of correlation (R^2=0.89) was observed between the expression values obtained by these two methods (fig. 2), validating the accuracy of our microarray-generated gene expression data.

3.1. Functional annotation analysis of SG-enriched transcripts

Based on available annotation for the known or predicted protein sequences encoded by the selected transcripts, 48 were identified as encoding putative components of the salivary secretions, 267 as corresponding to putative non-secreted proteins, and three as encoding proteins of unknown function. In accordance with the secretory nature of the SG, the average enrichment of probes associated with putative secreted proteins (11.1 fold vs. whole larva) was 2.8 times that of probes associated with putative non-secreted proteins (4 fold vs. whole larvae) (fig. 3).

3.2. Transcripts encoding putative secreted products

Probes associated with putative secreted gene products enriched in the SG were classified based on their available functional annotation. Among those with a known or predicted function, immune-related transcripts were found to be the most diverse and enriched (fig. 4 and table 1). To confirm that this enrichment of immune transcripts is a constitutive characteristic of the larval SG (and not the result of a microbial assault suffered by the particular cohort of insects used to obtain our SG samples) we measured, by qRT-PCR, the expression of five of these immune transcripts in the SG and whole body of a separate group of larvae reared specifically for this purpose. The enrichment in SG of all five transcripts observed in these independent samples (shown in supplementary fig. 3) supports the notion that immune-related gene products are constitutively enriched in the larval salivary secretions.

Other significantly enriched functional groups include digestive and detoxifying enzymes, members of the antigen 5 (AG5) family of genes, mucins and lipocalins (fig. 4 and table 1). Probes for 13 additional transcripts encoding putatively secreted proteins without any associated functional annotation were also found to be significantly enriched in the SG.

3.2.1. Immunity-related transcripts

3.2.1.1. Anti-microbial factors: Our analysis revealed enrichment in the SG of three functional groups associated with anti-microbial functions: (a) defensins, (b) lysozymes, and (c) proteins containing trypsin inhibitor-like (TIL) domains. Each one of these groups will be discussed separately. Additionally, salivary proteins assigned to other functional groups (such

as the mucins and chitinases, discussed in sections 3.2.2 and 3.2.5, respectively) have the potential to significantly contribute to the anti-microbial effect of the saliva.

Defensins are small anti-microbial peptides (usually 36–46 residues long), present in a wide diversity of plant, vertebrate and invertebrate species. They are mainly active against Gram-positive bacteria, but also exhibit activity against fungi, yeast and protozoa (Bulet et al., 1999; Zhou et al.,2007). Four defensin genes (*AgDef1* to 4) have been described to date in the *An. gambiae* genome (Christophides et al.,2002).

Our analysis identified the expression in larval SG of defensins AgDef2 and AgDef3, which have been previously reported to be present in the adult male and female SG, respectively (Arca et al.,2005; Calvo et al.,2006a). Additionally, we identified the expression of a previously undescribed defensin (henceforth referred to as AgDef5) which is encoded by the ENSEMBL gene AGAP007200, transcript AGAP007200-RA. We successfully cloned and sequenced the transcript encoding this novel defensin. The sequence of this transcript has been deposited in GenBank, with accession number EU273600.

The *AgDef5* gene contains a single exon and is located on the reverse strand of chromosome 2L (starting at position 44,248,847), immediately adjacent to the *AgDef3* gene. cDNA corresponding to the *AgDef5* transcript presents a 207 bp open reading frame that translates into a 68 amino-acid peptide (fig. 5). The first 23 residues of this peptide encode a signal sequence, indicating that it is a secreted protein. The mature 44 amino acid-long peptide contains the six conserved cystein residues characteristic of all insect defensins (fig. 6a; Bulet et al.,1999). A phylogenetic analysis (fig. 6b) places *AgDef5* closest to *AgDef3, AgDef4* and *AgDef2*, which have been previously classified as a highly divergent group of defensins (Christophides et al.,2002). The exact role of this new defensin in the larval mosquito's immune system remains to be determined.

Lysozymes are traditionally associated with antibacterial activity, which they exert either by catalytic activity (hydrolyzing glycosidic bonds in the peptidoglycan layer of the bacterial cell walls), or by non-catalytic mechanisms such as membrane perturbation and the stimulation of autolysin activity (During et al.,1999; Ibrahim et al.,2001). Additionally, it has been recently shown that the immune role of lysozyme in mosquitoes extends beyond its antibacterial activity, playing a role in the regulation of melanotic encapsulation of foreign bodies (Li and Paskewitz, 2006).

Eight different lysozyme-encoding genes (*Lys c-1* to *Lys c-8*) have been reported in the genome of *An. gambiae* (Li et al.,2005). Our analysis revealed one lysozyme, encoded by transcript AGAP007386-RA, to be significantly enriched in the larval SG. This transcript is encoded by the gene *Lys c-7*, and it has been reported to be expressed in all developmental stages, and in virtually all tissues of the adult female, with the exception of the ovaries (Li et al.,2005). Interestingly, the peptide encoded by this transcript contains the residues necessary to bind carbohydrates, but lacks two amino acids thought to be essential for catalytic function (Li et al.,2005). Therefore, this lysozyme is likely to exert its antibacterial activity via the aforementioned non-enzymatic functions.

Further support for the immune role of *Lys c-7* comes from a study recently published by our laboratory, in which we generated whole-genome expression data for each compartment of the larval digestive tract (Neira Oviedo et al.,2008). Analysis of this data set (available at http://www.ncbi.nlm.nih.gov/geo/, series number GSE7149), reveals abundant expression of *Lys c-7* in two regions of the digestive tract: the gastric caeca and hindgut / Malpighian tubules; both of these areas have been identified as being heavily involved in immune responses in larval Diptera (Neira Oviedo et al.,2008, McGettigan et al.,2005).

Four transcripts corresponding to proteins containing a trypsin inhibitor-like (TIL) domain were detected in the larval SG (transcripts AGAP006813-RA, AGAP002636-RA, AGAP002445-RA, and AGAP002450-RA). Proteins containing TIL domains fall within the larger category of serine-protease inhibitors, which have been associated with several functions, including the regulation of blood and hemolymph coagulation, activation of phenoloxidase and cytokines, inactivation of microbial proteases, regulation of endogenous proteases and inhibition of bacterial growth (Friedrich, 1993; Stubbs, 1997; Kanost, 1999; Fogaca, 2006). Several reports exist regarding the presence of serine protease inhibitors in the salivary glands of vertebrate and invertebrate organisms, including humans (Hochstrasser et al.,1993; Kolho et al.,2005), adult mosquitoes (Calvo et al.,2006a; Ribeiro et al.,2007; Arca et al.,2007) and hematophagous hemipterans (Santos et al.,2007; Assumpcao et al.,2008). Serine-protease inhibitors present in the salivary secretions of adult female mosquitoes have been shown to facilitate blood feeding by interfering with the host's inflammatory response and coagulation cascade (Stark and James, 1995; Lanfranciotti et al.,2002; Ribeiro et al.,2007). However, the presence of these transcripts in the non-hematophagous larvae suggests they play a role as part of the immune system, rather than acting as anticoagulant factors. A similar function has been proposed for serine-protease inhibitors found in adult male SG of *An. gambiae* (Calvo et al.,2006a).

3.2.1.2. Pattern recognition factors: Our results revealed significant enrichment in the SG of one probe (Ag.UNKN.836.0_CDS_s_at) associated with three transcripts (AGAP004998-RA, AGAP004999-RA, AGAP012651-RA) that encode proteins similar to the fibrinogen-related protein ficolin. Among these, transcripts AGAP004999-RA and AGAP012651-RA have been previously found to be expressed in the adult female SG of *An. gambiae* (Ribeiro, unpublished data).

Fibrinogen-related proteins (FREPs) are found in both vertebrates and invertebrates and are mainly associated with immune functions, although structural roles have also been reported (Wang et al.,2005). FREPs associated with immune reactions (such as ficolin) contain fibrinogen-like domains capable of recognizing and binding carbohydrates characteristic of the microbial surface (such as lipoteichoic acid, peptidoglycan and lipopolysaccharides). Once bound, they can act as opsonins, or trigger the complement cascade (Endo et al.,2007; Wang et al, 2004).

The presence of transcripts encoding ficolin-like products in the larval SG of mosquitoes is consistent with previous studies which have reported the expression of immune-related FREPs in different tissues of dipteran insects, including immature stages (Wang et al.,2005, 2004). Interestingly, it has been suggested that mosquitoes present a large expansion of FREP-encoding genes (when compared to other dipterans, such as *Drosophila*), probably reflecting the particularly diverse microbial challenges faced throughout their life cycle (Loker et al., 2004). Ribeiro *et al.* (2007) presented evidence of ficolins in the salivary secretions of adult female mosquitoes, and suggested they play a role related to blood-feeding. In the case of the larval salivary FREPs, however, the absence of blood-feeding implies that their role is likely to be either immune or structural.

3.2.1.3. Immune-related serine proteases: Several transcripts annotated as encoding immune-related serine proteases were found to be enriched in the larval SG. Transcripts AGAP011908-RA, AGAP011909-RA, AGAP011913-RA, AGAP011914-RA encode CUB - domain serine proteases, while transcript AGAP003057-RA encodes the clip-domain serine protease CLIPB8. With the exception of AGAP011909-RA, all these transcripts have been reported from the adult female SG of *An. gambiae* (Ribeiro, unpublished data).

The CUB domain consists of ~110 residues and is found almost exclusively in extracellular proteins. It was first discovered in the complement subcomponents C1s/C1r, and it has been reported to be present in several developmentally-regulated proteins (Bork and Beckmann, 1993), immune-related salivary proteins (Ligtenberg et al.,2007) and proteins involved in receptor-mediated endocytosis (Chrisrensen and Gburek, 2004), among others. Although the specific roles of this domain are not well understood, it has been suggested that it is involved in substrate recognition and oligomerization (Blanc et al.,2007). It is widely accepted that CUB domains present in complement-associated serine proteases mediate the interaction of these enzymes with non-self recognition proteins (such as ficolins), triggering the complement's characteristic cascade of enzymatic activations. A role in substrate-recognition has been suggested for enzymes of this kind present in the saliva of adult female mosquitoes (Ribeiro et al.,2007).

Clip-domain serine proteases are major regulators of developmental and immune-related signaling processes such as the Toll signaling pathway, and the activation of phenoloxidase (one of the principal components of the melanization immune response) (Jang et al.,2008; Barillas-Mury, 2007). The 'clip' domain receives its name from its unusual 'paper clip' configuration, resulting from unique disulfide bonds (Muta et al.,1990). The particular clip-domain serine protease found in our study (CLIPB8) has been reported to promote melanization in the G3 strain of *Anopheles gambiae* (Barillas-Mury, 2007; Paskevitz et al.,2006). Melanization-related enzymes have been reported to exist in the salivary secretions of larval insects, although their functional roles are unknown (Satoh et al.,1999).

3.2.2. Mucins—Mucins are extensively glycosilated proteins previously reported to be abundant in the salivary secretions of a wide diversity of animals, including adult male and female mosquitoes (Calvo et al.,2006a; Arca et al.,2005). Three mucin-encoding transcripts (AGAP006067-RA, AGAP010727-RA and AGAP008177-RA) were identified in our analysis as being significantly enriched in the larval SG. Interestingly, one of these (transcript AGAP010727-RA) has also been reported to exist in the adult salivary glands (Ribeiro, unpublished data), suggesting that it plays a role in aiding the digestive process throughout the mosquito's life cycle. Further support for the presence of mucins in the larval salivary secretions comes from the staining of the SG lumen contents with fluorescently-labeled wheat-germ agglutinin (fig. 7), a plant lectin that selectively binds sialic acid and N-acetylglucosamine, and can be used to identify mucin-type glycoproteins (Furukawa et al., 1986).

Traditionally, mucins have been exclusively associated with the formation of coats that protect and lubricate the epithelia where they are produced. However, recent studies have suggested that these proteins are also involved in a variety of complex physiological processes such as development, epithelial renewal, carcinogenesis and immunity (Corfield et al.,2000; Korayem et al.,2004; Moal et al.,2006; Wei and Bobek, 2005; Habte et al.,2006). In vertebrates, salivary mucins have shown potent antimicrobial activity, being able to inhibit viral replication and fungal growth (Wei and Bobek, 2005; Habte et al.,2006; Ogasawara et al.,2007). In *Drosophila*, Korayem *et al.* (2004) reported mucins to be co-expressed in larval SG and immune tissues, and suggested that they help form clots that immobilize potentially pathogenic microorganisms, preventing their dissemination and rendering them more susceptible to the insect's immune system.

Although previous studies have reported the presence of mucus in the lumen of the foregut (including the pharynx) and midgut of larval mosquitoes, they have failed to identify the glands responsible for its secretion (Fry, 1996; Dahl et al.,1990). Dahl *et al.* (1990) stated that the SG "would be an obvious structure" for the secretion of this mucus, but were unable to provide histochemical evidence in support of this hypothesis. On the other hand, our results provide

both transcriptomic and histochemical evidence supporting the secretion of mucin-type proteins by the larval SG. We believe this contradiction can be attributed to the technical limitations of the Alcian Blue / periodic acid Schiff's reagent staining methods used by Dahl et al.,which (as the authors of that study acknowledge) are very sensitive to pH conditions, and can be difficult to interpret.

In light of our results, it seems plausible for the larval SG to be the site of origin of at least some of the mucus reportedly present in the digestive tract of mosquito larvae. The roles of this mucus are likely to include, in addition to food bolus lubrication, the immobilization and neutralization of ingested microorganisms.

3.2.3. Lipocalins—Two transcripts (AGAP004799-RA and AGAP009281-RA) encoding proteins annotated as lipocalins were identified as significantly enriched in the larval SG; one of these (transcript AGAP004799-RA) has also been reported to be particularly abundant in the adult male antennae of *An. gambiae*, where its function is unknown (Justice et al.,2003).

Lipocalins are a large group of small, generally extracellular proteins characterized by their ability to bind small hydrophobic molecules. Recent studies have revealed diverse roles for lipocalins, including transport, metabolism, immune regulation, smell perception, tissue development and behaviour modification (Akerstrom et al.,2000). Lipocalins have been reported to be the most abundant proteins in the salivary secretions of blood-feeding hemipterans and ticks, where they act as anticoagulants, anti-complement, and vasodilators (Assumpcao et al.,2008; Santos et al.,2007; Mans *et al*, 2008); however, no evidence of their existence in the saliva of mosquitoes has been presented to date (Assumpcao et al.,2008), making this the first report of salivary lipocalins in the Culicidae. Although their exact function in the saliva of the non-blood feeding mosquito larvae is yet to be determined, their potential role in sensory perception (Akerstrom et al.,2000) and their ability to diffuse in aqueous environments makes it tempting to speculate that salivary lipocalins can act as pheromones, providing chemical cues to other individuals about factors such as larval density. Alternatively, they might serve to capture lipidic nutrients from the diet.

3.2.4. AG5 family—Three transcripts annotated as encoding proteins belonging to the AG5 family were found to be significantly enriched in the larval SG (transcripts AGAP007584-RA, AGAP006443-RA and AGAP006419-RA). All of these transcripts have also been reported to be expressed in the SG of adult *An. gambiae* (Arca et al.,2005; Calvo et al.,2006a; Ribeiro, unpublished data) (supplementary table 2), suggesting that their functions are not related to a particular feeding strategy.

The AG5 family of proteins is related to venom allergens found in social hymenopterans (Hoffman, 1993) and to antifungal proteins found in plants (Szyperski et al.,1998). Although members of the AG5 family have been reported to exist in the salivary secretions of several hematophagous insect species (Arca et al.,2005; Ribeiro et al, 2007; Santos et al.,2007), their exact function remains unknown. Megraw *et al.* (1998) reported the presence of AG5 proteins in the digestive tract of *Drosophila* larvae, where the authors suggested they function as protease inhibitors, either regulating the activity of digestive proteases, or exerting an antimicrobial effect.

3.2.5. Digestive and detoxifying enzymes—The list of digestive and detoxifying enzymes found to be enriched in the larval SG is presented in the supplementary materials, table 2; it includes enzymes involved in the metabolism of all major groups of nutrients (lipids, proteins and carbohydrates), as well as one apyrase-like nucleotidase (encoded by transcript AGAP011026-RA). Although the presence of digestive enzymes in the larval saliva is not surprising, the presence of an apyrase is rather puzzling: apyrases are a type of 5' nucleotidases

traditionally associated with the saliva of adult female mosquitoes, where they facilitate blood feeding by inhibiting platelet aggregation in the host (Ribeiro, 1987). In the non-blood feeding larva, the role of such an enzyme is unclear, and is probably related to the terminal digestion of nucleic acids ingested as part of the insect's normal diet (i.e. algal and microbial DNA). Interestingly, Lombardo *et al.* (2000) reported a complex developmental pattern of expression for transcript AGAP011026-RA (named *AgApyL1* by the authors of that study), being found in larvae, pupae, and both male and female adults. In agreement with our results, these findings support the notion that this enzyme plays a role unrelated to blood feeding.

Three transcripts annotated as putatively secreted chitinases (AGAP005339-RA, AGAP005634-RA and AGAP000789-RA) were found to be significantly enriched in the larval SG. Of these, transcript AGAP005339-RA has been previously detected in the SG of adult female *An. gambiae* (Ribeiro, unpublished data). Additionally, a chitinase-like protein has been previously reported from the saliva of adult *Anopheles* mosquitoes (Owhashi et al.,2008); unfortunately, neither its exact molecular identity nor its biological roles have been conclusively defined.

In vertebrates, salivary chitinases have been associated with protection against pathogenic fungi (Van Steijn et al.,1999). Shi and Paskewitz (2004) demonstrated that bacterial challenges triggered the production of chitinase-like proteins in the haemolymph of adult *An. gambiae*, suggesting a putative immune-related role. Although none of the chitinases identified in our study show a significant degree of identity with those reported by Shi and Paskewitz (table 3 and suplementary materials, fig. 4), the secretion of salivary chitinases as a line of defense against environmental microbes seems logical when we consider that chitin-containing structures (such as fungi) are likely to constitute a large fraction of the larval mosquito's regular diet (Clements, 1992). Further studies are required in order to confirm the enzymatic activity of the salivary chitinases identified by our study.

One probe (Ag.2L.1123.0_CDS_a_at) associated with three transcripts encoding flavin-containing monooxygenases (FMOs) was found to be significantly enriched in the larval SG. These enzymes have an important role in the metabolism of toxic compounds, often acting in parallel with (and on the same substrates as) cytochromes P450, another major group of detoxifying enzymes that are highly abundant in the digestive tract of *An. gambiae* larvae (Neira Oviedo et al.,2008, Testa and Kramer, 2007; Strode et al.,2006). The general metabolic function of FMOs consists in transforming toxic lipophilic substrates into more hydrophilic products, which are then easily transported and excreted with the urine (Testa and Kramer, 2007). The presence of this kind of enzymes in the saliva of larval *An. gambiae* might be related to the abundance of potentially toxic compounds (such as bacterial, algal and fungal toxins, as well as phenolic products of plant degradation) in their natural diet (Strode et al.,2006).

3.3. Transcripts encoding putative non-secreted and unknown proteins

The 186 probes (corresponding to 267 transcripts) associated with putative non-secreted proteins found to be significantly enriched in the larval SG are listed in the supplementary materials, table 2. Based on available functional annotation, these transcripts can be catalogued into 20 functional categories (table 2). Transcripts annotated as encoding hypothetical conserved proteins of unknown function are the most diverse, followed by transcripts associated with protein export machinery, and protein modification machinery. As shown in fig. 8, probes associated with extracellular matrix proteins show the highest average relative abundance (7.4 fold increase vs. whole larvae), followed by those associated with transcription machinery (6.6 fold increase vs. whole larvae) and lipid metabolism (5.6 fold increase vs. whole larvae).

Additionally, three transcripts encoding proteins not associated with any known function (AGAP007064-RA, AGAP003812-RA and AGAP008178-RA) were found to be significantly enriched in the SG. Interestingly, the protein encoded by transcript AGAP003812-RA has partial homology to a protein predicted to be a transposase in NCBI's KOG database (accession NP_566626). Transposable elements have been detected in the salivary transcriptome of adult mosquitoes, where they have been proposed to either be markers of active transposition, or to act as regulators of transposable elements recently included in the genome (Ribeiro et al., 2007).

3.4. Protein identification by 2D gel electrophoresis / MS

Nine proteins could be identified by 2D gel electrophoresis followed by MS of the most intense bands (fig. 9). One of these proteins corresponds to a putatively secreted trypsin (protein AGAP001246-PA), with the remaining nine corresponding to putative non-secreted products (i.e. products lacking a signal peptide). This virtual absence of secreted products detectable by 2D gel electrophoresis / MS in larval SG contrasts with the wider diversity of secreted proteins detected by similar methods in the salivary glands of adult female mosquitoes (Ribeiro et al., 2007). This discrepancy is most likely due to differences in the salivation strategies used by the different developmental stages: larval mosquitoes are constantly feeding and therefore require a permanent flow of saliva, which precludes the accumulation of secreted proteins in the gland's lumen at any point. In contrast, adult mosquitoes only discharge saliva while feeding (an event that normally occurs once every several hours or even days), allowing the glands to accumulate relatively large amounts of salivary secretions between discharges.

Transcripts corresponding to three putative non-secreted proteins detected by MS (protein disulfide isomerases 1 and 2, and heat shock protein 70), were identified in our analysis as significantly enriched in the salivary glands (transcripts AGAP012407-RA, AGAP007393-RA and AGAP004192-RA, respectively). Heat shock protein 70 (Hsp70) and at least one protein disulfide isomerase have also been reported in 2D gel studies in adult female *Aedes aegypti* mosquitoes (Ribeiro et al.,2007), suggesting a role common to larval and adult stages.

The sequence of Hsp70 lacks a signal peptide and is therefore considered to be intracellular in our study; however, recent studies have reported its ability to be secreted via alternative mechanisms involving lipid rafts and/or exosomes (Lancaster and Febbraio, 2005) and have provided evidence linking salivary Hsp70 to diverse immune functions including complement activation, antigen-display, and the prevention of bacterial adhesion to mucosal surfaces (See Fabian et al.,2007 for an extensive review). Considering the abundance of this protein revealed by our 2D gel analysis, it might represent an important component of the mosquito's salivary immune arsenal.

Protein AGAP001246-PA (the aforementioned secreted trypsin) is unfortunately not associated with any probe in the commercial microarray design used in this experiment, and therefore its transcript could not be detected. Other putative non-secreted proteins detectable by MS whose transcripts were not found to be enriched in the SG (actin, acyl-coenzyme A dehydrogenase, ATP synthase sub-units A and B, and the receptor for activated protein kinase c) are at least equally abundant in other tissues of the whole larvae as they are in the SG, which explains why our experimental design did not detected them as significantly enriched in the SG tissue.

3.5. Comparison to adult sialomes

In clear contrast with the diet of adult mosquitoes (which consists of blood and/or sugary plant secretions), the diet of mosquito larvae consists mainly of microorganisms and various organic detritus collected from their aquatic environment (Clements, 1992). This radical difference in

food sources is reflected in the different physiological roles played by the salivary secretions of the two life stages: while the saliva of adult female mosquitoes is used as a potent chemical cocktail aimed at countering the hemostatic and immune responses of their hosts (Ribeiro, 1987), the saliva of larval mosquitoes is unlikely to possess any particular anti-hemostatic activity, and is instead likely to be rich in elements that facilitate the ingestion and digestion of microbes.

In accordance with this, a comparative analysis of the larval salivary transcripts found in our study with that of adult *An. gambiae* available from previous studies (Arca et al.,2005; Calvo et al.,2006a, Ribeiro, unpublished data) (Supplementary materials, table 2) reveals some interesting differences and similarities. Notably, proteins of the D7 family (which are among the most abundant in the saliva of adult female mosquitoes, and are believed to act as anti-hemostatic factors that facilitate blood feeding) (Valenzuela et al.,2002; Arca et al.,2005; Calvo et al.,2006 a and b) were not detected in the larval SG. Similarly, proteins of the SG1 family were not detected in the larval saliva. SG1-type proteins are abundant in the saliva of adult female anopheline mosquitoes, where they play a yet undetermined role (Arca et al.,2005). The absence of this type of proteins in the non blood-feeding adult males (Calvo et al., 2006a) and larvae (this study) is strongly suggestive of a role related to the blood-feeding process.

On the other hand, our study suggests that the saliva of *An. gambiae* larvae is particularly rich in proteins with antimicrobial activity, such as chitinases, defensins and lysozyme, as well as other components that can help immobilize microorganisms (mucins), make them recognizable by the immune system (FREPs), or detoxify their potentially dangerous byproducts (FMOs). Additionally, other proteins for which an immune function is suspected (such as Hsp70 and members of the AG5 family) could increase the antimicrobial potency of the larval mosquito saliva. As previously mentioned, it is not surprising to find constitutive expression of antimicrobial factors in the saliva of an organism that feeds mostly on microorganisms. Larval mosquito saliva is discharged directly in the mouth (Clements, 1992), allowing for its components to start interacting with (and possibly neutralizing) any microbes immediately upon ingestion, and before they reach the metabolically-active midgut tissue. The fact that some of these antimicrobial factors are also present in the salivary secretions of adult mosquitoes (Supplementary materials, table 2) highlights the need to control microbes inevitably ingested with the regular diet of all developmental stages.

Our data supporting the presence of lipocalins in the larval saliva of *An. gambiae* is quite interesting, since proteins of this kind are reportedly absent in the salivary transcriptome of adult mosquitoes (Assumpcao et al.,2008), but are highly abundant the saliva of other blood-sucking arthropods (Montfort et al.,2000). It remains to be established why evolutionary forces have restricted their expression to the larval stages in *An. gambiae*, and what the physiological role of these proteins is.

Finally, we found an extensive set of transcripts encoding putative non-secreted proteins that are present in both the larval and adult SG (supplementary materials, table 2), suggesting that both developmental stages use, at least to some extent, similar molecular mechanisms for the production and secretion of salivary proteins.

4. Conclusions

Our study represents, to the best of our knowledge, the first report of the larval salivary transcriptome of any species in the Culicidae. By identifying the most abundant transcripts and proteins in the larval SG of *An. gambiae*, and analyzing their available functional annotation, we are able to provide an insight into the functional roles of the salivary secretions of mosquito

larvae. Based on our results, we propose that these roles include: (a) immune response, (b) food bolus lubrication, (c) nutrient metabolism, and (d) xenobiotic detoxification.

In agreement with the feeding habits of the *An. gambiae* larvae, a clear abundance and diversity of immune-related gene products was observed, highlighting the crucial role of saliva as the first line of defense against the ingestion of potentially pathogenic microorganisms. In addition, some of the most abundant proteins in the salivary transcriptome of adult female *An. gambiae* (such as those of the D7 and SG1 families) were not detected by our study, supporting the notion that these proteins' functions are mainly related to the blood feeding process.

Finally, the abundance of salivary transcripts that have no associated functional annotation (or for which a clear functional role in the saliva cannot be discerned) is a strong reminder of the large gaps that still exist in our understanding of the basic biology of one of the most important vectors of human disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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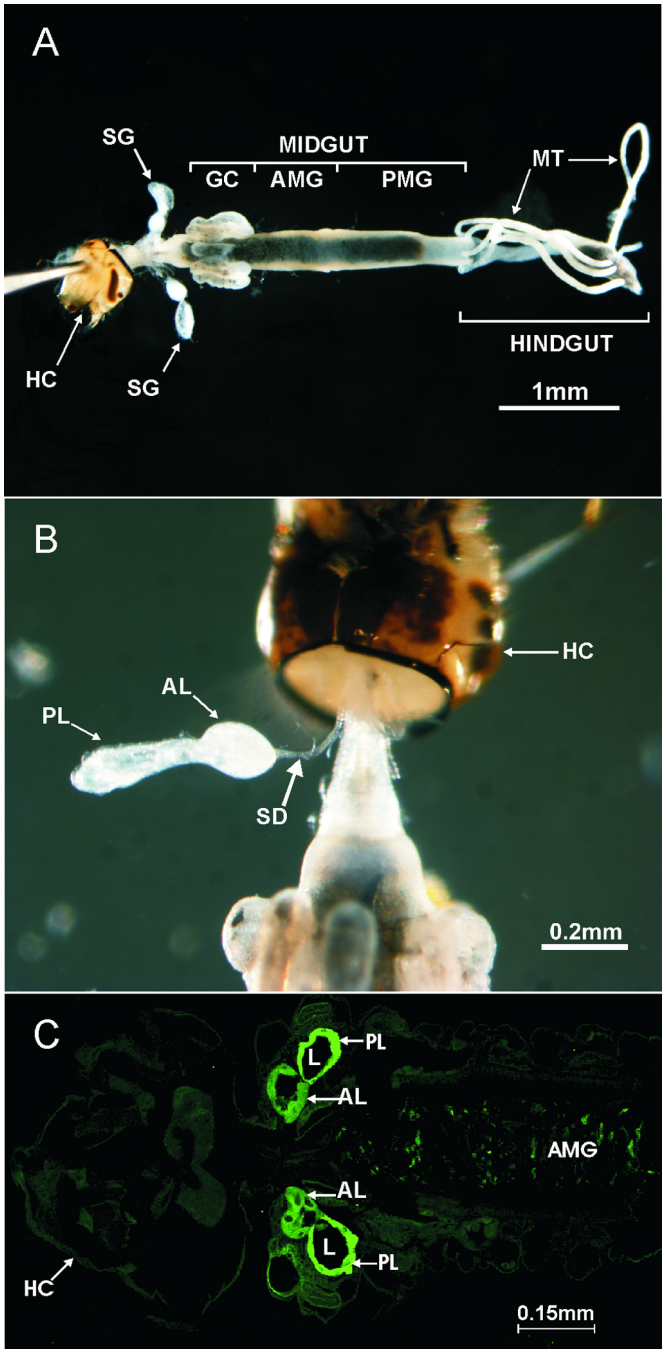

Fig. 1.

Location and structure of the salivary glands in the fourth-instar larva of *An. gambiae.* A) Micrograph showing the location of the salivary glands in relation to the digestive tract. Exoskeleton, fat body and other tissues were previously removed. B) External structure of the salivary gland's lobes and the salivary duct (only one gland shown). C) Internal structure of the salivary glands. The lumen of both lobes can be visualized in this laser confocal micrograph of a paraffin section labeled with FITC-conjugated *Erythrina cristagalli* lectin, which displays high binding affinity for the salivary gland tissue (shown in bright green). AL, anterior lobe. AMG, anterior midgut. GC, gastric caeca. HC, head capsule. L, lumen. MT, Malpighian tubes. PL, posterior lobe. PMG, posterior midgut. SD, salivary duct. SG, salivary gland.

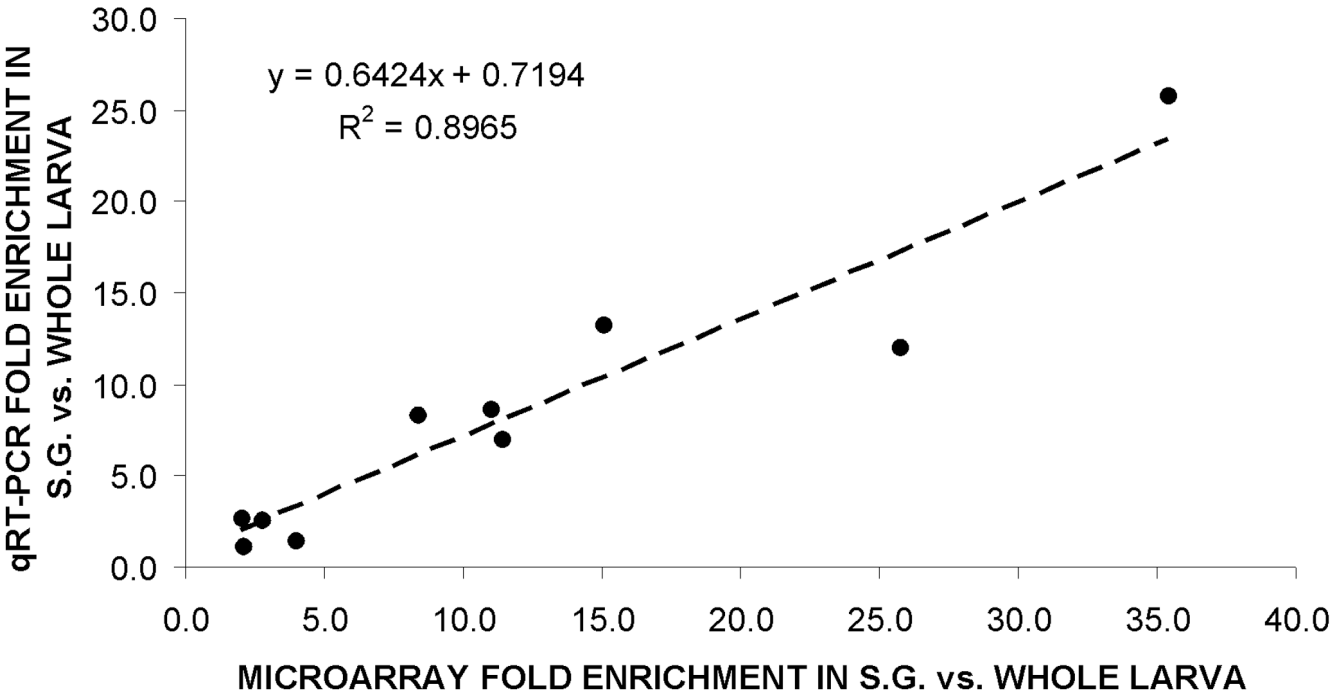

Fig. 2.

Validation of microarray data. A linear regression model (equation and dashed line) revealed a high degree of correlation (R^2>0.89) between results obtained by microarray analysis (x-axis) and qRT-PCR analysis (y-axis).

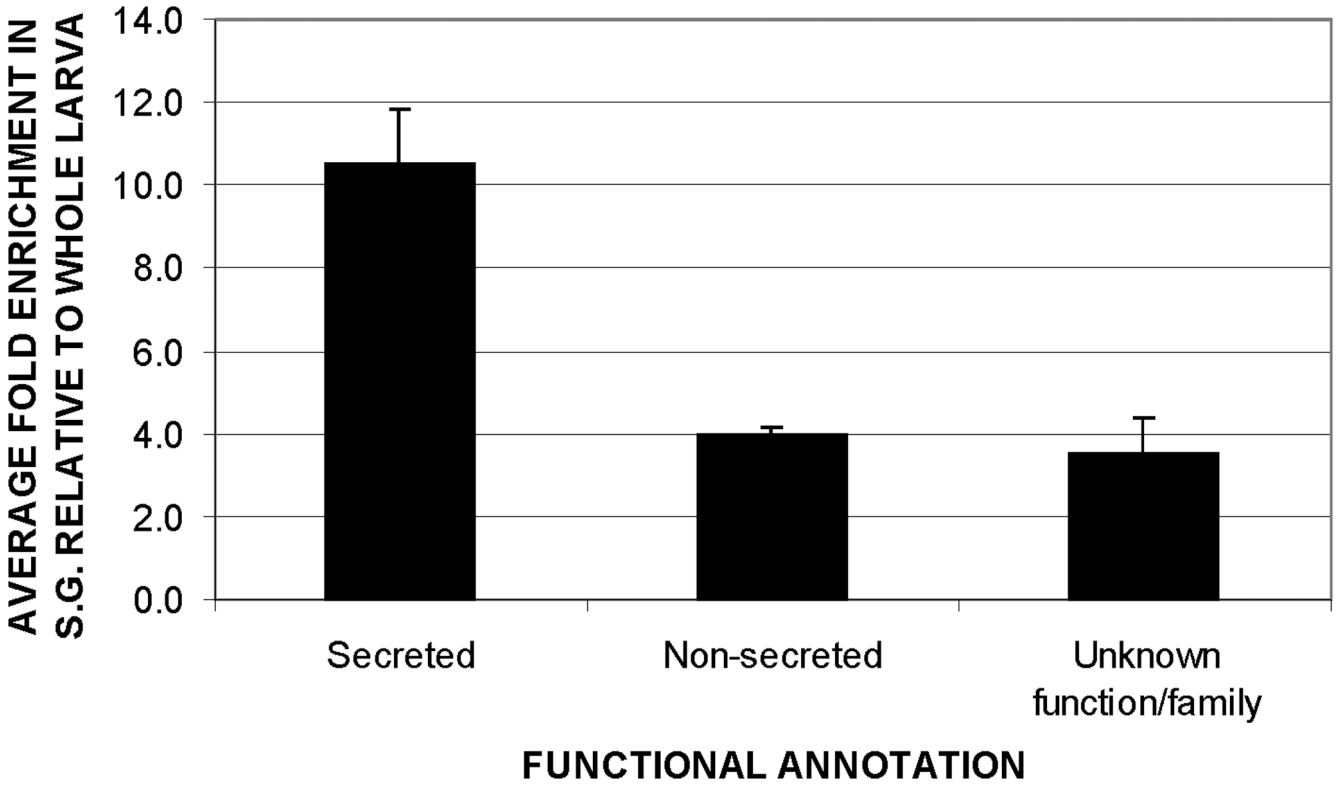

Fig. 3.

Relative abundance of transcripts encoding putative secreted, non-secreted and unknown proteins in the larval SG of *An. gambiae*, as suggested by microarray analysis. Transcripts encoding secreted proteins were 2.8 times more abundant than those encoding non-secreted proteins. Bar height represents the mean enrichment of transcripts within each functional group. Error bars show the standard error of the mean.

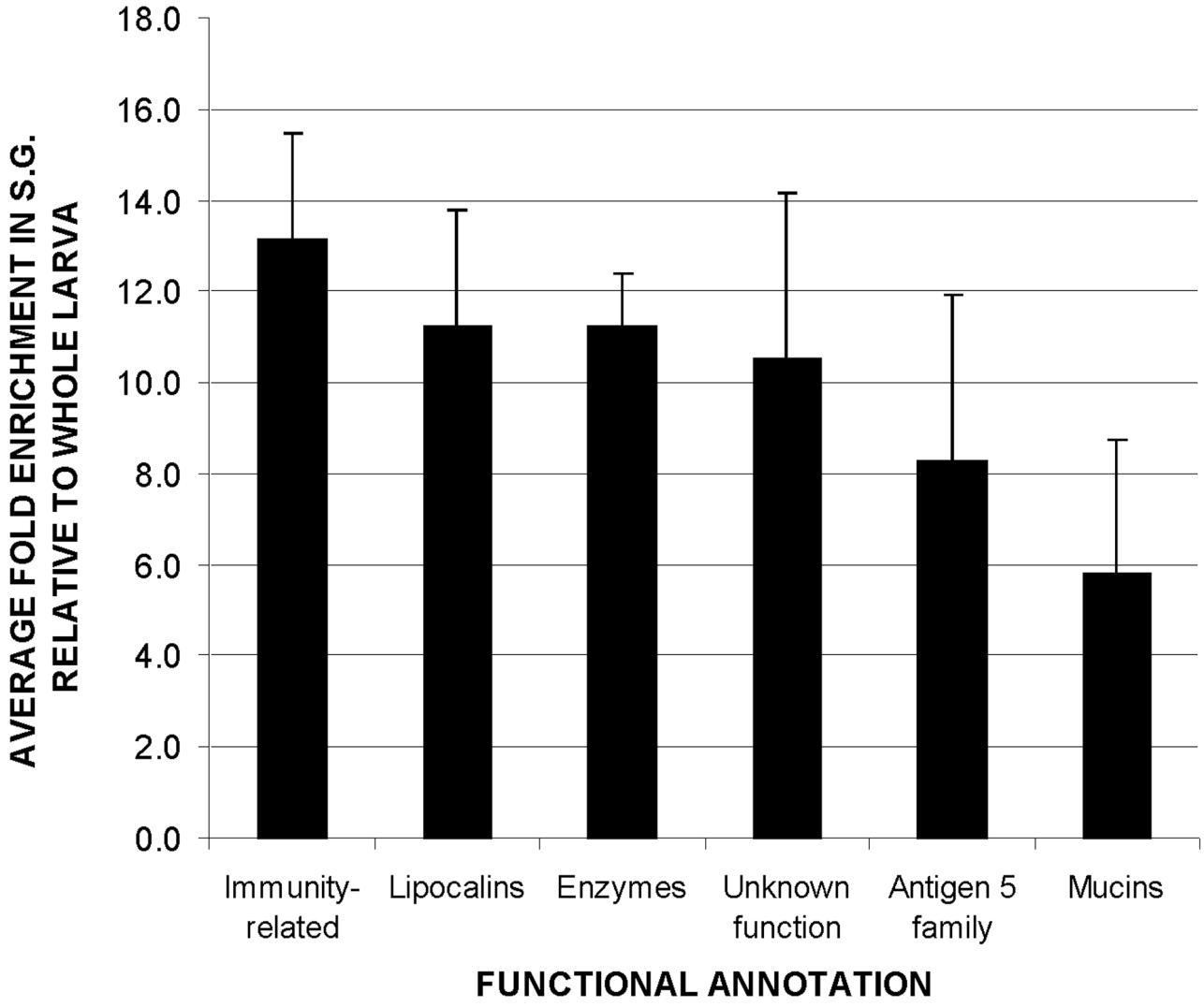

Fig. 4.

Relative abundance of transcripts associated with putative secreted proteins, grouped by functional annotation. Transcripts associated with immune functions were the most abundant, followed by lipocalins and digestive/detoxifying enzymes. Bar height represents the mean enrichment of transcripts within each functional group. Error bars show the standard error of the mean.

Fig. 5.

Nucleotide and deduced amino acid sequence of *AgDef5*. The first 23 amino acids (underlined) form a signal sequence, suggesting a secreted protein. The asterisk indicates the position of the stop codon.

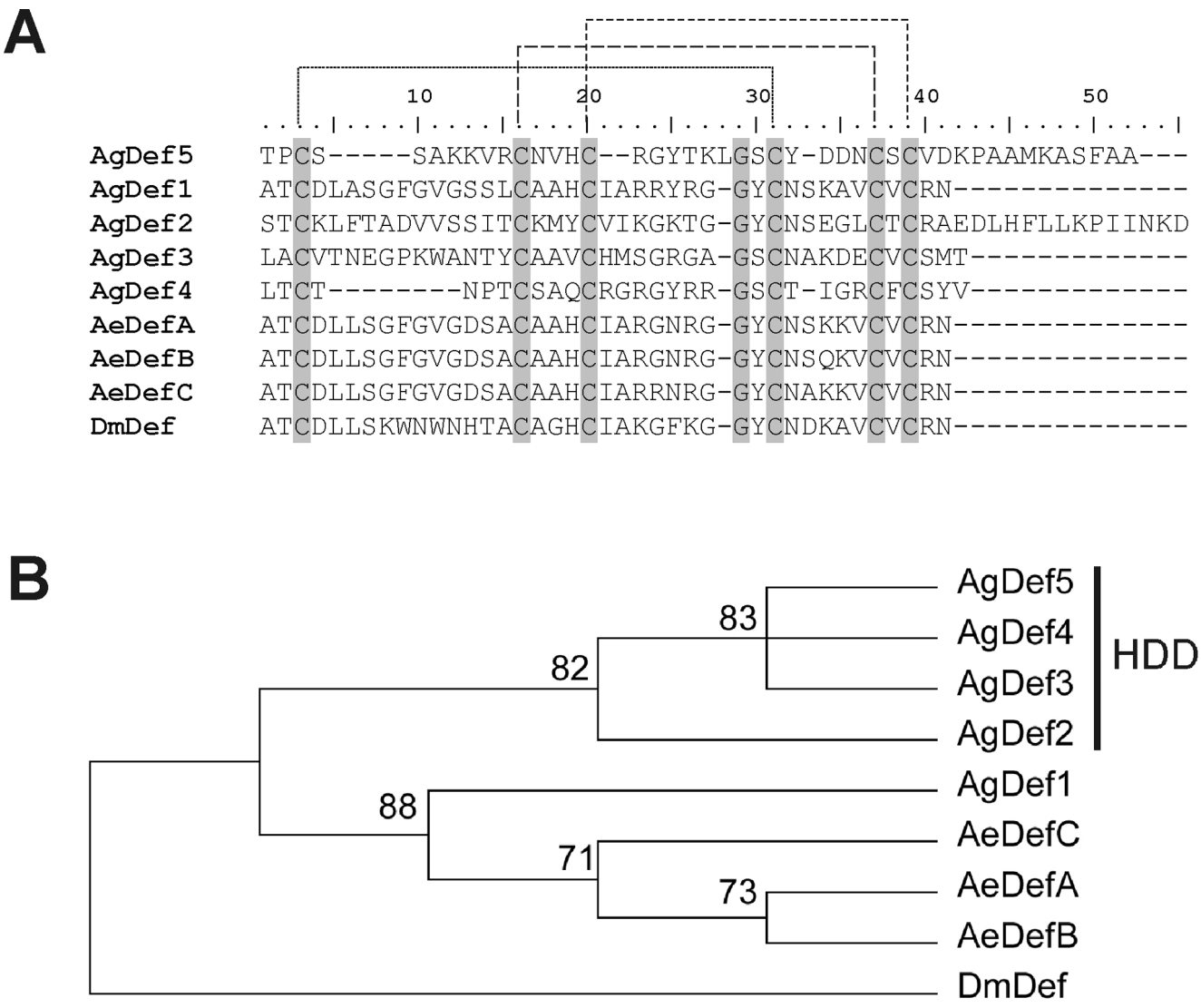

Fig. 6.

Comparison of the amino acid sequence of *AgDef5* with other known dipteran defensins. A) The predicted mature peptide sequence of *Anopheles gambiae AgDef5* is aligned with those of *Anopheles gambiae AgDef1-4* (ENSEMBL accessions AGAP011294-PA, AGAP004632-PA, AGAP007199-PA, AGAP005416-PA, respectively), *Aedes aegypti AeDefA-C* (ENSEMBL accessions AAEL003841-PA, AAEL003857-PA, AAEL003832-PA, respectively), and *Drosophila melanogaster DmDef* (ENSEMBL accession FBgn0010385). Conserved residues are shown in grey background; the peptide sequence of *AgDef5* contains the six conserved cystein residues characteristic of all insect defensins. Corresponding cysteins for disulfide bridge formation are connected with dashed lines. Homology annotation of these residues was performed using the known structure of sapecin from the flesh fly *Sarcophaga peregrina* (GenBank accession number P18313) as a model. B) Neighbor-joining phylogenetic analysis of dipteran defensin sequences using 100,000 bootstrap replicates and pairwise comparison. *AgDef5* is placed in the cluster of highly divergent defensins (HDD) formed by *AgDef3, AgDef4* and *AgDef2* (Christophides *et al.*, 2002). Numbers in the phylogram nodes indicate the percent bootstrap support. Overall tree topology was further confirmed using maximum parsimony analysis (data not shown).

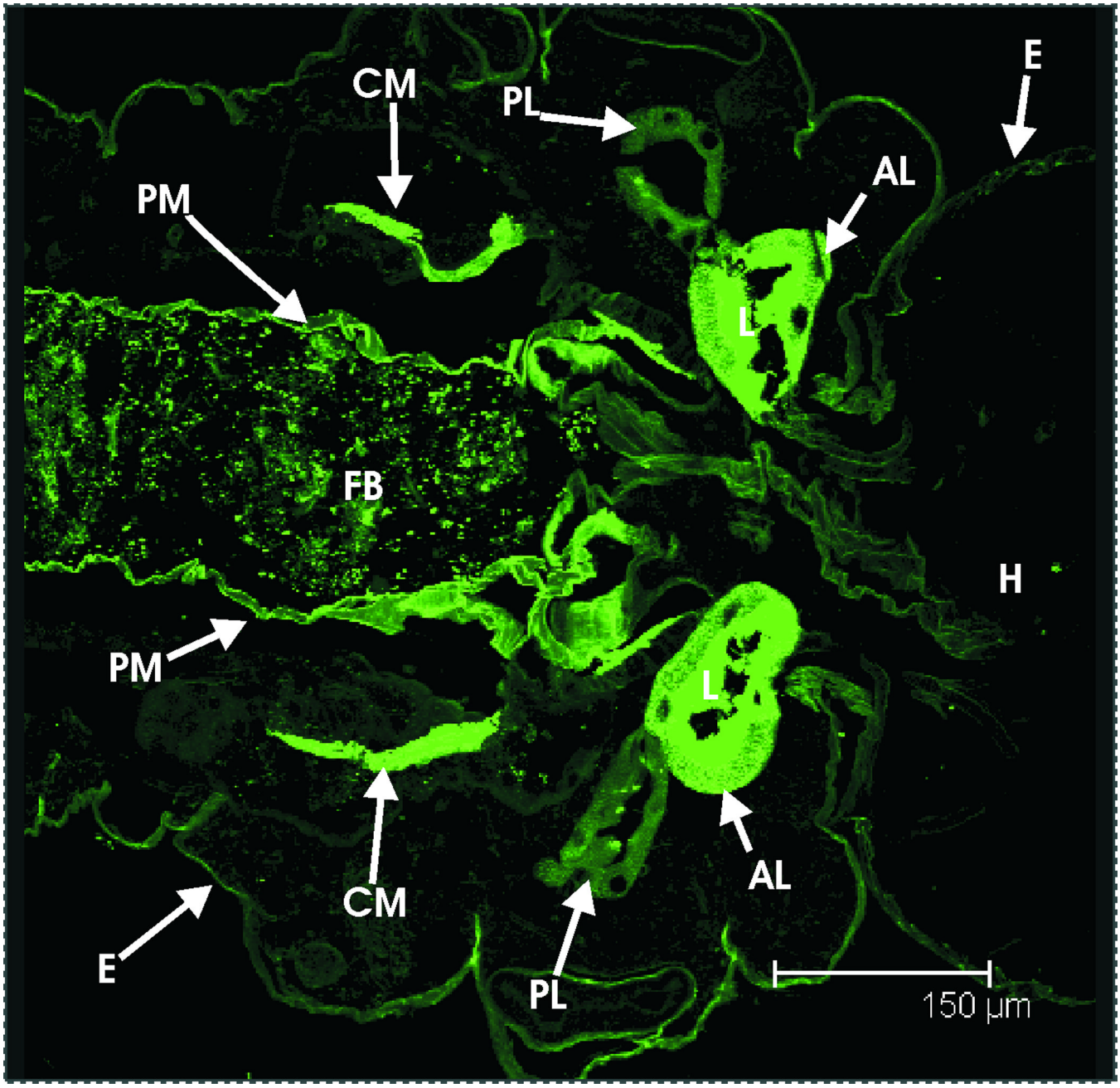

Fig. 7.

Histochemical detection of mucins in the larval SG. Confocal micrograph showing a section of the thorax of a 4th instar *An. gambiae* larva stained with fluorescently-labeled wheat-germ agglutinin. Clear staining can be seen in the anterior lobe of both salivary glands (lumen), as well as in the food bolus, caecal membranes and perithtophic membrane. AL, anterior lobe of salivary gland. CM, caecal membrane. E, exosqueleton. FB, food bolus. H, head. L, lumen of salivary gland's anterior lobe. PL, posterior lobe of salivary gland. PM, peritrophic matrix.

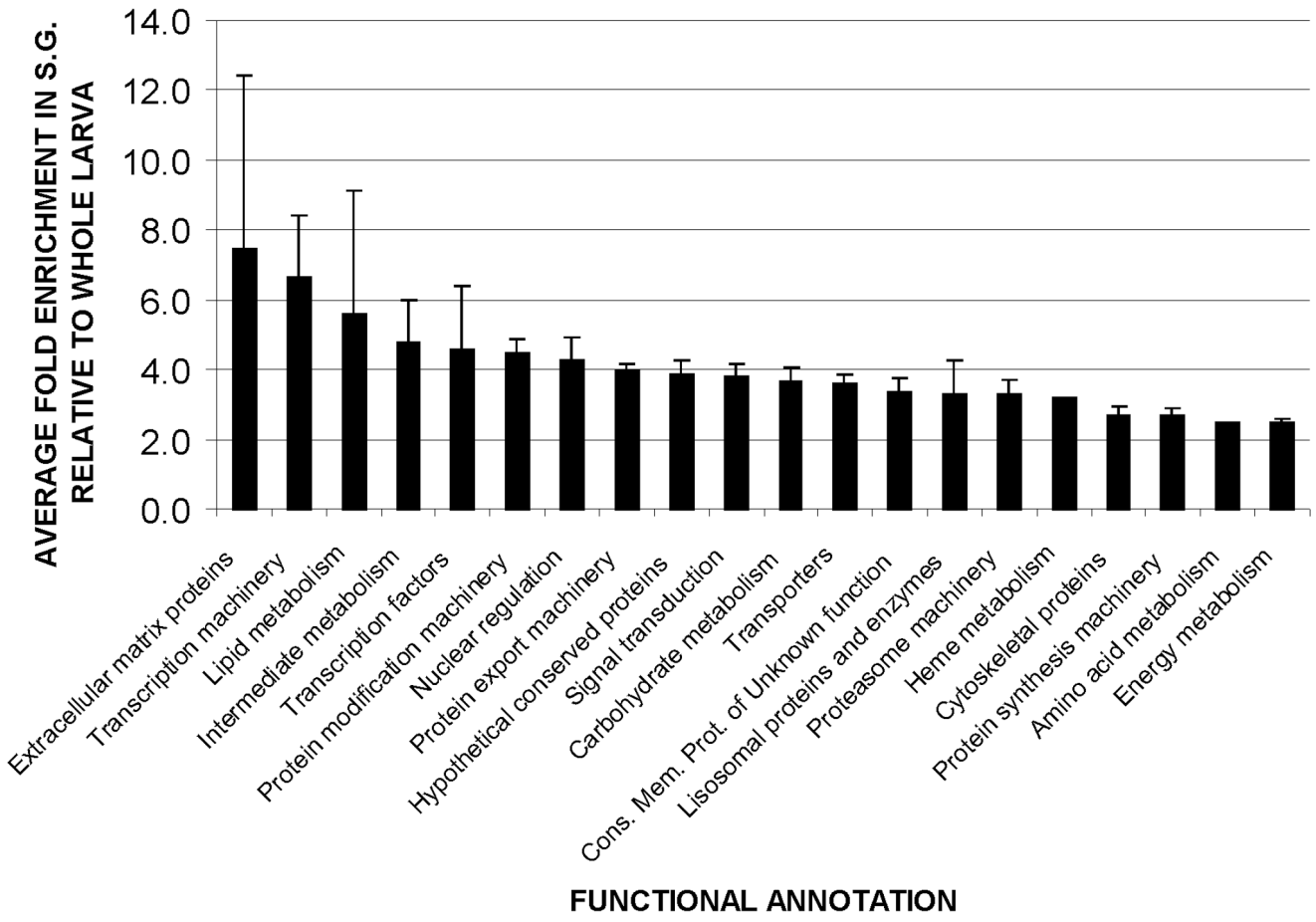

Fig. 8.

Relative abundance of transcripts associated with putative non-secreted proteins, grouped by functional annotation. Bar height represents the mean enrichment of transcripts within each functional group. Error bars show the standard error of the mean.

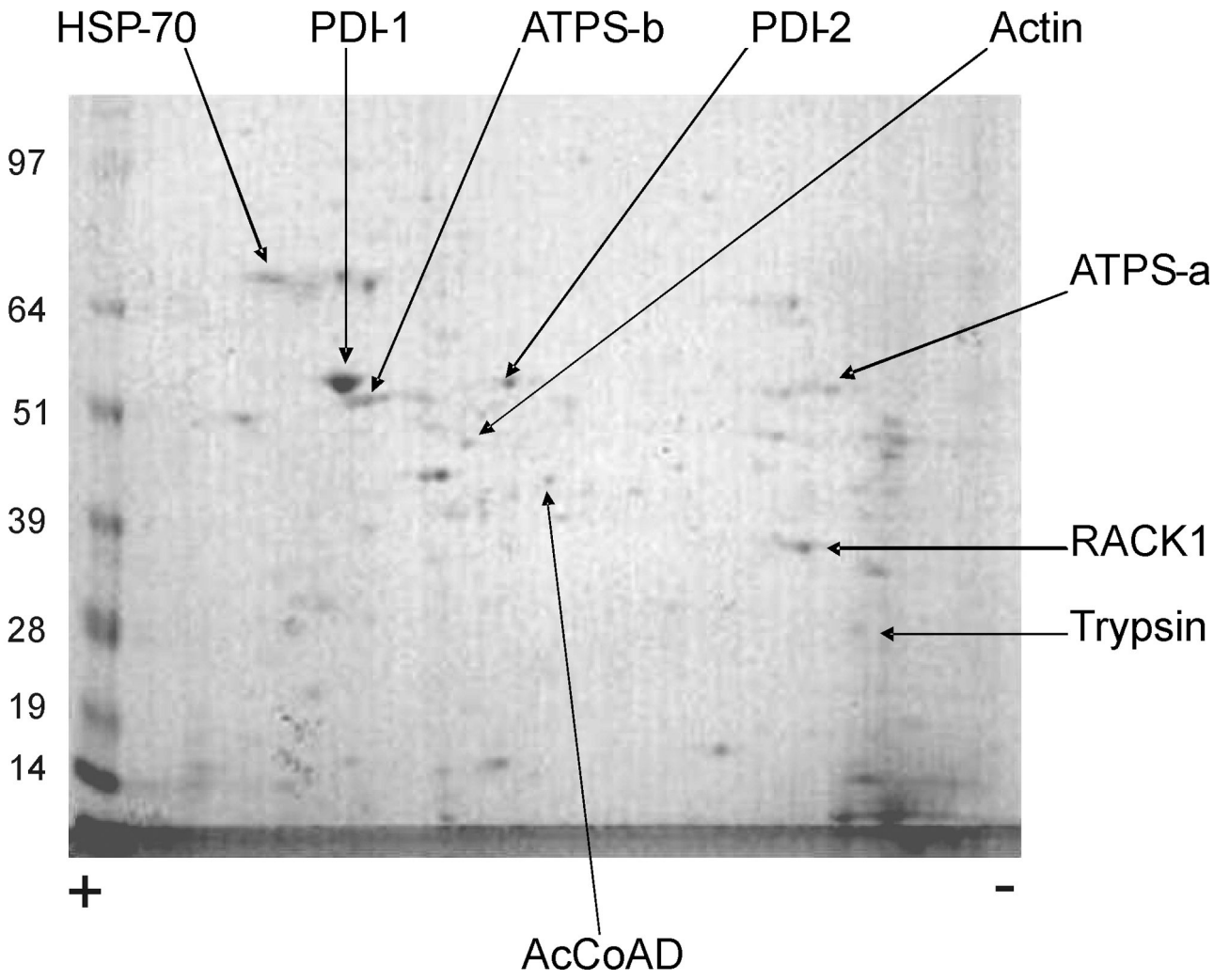

Fig. 9.

Two-dimensional gel electrophoresis of larval SG. Bands identified to a protein by triptic digestion / MS are shown. Numbers on the left correspond to molecular weight marker bands shown in the gel. The positive (+) and negative (−) signs below the gel mark the polarity of the isoelectrofocusing dimension. AcCoAD, acyl-coenzyme A dehydrogenase. ATPS-a, ATP synthase alpha subunit. ATPS-b, ATP synthase beta subunit. HSP-70, heat shock protein 70. PDI-1, protein disulfide isomerase 1. PDI-2, protein disulfide isomerase 2. RACK1, receptor for activated protein kinase c. ENSEMBL protein identifiers associated with each band are listed in the supplementary materials, table 3.

Table 1

Diversity of secreted gene products found to be enriched in the larval SG, grouped by functional annotation.

Functional category	No. of probes	No. of transcripts
Immune-related gene products		
Antimicrobial factors		
Defensins	2	3
Lysozyme	1	1
TIL-domain containing proteins	4	4
Pattern-recognition factors		
Fibrinogen-related proteins	1	3
Immune-related serine proteases	4	5
Total- immune related gene products	*12*	*16*
Mucins	3	3
Lipocalins	2	2
AG5 Family	3	3
Digestive and detoxifying enzymes		
Lipid digestion	2	2
Carbohydrate digestion	4	4
Protein digestion	1	1
Nucleotide digestion	1	1
Xenobiotic detoxification	1	3
Total- digestive and detoxifying enzymes	9	11
Gene products of unknown function		
Conserved family	3	3
Other	9	10
Total – gene products of unknown function	*12*	*13*

Table 2

Diversity of housekeeping gene products found to be enriched in the larval SG, grouped by functional annotation.

Functional category	No. of probes	No. of transcripts
Hypothetical conserved proteins of unknown function	36	43
Protein export machinery	35	37
Protein modification machinery	31	36
Signal transduction	28	35
Transporters	19	20
Conserved membrane proteins of unknown function	17	20
Carbohydrate metabolism	12	14
Proteasome machinery	10	10
Protein synthesis machinery	9	9
Transcription machinery	5	5
Lipid metabolism	5	6
Cytoskeletal proteins	5	8
Transcription factors	4	5
Nuclear regulation	4	4
Intermediate metabolism	3	3
Lisosomal proteins and enzymes	3	5
Energy metabolism	3	3
Extracellular matrix proteins	2	2
Heme metabolism	1	1
Amino acid metabolism	1	2

Table 3

Percent amino acid identity between putative salivary chitinases found in our study (AGAP000789-PA, AGAP005339-PA, AGAP005634-PA) and putatively immune chitinases previously reported from *An. gambiae* (AgBR1 and AgBR2).

	AgBR1	AgBR2	AGAP000789-PA	AGAP005339-PA	AGAP005634-PA
AgBR1	-	61.9%	5.8%	20.5%	17.9%
AgBR2		-	6%	20.9%	21.6%
AGAP000789-PA			-	5.1%	4.4%
AGAP005339-PA				-	25.6%
AGAP005634-PA					-