RESEARCH ARTICLE

Blood-feeding and immunogenic *Aedes aegypti* saliva proteins

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Mosquito-transmitted pathogens pass through the insect's midgut (MG) and salivary gland (SG). What occurs in these organs in response to a blood meal is poorly understood, but identifying the physiological differences between sugar-fed and blood-fed (BF) mosquitoes could shed light on factors important in pathogens transmission. We compared differential protein expression in the MGs and SGs of female *Aedes aegypti* mosquitoes after a sugar- or blood-based diet. No difference was observed in the MG protein expression levels but certain SG proteins were highly expressed only in BF mosquitoes. In sugar-fed mosquitoes, housekeeping proteins were highly expressed (especially those related to energy metabolism) and actin was up-regulated. The immunofluorescence assay shows that there is no disruption of the SG cytoskeletal after the blood meal. We have generated for the first time the 2-DE profiles of immunogenic *Ae. aegypti* SG BF-related proteins. These new data could contribute to the understanding of the physiological processes that appear during the blood meal.

Keywords:
Aedes / Animal proteomics / Blood meal / Dengue / Midgut / Salivary glands

1 Introduction

Dengue virus (DV) causes dengue fever and the more severe conditions dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The World Health Organization estimates that there are 50 million dengue infections every year worldwide. *Aedes aegypti* and *Aedes albopictus*, common vectors of DV, are both found in Thailand together in some areas where they pose a major risk of epidemic DHF [1]. An understanding of the mosquito's normal physiology could help with controlling its pathogenic state. Mosquitoes feed on sugar to sustain life, and the females also feed on blood to obtain the nutrients necessary to produce yolk proteins and eggs [2]; during such blood meals, pathogens can be transmitted from the insect vector to a vertebrate host. Pathogens such as DV pass through the insect's midgut (MG) epithelial cells into the lymph and thence to other organs. They ultimately reach the salivary gland (SG) where virus propagation takes place, with subsequent transfer to a vertebrate host at the next feeding.

The MG, a site of nutrient digestion and absorption, is also an important immune site, but the molecular mechanisms that prevent pathogen infection in mosquito MG is less understood [3]. It has also been proposed that the

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Abbreviations: BF, blood-fed; **DHF,** dengue hemorrhagic fever; **DPI,** days post infection; **DSS,** dengue shock syndrome; **DV,** dengue virus; **IF,** immunofluorescence; **MG,** midgut; **NL,** non-linear; **SF,** sugar-fed; **SG,** salivary gland

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infection barriers in the mosquitoes are the MG infection barrier, which prevents DV invasion of MG epithelial cells, and the MG escape barrier, which prevents the dissemination of DV to other tissues [4]. Currently, only the proteomic analysis of larvae *Ae. aegypti* MG brush border membrane vesicles is available [5, 6]. Knowing that only adult females can transmit pathogens, the MG protein profile of the adult females must be further characterized in order to understand the physiological function of this organ.

The mosquito SG is the last organ in the vector with which viruses are in contact before being inoculated into a vertebrate host, and SG-derived factors may enhance virus transmission. The SG plays an important role in food digestion as well as transmission of pathogens. Mosquito saliva is vital for successful blood feeding because it contains anticoagulant, anti-inflammatory, and immunosuppressive factors [7, 8]. It has also been demonstrated that apyrase, an enzyme affecting blood feeding by inhibiting platelet aggregation, was reduced in *Plasmodium berghei*-infected *Anopheles gambiae* mosquito SGs [9]. Saliva proteins are also antigenic and immunogenic, involving immunoglobulin E, immunoglobulin G, and T-lymphocyte–mediated hypersensitivity response [10]. The MG and SG proteomes of the *Ae. aegypti* adult female have not been extensively studied.

Proteomics, which enables investigating the translation of genomic information, offers an approach for studying the MG and SG proteomes of any mosquito species and for revealing the meal-associated global changes in protein expression in these organs. Here, we have used proteomics, first, to elaborate an *Ae. aegypti* protein map of MG and SG by using 2-D PAGE coupled to MS, and second, to compare blood-fed (BF) and sugar-fed (SF) mosquitoes to identify candidate proteins potentially involved in pathogen invasion of mosquitoes and transmission to vertebrate hosts. Thus, 2-D DIGE coupled with MS was employed to analyze and compare SG and MG proteomes of *Ae. aegypti* adult females to identify proteins with altered levels within these two organs between BF and SF female mosquitoes.

It has been reported that pre-exposure to blood-feeder bites or injection of saliva into the host generates a strong immune response to salivary proteins that affects the establishment of the pathogens delivered by the vector [11]. The protein profiling of immunogenic molecules of *Ae. aegypti* saliva is currently unknown. The immunogenicity of *Ae. aegypti* salivary proteins was estimated in our study by immunoblotting with IgM/IgG Dengue negative sera from *Ae. aegypti* bitten individuals.

2 Materials and methods

2.1 Mosquitoes and protein sample preparation

Ae. aegypti female mosquitoes (Liverpool strain) were reared and maintained at 26°C ± 0.5°C with 75–80% relative humidity and a 12:12 h (light:dark) photoperiod. For female mosquitoes, MG were dissected at 3 and 10 days after feeding in order to have early and late physiological status of the MG. Previous study have demonstrated that DV infection was detected in MG epithelial cells at 2 days post infection (DPI) and viral titers then increased until 10 DPI [12]. SG were sampled 10 days after feeding, a time frame corresponding to the extrinsic incubation period of DV, *i.e.* the time from initial infection of the mosquito to transmission [12]. In addition, Liverpool mosquitoes SG viral titer was detected at 10 DPI in our experiments (unpublished data). For the two groups of *Ae. aegypti* female : (i) fed exclusively on 10% sugar, *i.e.* "control" (SF) and (ii) fed with defibrinated human blood, the experimental group (BF), MG and SG were dissected in PBS and the tissues were subjected to three freeze–thaw cycles (liquid nitrogen) then centrifuged for 45 min at 4°C and 13 000 rpm before collection of the supernatant. Protein extracts were then desalted using a 2-D Clean-Up Kit (GE Healthcare, Germany). The Pellet was resuspended in IEF buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.8% IPG buffer, 0.2% Tergitol, 100 mM DTT) for 2-D PAGE or DIGE buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, and 40 mM Tris-HCl) for 2-D DIGE. Protein concentrations were measured using a 2-D Quant kit (GE Healthcare).

2.2 Protein map of *Ae. aegypti* MG and SG

2-DE was carried out with 100 μg of *Ae. aegypti* MG or SG protein extracts on 18 cm Immobiline™ DryStrips pH 3–11 non-linear (NL) (GE Healthcare). Strips were rehydrated for 10–12 h at 20°C with protein samples made up to 350 μL by adding IEF buffer 1.22% DeStreak (GE Healthcare). Run conditions were as follows: temperature at 20°C; current at 50 μA *per* strip; 300 V (gradient) for 5 min; 300 V (step) for 30 min; 5000 V (gradient) for 3 h and then 5000 V steps up to 60 000 Vh. 2-DE was performed on 12% SDS-PAGE gel at 40 V for 45 min and then at 45 mA/gel until the bromophenol blue front had reached the end of the gel.

2.3 2-D DIGE, image scanning, and statistical analysis

For each sample, 40 mosquitoes were dissected for SG and MG in triplicate from which 30 μg of proteins were used. For analytical 2-D DIGE, control and treated protein samples were compared using the CyDye™ DIGE Fluors for Ettan DIGE (Cy2, Cy3, and Cy5). Proteins were labeled according to the Ettan DIGE minimal labeling protocol (Ettan DIGE User Manual, GE Healthcare). For each sample, 20 μg of protein was labeled with 150 pmol of CyDye. An equal amount of each sample was labeled with Cy2 and added to each gel as an internal standard. The labeled samples were then pooled (according to the experimental design) and the sample volume was made up to

450 μL by adding rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, 40 mM Tris-HCl, 1% IPG buffer, and 1.2% DeStreak) prior to separation by IEF. IEF was performed with 24 cm Immobiline™ DryStrip, pH 3–10 NL. The run conditions were as followed: rehydration for 14 h at 20°C; current 50 μA *per* strip; 60 V (step) for 3 h; 1000 V (gradient) for 4 h; 8000 V (gradient) for 4 h; and 8000 V (step) until reaching total 64 000 Vh. 2-DE was performed on 12% SDS-PAGE gel at 15 mA/gel for 6 h and then at 30 mA/gel until the bromophenol blue front reached the end of the gel. Gels were scanned using a Typhoon™ 9400 imager (Amersham Biosciences). All gel images were acquired at 100 μm pixel resolution under nonsaturating conditions. 2-D DIGE images were analyzed using Progenesis SameSpots 3.1 software. Statistical analysis and protein quantification were carried out using the same software with a cut-off of 1.8-fold up- or down-regulated and ANOVA (p) < 0.05. Protein spots with a significant altered expression ($p < 0.05$) were trypsin digested and identified with MS.

2.4 Protein identification by MALDI-TOF MS

For 2-D PAGE protein map, the gels were stained with PageBlue™ protein Staining Solution overnight. After washes, visible spots were then excised manually in a laminar flow hood. For 2-D DIGE, two 2-DE gels were run with 100 μg of a mix of protein from the different samples for the MG and SG, and stained with CBB. Spots of interest in MG and SG were localized on the gel by comparing the CBB-stained spot pattern with the 2-D DIGE protein pattern. Spots were then excised. Enzymatic in-gel digestion and peptide spotting was performed automatically (Tecan freedom evo® proteomics) as previously described [13]. Briefly, protein spots were digested using 150 ng of trypsin, peptide extraction was performed using five sonication cycles of 2 min each and peptides were concentrated 1 h at 50°C; 0.5 μL of sample peptide and 0.5 μL of CHCA were deposited on a 384-well MALDI anchorship target using the dry-droplet procedure. Peptide sample were then desalted on target using a 10 mM phosphate buffer. Analyses were performed using an UltraFlex I MALDI TOF-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in the reflectron mode with a 26 kV accelerating voltage and a 50 ns delayed extraction. Mass spectra were acquired manually or in the automatic mode using the AutoXecute™ module of Flexcontrol™ (Bruker Daltonics) (laser power ranged from 30 to 50%, 600 shots). Spectra were analyzed using FlexAnalysis™ software (Bruker Daltonics) and calibrated internally with the autoproteolysis peptides of trypsin (m/z 842.51; 1045.56; 2211.10). Peptides were selected in the mass range of 900–3000 Da. Peptide Mass Fingerprint identification of proteins was performed by searching against the Insecta entries of either Swiss-Prot or TrEMBL databases (http://www.expasy.ch) and by using the MASCOT v 2.2 algorithm (http://www.matrixscience.com) with trypsin enzyme specificity and one trypsin missed cleavage [14]. Carbamidomethyl was set as fixed cystein modification and oxidation was set as variable methionine modification for searches. A mass tolerance of 50 ppm was allowed for identification. Matching peptides with one missed cleavage were considered as pertinent when there were two consecutives basic residues or when arginine and lysine residues were in an acidic context. MASCOT scores higher than 65 were considered as significant ($p < 0.05$) for Swiss-Prot and TrEMBL databases interrogation.

2.5 SG immunolabeling

Ten-day BF and SF SGs of *Ae. aegypti* females were dissected in PBS and fixed in 4% paraformaldehyde, PBS, pH 7.4, for 1 h. The tissue was then blocked for 30 min in 10% goat serum 0.3% Triton X-100 and incubated with tetramethyl rhodamine iso-thiocyanate-phalloidin for 2 h at room temperature. Hoechst dye was used to visualize the nucleus. Preparations were examined with a confocal microscope (Zeiss 5 Live Duo).

2.6 Immunogenicity test

SG proteins (80 μg protein) from BF mosquitoes were loaded on two Immobiline DryStrips (11 cm, pH 3–10 NL). Each sample was run in duplicate: one was stained with colloidal blue while the other was transferred to a PVDF membrane for Western blotting. The membrane was blocked in TBS Tween (0.1% v/v), milk (5% v/v) and incubated with a pool of serum from ten patients with DHF. An alkaline phosphatase–conjugated monoclonal mouse anti-human IgG was used as secondary antibody and detected using the Lumi-Phos™ WB Chemiluminescent substrate (Pierce). For reblotting with pooled sera from bitten and non-infected individuals, membranes were stripped as previously reported [15].

All serum samples were kindly provided by Miss. Akanitt Jittmittraphap, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University.

3 Results and discussion

3.1 Protein map of MG and SG on 2-D PAGE

The separation and identification of 10 days post feeding *Ae. aegypti* SF and BF pooled proteins revealed 220 MG protein spots and 102 SG protein spots for a pI scale of 3–11 and a Mw scale of 15–278 kDa. Proteins from each organ were classified according to function in the MG (Table S1, Fig. S1, Supporting Information) and SG (Table S2, Fig. S2, Supporting Information). The spot numbers in Tables S1 and S2 correspond to the MG and SG proteins shown in

Fig. S1 and S2, respectively. Almost all of the characterized MG proteins were classified as housekeeping types, *i.e.* cytoskeletal proteins; proteins involved in protein modification machinery, transcription machinery, and translation machinery; transcription factors; ribosomal proteins; proteasome machinery and proteolysis proteins; transporters, and many proteins implicated in energy generation and signal transduction. Proteins involved in metabolism were also characterized, *i.e.* in oxidant metabolism, carbohydrate metabolism, amino acid metabolism, lipid metabolism, and transport or intermediate metabolism. Two secreted protease inhibitors (serpins) were recognized: Q0IEW2_AEDAE and Q0IEW4_AEDAE. One phosphatidylethanolamine-binding protein was identified as a secreted carrier-like protein belonging to the D7 family. One enzyme involved in sugar digestion (α-amylase), one immunity-related protein (galectin) [16]; and 16 secreted and non-secreted proteins with unknown function were also detected in the MG. Similar types of housekeeping genes were expressed in the two organs, except that transcription factors, ribosomal proteins, proteasome elements, proteases, and proteins involved in signal transduction were not identified in SGs. However, this does not necessarily mean that these proteins were entirely nonexpressed in the SG; it is possible that differential expression levels exist in the two organs and there was a failure to detect the proteins in the SG. Other groups of protein were also identified in the SG such as secreted carrier-like proteins including D7 family, serpins, enzymes, immunity-related proteins, and proteins with unknown function. Furthermore, *Aedes*-specific proteins and proteins with unknown function found in hematophagous dipteran saliva were also detected on the SG 2-DE gels. In addition to a number of major, previously characterized SG proteins (D7 family, apyrase, serpin family, angiopoietin, antigen 5 protein, 30 kDa protein, F0F1 ATPase subunit, protein disulfide isomerase, adenosine deaminase, amylase, HSP70, and actin) [17], many other proteins that have never been characterized on 2-DE analysis of SG were detected (various proteins belonging to the apyrase, serpin, and D7 families as well as housekeeping proteins such as aconitase, paramyosin, saccharopine dehydrogenase, carbonic anhydrase, and myosin heavy chain). Spot numbers in Table S2 (Supporting Information) correspond to the SG proteins shown in Fig. S2 (Supporting Information). Among the identified proteins, we recognized four of them from another *Aedes* species (*Ae. albopictus*): (i) long form D7 salivary protein D7l2 (Q5MIW6_AEDAL), (ii) long form D7Bclu1 salivary protein d7l1 (Q5MIW7_AEDAL), (iii) salivary apyrase (Q5MIX3_AEDAL), and (iv) serpin protein (Q5MIW0_AEDAL). Such homologies would be expected between two mosquitoes of the same subgenus [17, 18]. A comprehensive understanding of the normal MG and SG proteomes could be useful when it comes to pathogenesis, which is why we investigated the differential expression of protein by 2-D DIGE in each organ with regard to diet.

3.2 Differential protein expression between SF and BF groups in MG and SG

These results extend our understanding of the 2-D profile of salivary proteins and provide the first comparison by 2-D DIGE of the level of SF and BF protein expression in female *Ae. aegypti* mosquitoes at 3 and 10 days post feeding for MG and 10 days for SG. After detection of protein spots, image gels were ''cleaned off'' for speckling and artefact spots. In total, 2000 spots were detected on the MG gel (Fig. 1A and Fig. S3, Supporting Information) and 706 on the SG gel (Fig. 1B). The MG protein profile is more complex than the SG one, considering the number of proteins present in each organ (see Fig. 1). No differences were detected between the MG profiles of SF and BF mosquitoes at 3 and 10 days after feeding (Table S3 and S4, Supporting Information). One of the most important parameters in the 2-DE technique is the quantity of proteins loaded onto each gel (which must be the same). There may have been some differences but not in these p*I* and Mw ranges. It is also well known that the molecular weight of proteins detected in 2-DE experiments is generally limited to the range of 150–15 kDa so very high and low molecular weight proteins as well as peptides are missed. Hamilton *et al.* [19] used ELISA to show that the level of MG defensin, an antimicrobial peptide, increased up to 40-fold in response to a blood meal but not to a sugar meal. However, in the SG, 84 protein spots were detected as being differentially expressed: 37 spots were denser in the BF insects and 47 spots were weaker (Fig. 1B). The absence of any difference in global protein profile suggests that all these proteins are normally expressed in each organ but the nature of the ingested meal can modulate their expression levels.

A total of 32 protein spots were characterized in *Ae. aegypti* SG: 17 were up-regulated and 15 down-regulated in the BF group compared with the SF group (Table 1, Fig. 1B). From the 17 spots that were up-regulated, 13 proteins could be identified (see Table 1). Among the up-regulated SG proteins, three D7 proteins were highly expressed in BF SG: long form D7 salivary protein D7l2 (Q5MIW6_AEDAL), D7 protein, and putative (Q0IF93_AEDAE) and D7 protein precursor (D7_AEDAE). The long form D7 salivary protein D7l2 is one type of *Ae. albopictus* D7 protein that is best matched with the 37 kDa SG allergen Aed a 2 (D7 protein precursor: ALL2_AEDAE) of *Ae. aegypti* D7 protein. The D7 protein family is related to odorant-binding proteins that are able to bind host biogenic amine to antagonize vasoconstictor, platelet-aggregating, and pain-inducing properties [20]. Additionally, the long form D7Bclu1 salivary protein d7l1 (*Ae. albopictus* protein), also identified in our protein map, matches best with a putative D7 protein, Q0IF93_AEDAE. These similarities were discussed above in the part of protein map. Aldehyde dehydrogenase possesses oxidoreductase activity that catalyzes the NAD^+-dependent oxidation of aldehyde to carboxylic acid, and this ubiquitous enzyme is related to

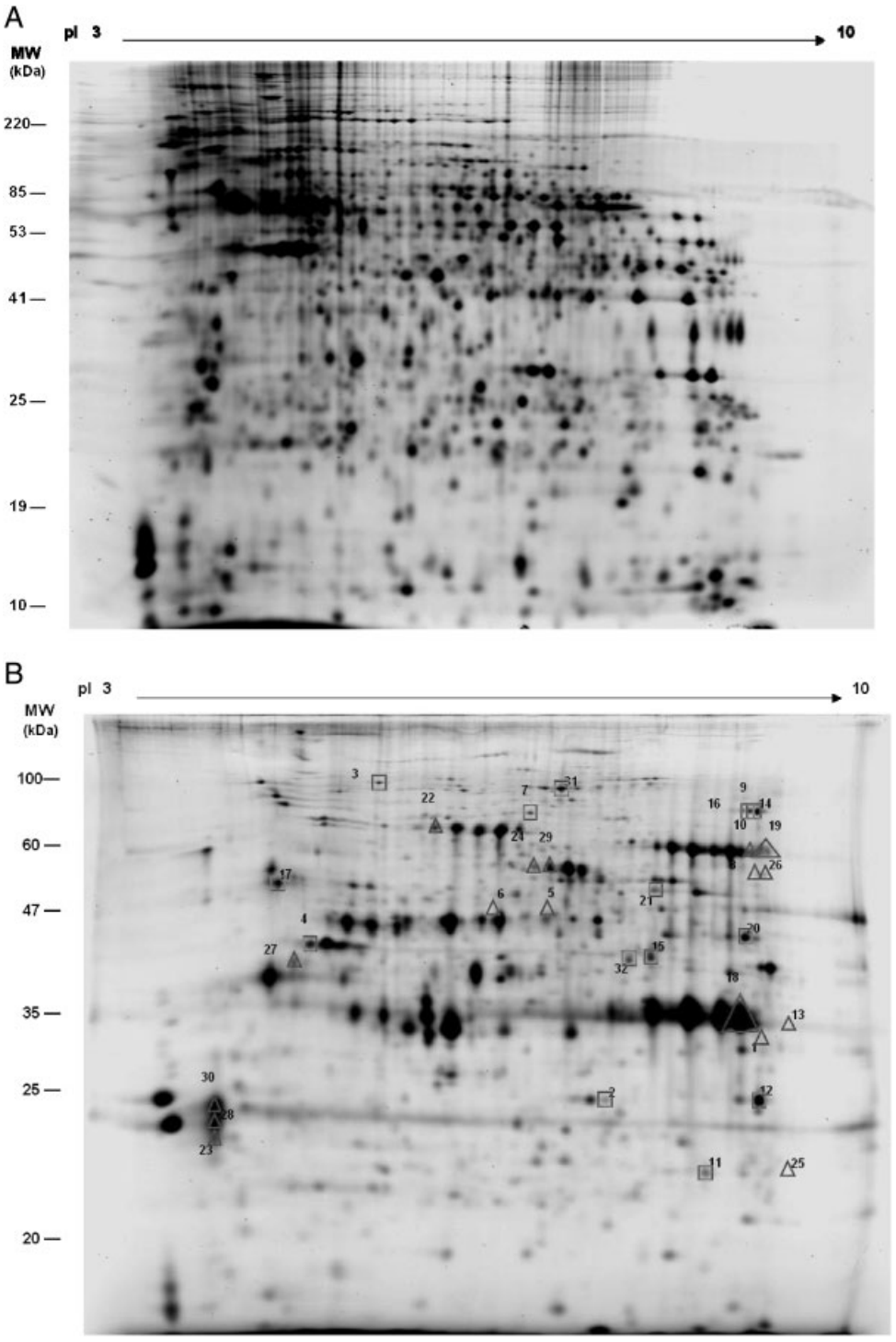

Figure 1. 2-D DIGE synthetic gel of *Ae. aegypti* MG (day 3) (A) and SG (day 10) (B) proteins for the SF and BF groups with a p*I* scale of 3–10. The molecular weight scales are indicated in the figures. Protein spots differentially expressed in MG and SG are indicated by number and symbols: SF (□), BF (Δ).

energy metabolism [21]. Fry *et al.* [22] demonstrated that aldehyde dehydrogenase converts toxic acetaldehyde to the nontoxic acetate. Adenosine deaminase was also up-regulated in SG after a blood meal. This enzyme hydrolyzes adenosine to inosine and ammonia, and the removal of adenosine may inhibit platelet aggregation and mast cell degranulation. Inosine interferes with host coagulation at the bite site to inhibit the production of inflammatory cytokines. All these processes may enhance blood feeding [23–25]. Salivary serpin putative anticoagulant (Q5MIW0_AEDAL: *Ae. albopictus*–specific protein) was expressed 3.5 times more in BF SG than SF SG. The serpin superfamily is reported to inhibit endogenous proteases like the serine proteases involved in regulating coagulation, such

Table 1. Differential protein expression between SF and BF groups in Ae. aegypti salivary gland

Spot number	ANOVA (*p*-value)	X-fold average	ANV[a]		Accession no.	Name of protein[b]	MASCOT score[c]	Coverage (%)
			Sugar	Blood				
Up-regulated								
1	0.002	8.1	0.188	1.528	Q5MIW6_AEDAL	Long form D7 salivary protein D7I2	73	22
5	9.96E-04	3.7	0.46	1.715	Q16P57_AEDAE	Aldehyde dehydrogenase	85	18
6	0.003	3.5	0.461	1.626	Q5MIW0_AEDAL	Salivary serpin putative anticoagulant	98	24
8	0.001	3.1	0.45	1.375	Q5MIX3_AEDAL	Salivary apyrase	142	30
10	0.002	2.9	0.506	1.487	APY_AEDAE	Apyrase precursor	260	48
13	5.89E-04	2.7	0.595	1.602	Q0IF93_AEDAE	D7 protein, putative	69	17
18	1.66E-04	2.2	0.762	1.701	D7_AEDAE	D7 protein precursor	83	30
19	4.43E-04	2.2	0.651	1.435	APY_AEDAE	Apyrase precursor	253	42
22	0.002	2.1	0.694	1.439	Q17NC3_AEDAE	Putative uncharacterized protein	203	40
23	0.008	2.1	0.966	1.987	Q58HB7_AEDAE	Putative 19.6 kDa secreted protein precursor	90	23
24	8.04E-04	2.0	0.693	1.395	Q179D4_AEDAE	Adenosine deaminase	142	31
25	0.004	2.0	0.635	1.254	Q8T9W0_AEDAE	Putative secreted protein	59	27
26	0.006	2.0	0.657	1.282	Q5MIX3_AEDAL	Salivary apyrase	152	31
27	6.71E-05	1.9	0.792	1.53	Q8T9V0_AEDAE	Putative secreted protein	76	23
28	0.009	1.9	0.91	1.685	Q8T9T8_AEDAE	Putative 30 kDa allergen-like protein (putative uncharacterized protein)	81	28
29	6.16E-04	1.8	0.749	1.334	Q179D4_AEDAE	Adenosine deaminase	159	29
30	0.019	1.8	0.904	1.591	Q58HB7_AEDAE	Putative 19.6 kDa secreted protein precursor	90	23
Down-regulated								
2	0.009	7.5	2.482	0.331	Q16QU2_AEDAE	Putative uncharacterized protein	72	23
3	7.05E-05	6.5	2.124	0.327	Q16RF4_AEDAE	Paramyosin, long form	169	20
4	7.28E-04	4.5	2.181	0.49	ACT1_AEDAE	Actin-1	57	21
7	4.91E-04	3.2	1.975	0.617	Q16LR5_AEDAE	NADH-ubiquinone oxidoreductase 75 kDa subunit	149	23
9	0.003	3.0	1.772	0.582	Q16KR4_AEDAE	Aconitase, mitochondrial	255	35
11	0.004	2.9	1.617	0.558	Q179J9_AEDAE	Mitochondrial ATP synthase b chain	70	33
12	0.002	2.9	1.795	0.622	Q1HR57_AEDAE	Mitochondrial porin	160	42
14	0.012	2.4	1.698	0.718	Q16KR4_AEDAE	Aconitase, mitochondrial	255	35
15	0.001	2.3	1.707	0.749	Q17AK0_AEDAE	Ubiquinol-cytochrome *c* reductase complex core protein	131	34
16	0.011	2.3	1.631	0.72	Q16KR4_AEDAE	Aconitase, mitochondrial	255	35
17	0.003	2.2	1.68	0.748	Q17FL3_AEDAE	ATP synthase subunit β	266	45
20	0.008	2.1	1.675	0.781	Q17GM7 CISY1_AEDAE	Probable citrate synthase 1, mitochondria	136	35
					Q16P20 CISY2_AEDAE	Probable citrate synthase 2, mitochondrial precursor	134	35
21	0.003	2.1	1.555	0.746	Q16LP4_AEDAE	Pyruvate kinase	123	22
31	8.18E-04	1.8	1.38	0.786	Q16FJ9_AEDAE	Saccharopine dehydrogenase	127	19
32	0.002	1.8	1.467	0.837	Q16GA1_AEDAE	Acyl-CoA dehydrogenase	202	40

a) Average normalized volume.
b) Accession number in Swiss-Prot and TrEMBL protein databases (http://www.expasy.org/).
c) MASCOT scores >50 indicate significant identity or extensive homology.

as thrombin and factor Xa [26]. In addition, a serine protease cascade is one innate insect immune response that activates prophenoloxidase in plasma [27]. *Drosophila* serpins have been shown to be inhibitory and involved in regulating Toll and prophenoloxidase signaling pathways [28]. We also observed the up-regulation of salivary apyrase and its precursor in BF SG. Two spots of differentially expressed salivary apyrase (Q5MIX3_AEDAL) were identified as

Ae. albopictus specific: they were found at levels 2–3.1 times higher in BF SG compared with SF one. Apyrase is an enzyme that inhibits ADP-dependent platelet aggregation during blood-feeding [18] and prevents neutrophil activation [29]. Apyrase is synthesized in the adult female SG and accumulates in the distal lateral lobes [30]. Smartt *et al.* [31] have shown that apyrase protein levels peak in the SG about 4 days after adult emergence and remain high after a blood meal. A putative 30 kDa allergen-like protein (accession number UniProtKB/TrEMBL: Q8T9T8_AEDAE) previously identified as a 30 kDa antigen (gi: 18568322) by Ribeiro *et al.* [17], a species of unknown function, is ubiquitously found in the SGs of adult mosquitoes [32]. Studying saliva allergens could provide valuable information on immune responses, with a view to developing new diagnostic tests for allergies to mosquito bites [10, 33, 34]. We identified four secreted proteins: Q8T9V0_AEDAE, which binds a receptor involved in signal transduction; a 19.6 kDa secreted protein precursor (Q58HB7_AEDAE); a putative secreted protein (Q8T9W0_AEDAE); and a putative uncharacterized protein (Q17NC3_AEDAE). The functions of the last three proteins are unknown. Almost all the proteins that were up-regulated in BF SG seem to have an important role in successful blood feeding.

The proteins that were down-regulated in BF group (up-regulated in SF mosquitoes) tended to have housekeeping functions (Table 1): paramyosin, long form, and actin-1 are cytoskeletal proteins. Paramyosin is a component of the thick filament core-regulating complex of invertebrate muscle [35]. Actin, the main protein of muscle and cytoskeleton [36], is the major component of thin filament in muscle. It is also one of three subclasses of the cytoskeleton (microfilament, intermediate filament, and microtubule) found in the cytoplasm of all cells. It plays important roles in cellular dynamics such as the maintenance of cell shape, cell growth, cellular motion, intracellular transport, and cell division and differentiation. Differential actin expression of actin was also studied by immunofluorescence (IF) and will be discussed below in detail in the section of IF assay. Many proteins that were up-regulated in the SG of SF *Ae. aegypti* have a function in metabolism, ion transport, and energy generation. Mitochondrial porin and ATP synthase subunit β are housekeeping products involved in anion and hydrogen ion transport, respectively. Porins are major channel-forming proteins found in the mitrochondrial outer membrane. All metabolites as well as ATP and ADP pass through these channels [37]. Mitochondrial aconitase, mitochondrial ATP synthase β chain, NADH-ubiquinone oxidoreductase 75 kDa subunit, ubiquinol–cytochrome *c* reductase complex, pyruvate kinase, and probable citrate synthase 1 and 2 are enzymes involved in the cell's energy-generating machinery. Oxidative phosphorylation occurs at the inner membrane of the mitochondrion depending on the relative proportions of NADH, oxygen, ATP, ADP, and inorganic phosphate [38]. Both pyruvate kinase and citrate synthase are involved in glycolysis [39] and the tricarboxylic acid cycle [40]. Saccharopine dehydrogenase plays a role in conversion of lysine to glutamate. Two enzymes of lysine catabolism are synthesized from the lysine-ketoglutarate reductase/saccharopine dehydrogenase gene and this bifunctional enzyme is conserved from plants through animals [41, 42]. Acyl-CoA dehydrogenase catalyzes the initial mitochondrial step in the β-oxidation fatty acid cycle [43]. Apart from pyruvate kinase, all these proteins are mitochondria-specific [44] and their preferential expression in SF mosquitoes points to their role in basic metabolism. One uncharacterized protein (Q16QU2_AEDAE) of unknown function showed highly differential protein expression.

Two groups of proteins were distinct: those proteins that were up-regulated in SF mosquitoes seemed to be specific to the maintenance of physiological functions, whereas those that were up-regulated in BF mosquitoes were proteins that facilitate the process of blood-feeding. All those sequences that up-regulated in BF mosquitoes contained a cleavage site [45] meaning that they were secreted proteins in saliva. This characteristic confirms their important role in facilitating blood feeding when the mosquito takes a blood meal. We suggest that the production of these molecules is strongly stimulated after mosquitoes have had their first blood meal because they are only weakly expressed when mosquitoes are feeding exclusively on sugar.

To supplement our data on the effect of blood feeding on the expression of the SG proteome, it would be interesting to do a survey on the less-abundant salivary proteins recently identified [46].

3.3 IF assay

As described above in the section of differential protein expression between SF and BF groups in MG and SG, actin

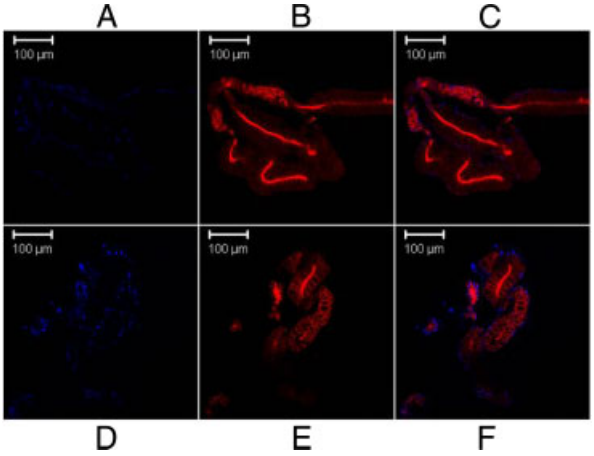

Figure 2. Expression of actin in *Ae. aegypti* SG for SF and BF groups. Actin was detected in red by IFA at 10 days post sugar meal (A–C) or blood meal (D–F). Nuclei were marked with Hoechst in blue. Similar results were observed in three separate experiments.

and paramyosin were more highly expressed in SF SG (Table 1). These proteins are constituents of the cytoskeleton and muscle fibers. The disruption of cytoskeletal links leads to changes in cell shape [47]. We hypothesized that blood-feeding probably modifies the cytoskeleton and/or the shape of SG cells. For these reasons, SG morphology was checked by IF. Whole SG from BF and SF mosquitoes were dissected out, actin expression was labeled using tetramethyl rhodamine iso-thiocyanate-phalloidin, and nuclei were stained with Hoechst dye (Fig. 2). Actin seemed to localize to the cell boundary and along the duct of each lobe in both SF and BF SG. Nuclei were located apically. No difference was observed between the two SG groups morphology, condition, or cytoskeletal organization. These results suggest that after a blood meal there is no disruption of the SG cytoskeletal.

3.4 Immunogenic proteins

The major risk of DHF is exacerbation to fatal DSS. The major pathophysiologic hallmarks that determine disease severity and distinguish DHF from dengue fever are plasma leakage (as a result of increased vascular permeability) and abnormal coagulation. The mechanisms that lead to DHF/DSS are a subject of intense debate [48–52]. The possible role of mosquito saliva in these mechanisms has never been evaluated. Remoue *et al.* [53] demonstrated that some salivary proteins are immunogenic and can initiate a specific antibody response. In addition, the anti-saliva antibody response could be a reliable marker of exposure to vector-borne diseases in individuals exposed to bites of arthropod vectors [33, 54, 55]. As described for maxadilan, which enhances *Leishmania* infection [56, 57], the SG molecules detected by serum of DHF patients may enhance virus infection so they could constitute vaccine targets. In this study, we identified for the first time the immunogenic salivary molecules of *Ae. aegypti*, with sera of DHF patients and bitten non-infected individuals (Fig. 3). Within the BF SG proteins of *Ae. aegypti*, 15 immunogenic proteins were identified in our study: heat shock cognate 70, ATP synthase subunit β vacuole, putative adenosine deaminase, salivary apyrase and its precursor, putative serpin, salivary anti–FXa serpin, fibrinogen and fibronectin, angiopoietin-like protein variant, putative secreted salivary 34 kDa family, D7 protein precursor, long form D7Bclu1 salivary protein d7l1, and two putative uncharacterized proteins (62kDa, Q17NC2_AEDAE and Q17F11_AEDAE). These results show that there is a strong immune response to *Ae. aegypti* salivary proteins. The probing of salivary proteins blot with sera from bitten non-infected individuals shows that there is few group of proteins (see green circle on Fig. 3C) that are not revealed as immunogenic in DHF patients. This protein spots are in lesser quantity; thus to identify them it will be necessary to develop a purification protocol to recuperate enough protein volume for each candidate spots to identify them with MS and to assert their potential function in dengue infection.

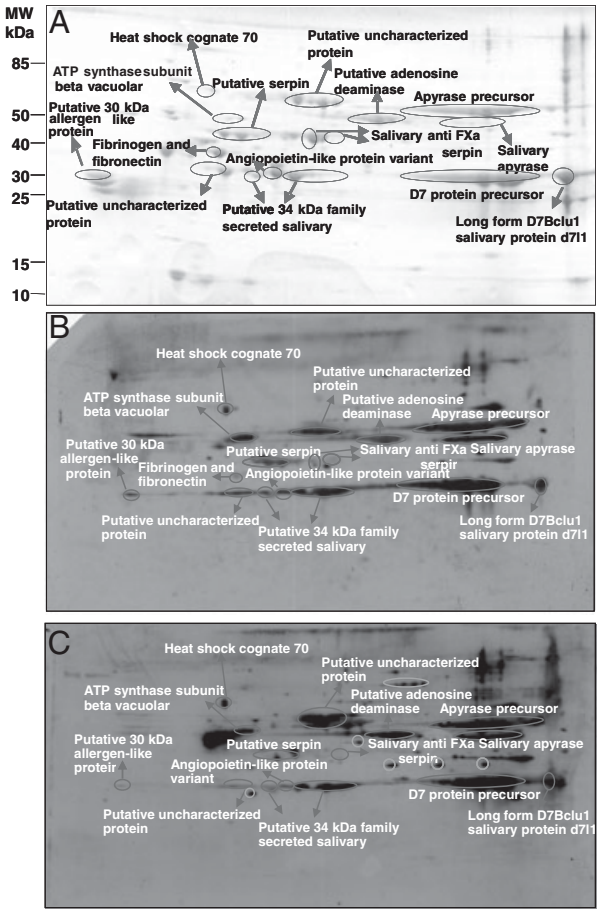

Figure 3. Immunogenic proteins of *Ae. aegyti*. 2-DE followed by Western blot of BF SG extracts of *Ae. aegypti* was carried out on Immobiline DryStrips 11 cm, pH 3–10. (A) The gels were stained with PageBlue protein staining, and (B) IgG responsive proteins on PVDF membrane were detected with the pool of ten DHF patients' sera. (C) The immunoblot was then stripped and reblotted with the pool of ten *Ae. Aegypti*-bitten and non-infected individuals'sera. Light circles represent proteins that are not immunogenics in DHF patients. Similar results were observed in two separate experiments.

4 Concluding remarks

Although 2-DE offers a high-quality approach for studying the host and/or parasite proteomes, only a very little part of the total proteome of a tissue or a cell can be seen. For instance, the mosquito genes could encode at least 61 421 non-redundant proteins (see http://www.ebi.ac.uk/GOA/proteoms.html). For tools as 2-DE and 2-D DIGE, the number of proteins revealed is limited (*i.e.* ~1500–2000 protein spots) [58]. Thus, this study is a preliminary step towards understanding the role of MG and SG proteins in

physiological processes that appear during the blood meal. In this paper, we presented the first differentially expressed *Aedes* SG proteins analysis by 2-D DIGE relative to blood feeding. Additionally, we completed the protein map of *Ae. aegypti* MG and SG. We have also for the first time identified *Ae. aegypti* saliva proteins that exhibit immunogenic properties in *Ae. aegypti*-bitten individuals. These proteins could play a role in DV infection or could be epidemiological indicators of exposure to dengue. Understanding the interactions between vectors and vertebrate hosts will promote the development of effective and novel control strategies for this major human emerging disease.

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5 References

[1] Thavara, U., Siriyasatien, P., Tawatsin, A., Asavadachanukorn, P. *et al.*, Double infection of heteroserotypes of dengue viruses in field populations of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) and serological features of dengue viruses found in patients in southern Thailand. *Southeast Asian J. Trop. Med. Public Health* 2006, *37*, 468–476.

[2] Raikhel, A. S., Dhadialla, T. S., Accumulation of yolk proteins in insect oocytes. *Annu. Rev. Entomol.* 1992, *37*, 217–251.

[3] Sánchez-Vargas, I., Scott, J. C., Poole-Smith, B. K., Franz, A. W. *et al.*, Dengue virus type 2 infections of *Aedes aegypti* are modulated by the mosquito's RNA interference pathway. *PLoS Pathog.* 2009, *5*, e1000299.

[4] Black, W. C., IV, Bennett, K. E., Gorrochótegui-Escalante, N., Barillas-Mury, C.V. *et al.*, Flavivirus susceptibility in *Aedes aegypti*. *Arch. Med. Res.* 2002, *33*, 379–388.

[5] Bayyareddy, K., Andacht, T. M., Abdullah, M. A., Adang, M. J., Proteomic identification of *Bacillus thuringiensis* subsp. israelensis toxin Cry4Ba binding proteins in midgut membranes from *Aedes (Stegomyia) aegypti* Linnaeus (Diptera, Culicidae) larvae. *Insect Biochem Mol. Biol.* 2009, *39*, 279–286.

[6] Popova-Butler, A., Dean, D. H., Proteomic analysis of the mosquito *Aedes aegypti* midgut brush border membrane vesicles. *J. Insect Physiol.* 2009, *55*, 264–272.

[7] Ribeiro, J. M., Francischetti, I. M., Role of arthropod saliva in blood feeding: Sialome and post-sialome perspectives. *Annu. Rev. Entomol.* 2003, *48*, 73–88.

[8] Ribeiro, J. M., Blood-feeding arthropods: Live syringes or invertebrate pharmacologists? *Infect. Agents Dis.* 1995, *4*, 143–152.

[9] Choumet, V., Carmi-Leroy, A., Laurent, C., Lenormand, P. *et al.*, The salivary glands and saliva of *Anopheles gambiae* as an essential step in the Plasmodium life cycle: a global proteomic study. *Proteomics* 2007, *7*, 3384–3394.

[10] Peng, Z., Simons, F. E., Advances in mosquito allergy. *Curr. Opin. Allergy Clin. Immunol.* 2007, *7*, 350–354.

[11] Valenzuela, J. G., Blood-feeding arthropod salivary glands and saliva, in: Marquardt, W. C., Kondratieff, B. C., Black, W. C, IV., Moore, C. G., *et al.* (Eds.), *Biology of Diseases Vectors*, Elsevier Academic Press, Amsterdam 2005, pp. 377–385.

[12] Salazar, M. I., Richardson, J. H., Sánchez-Vargas, I., Olson, K. E., Beaty,B. J., Dengue virus type 2: replication and tropisms in orally infected *Aedes aegypti* mosquitoes. *BMC Microbiol.* 2007, *30*, 7–9.

[13] Lefevre, T., Thomas, F., Schwartz, A., Levashina, E. *et al.*, Malaria *Plasmodium* agent induces alteration in the head proteome of their *Anopheles* mosquito host. *Proteomics* 2007, *7*, 1908–1915.

[14] Wilkins, M. R., Williams, K. L., Cross-species protein identification using amino acid composition, peptide mass fingerprinting, isoelectric point and molecular mass: a theoretical evaluation. *J. Theor. Biol.* 1997, *186*, 7–15.

[15] Missé, D., Cerutti, M., Noraz, N., Jourdan, P. *et al.*, A CD4-independent interaction of human immunodeficiency virus-1 gp120 with CXCR4 induces their cointernalization, cell signaling, and T-cell chemotaxis. *Blood* 1999, *93*, 2454–2462.

[16] Kamhawi, S., Ramalho-Ortigao, M., Pham, V. M., Kumar, S. *et al.*, A role for insect galectins in parasite survival. *Cell* 2004, *119*, 329–341.

[17] Ribeiro, J. M., Arcá, B., Lombardo, F., Calvo, E. *et al.*, An annotated catalogue of salivary gland transcripts in the adult female mosquito, *Aedes aegypti*. *BMC Genomics* 2007, *8*, 6.

[18] Champagne, D. E., Smartt, C. T., Ribeiro, J. M., James, A. A., The salivary gland-specific apyrase of the mosquito *Aedes aegypti* is a member of the 5′-nucleotidase family. *Proc. Natl. Acad. Sci. USA* 1995, *92*, 694–698.

[19] Hamilton, J. V., Munks, R. J., Lehane, S. M., Lehane, M. J., Association of midgut defensin with a novel serine protease in the blood-sucking fly *Stomoxys calcitrans*. *Insect Mol. Biol.* 2002, *11*, 197–205.

[20] Calvo, E., Mans, B. J., Andersen, J. F., Ribeiro, J. M., Function and evolution of a mosquito salivary protein family. *J. Biol. Chem.* 2006, *281*, 1935–1942.

[21] Santos, K. S., dos Santos, L. D., Mendes, M. A., de Souza, B. M. *et al.*, Profiling the proteome complement of the secretion from hypopharyngeal gland of Africanized nurse-honeybees (*Apis mellifera* L.). *Insect Biochem. Mol. Biol.* 2005, *35*, 85–91.

[22] Fry, J. D., Donlon, K., Saweikis, M., A worldwide polymorphism in aldehyde dehydrogenase in *Drosophila melanogaster*: evidence for selection mediated by dietary ethanol. *Evolution* 2008, *62*, 66–75.

[23] Valenzuela, J. G., Pham, V. M., Garfield, M. K., Francischetti, I. M., Ribeiro, J. M., Toward a description of the sialome of the adult female mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 2002, *32*, 1101–1122.

[24] Ribeiro, J. M., Charlab, R., Valenzuela, J. G., The salivary adenosine deaminase activity of the mosquitoes *Culex quinquefasciatus* and *Aedes aegypti*. *J. Exp. Biol.* 2001, *204*, 2001–2010.

[25] Haskó, G., Kuhel, D. G., Németh, Z. H., Mabley, J. G. *et al.*, Inosine inhibits inflammatory cytokine production by a posttranscriptional mechanism and protects against endotoxin-induced shock. *J. Immunol.* 2000, *164*, 1013–1019.

[26] Pike, R. N., Buckle, A. M., le Bonniec, B. F., Church, F. C., Control of the coagulation system by serpins. Getting by with a little help from glycosaminoglycans. *FEBS J.* 2005, *272*, 4842–4851.

[27] Tong, Y., Jiang, H., Kanost, M. R., Identification of plasma proteases inhibited by *Manduca sexta* serpin-4 and serpin-5 and their association with components of the prophenol oxidase activation pathway. *J. Biol. Chem.* 2005, *280*, 14932–14942.

[28] Suwanchaichinda, C., Kanost, M. R., The serpin gene family in *Anopheles gambiae*. *Gene* 2009, *442*, 47–54.

[29] Sun, D., McNicol, A., James, A. A., Peng, Z., Expression of functional recombinant mosquito salivary apyrase: a potential therapeutic platelet aggregation inhibitor. *Platelets* 2006, *17*, 178–184.

[30] Marinotti, O., de Brito, M., Moreira, C. K., Apyrase and alpha-glucosidase in the salivary glands of *Aedes albopictus*. *Comp. Biochem. Physiol. B.* 1996, *113*, 675–679.

[31] Smartt, C. T., Kim, A. P., Grossman, G. L., James, A. A., The apyrase gene of the vector mosquito, *Aedes aegypti*, is expressed specifically in the adult female salivary glands. *Exp. Parasitol.* 1995, *81*, 239–248.

[32] Francischetti, I. M., Valenzuela, J. G., Pham, V. M., Garfield, M. K., Ribeiro, J. M., Toward a catalog for the transcripts and proteins (sialome) from the salivary gland of the malaria vector *Anopheles gambiae*. *J. Exp. Biol.* 2002, *205*, 2429–2451.

[33] Remoue, F., Alix, E., Cornelie, S., Sokhna, C. *et al.*, IgE and IgG4 antibody responses to *Aedes saliva* in African children. *Acta Trop.* 2007, *104*, 108–115.

[34] Peng, Z., Simons, F. E., Mosquito allergy: immune mechanisms and recombinant salivary allergens. *Int. Arch. Allergy Immunol.* 2004, *133*, 198–209.

[35] Maroto, M., Arredondo, J. J., San Román, M., Marco, R., Cervera, M., Analysis of the paramyosin/miniparamyosin gene. Miniparamyosin is an independently transcribed, distinct paramyosin isoform, widely distributed in invertebrates. *J. Biol. Chem.* 1995, *270*, 4375–4382.

[36] Khaitlina, S. Y., Functional specificity of actin isoforms. *Int. Rev. Cytol.* 2001, *202*, 35–98.

[37] Aiello, R., Messina, A., Schiffler, B., Benz, R. *et al.*, Functional characterization of a second porin isoform in *Drosophila melanogaster*. DmPorin2 forms voltage-independent cation-selective pores. *J. Biol. Chem.* 2004, *279*, 25364–25373.

[38] Leverve, X., Batandier, C., Fontaine, E., Choosing the right substrate. *Novartis Found. Symp.* 2007, *280*, 108–121.

[39] Thompson, S. N., Pyruvate cycling and implications for regulation of gluconeogenesis in the insect, *Manduca sexta* L. *Biochem. Biophys. Res. Commun.* 2000, *274*, 787–793.

[40] Cheng, T. L., Liao, C. C., Tsai, W. H., Lin, C. C. *et al.*, Identification and characterization of the mitochondrial targeting sequence and mechanism in human citrate synthase. *J. Cell. Biochem.* 2009, *107*, 1002–1015.

[41] Benevenga, N. J., Blemings, K. P., Unique aspects of lysine nutrition and metabolism. *J. Nutr.* 2007, *137*, 1610S-1615S.

[42] Stepansky, A., Less, H., Angelovici, R., Aharon, R. *et al.*, Lysine catabolism, an effective versatile regulator of lysine level in plants. *Amino Acid* 2006, *30*, 121–125.

[43] Thorpe, C., Kim, J. J., Structure and mechanism of action of the acyl-CoA dehydrogenases. *FASEB J.* 1995, *9*, 718–725.

[44] Stuart, R. A., Supercomplex organization of the oxidative phosphorylation enzymes in yeast mitochondria. *J. Bioenerg. Biomembr.* 2008, *40*, 411–417.

[45] Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 1997, *10*, 1–6.

[46] Almeras, L., Fontaine, A., Belghazi, M., Bourdon, S. *et al.*, Salivary gland protein repertoire from *Aedes aegypti* mosquitoes. *Vector Borne Zoonotic Dis.* 2009, online ahead of print.

[47] Woods, D. F., Wu, J. W., Bryant, P. J., Localization of proteins to the apico-lateral junctions of *Drosophila* epithelia. *Dev. Genet.* 1997, *20*, 111–118.

[48] Halstead, S. B., Pathogenesis of dengue: challenges to molecular biology. *Science* 1988, *239*, 476–481.

[49] Sierra, B., Alegre, R., Pérez, A. B., García, G. *et al.*, HLA-A, -B, -C, and -DRB1 allele frequencies in Cuban individuals with antecedents of dengue 2 disease: advantages of the Cuban population for HLA studies of dengue virus infection. *Hum. Immunol.* 2007, *68*, 531–540.

[50] Diamond, M. S., Roberts, T. G., Edgil, D., Lu, B. *et al.*, Modulation of dengue virus infection in human cells by alpha, beta, and gamma interferons. *J. Virol.* 2000, *74*, 4957–4966.

[51] Vaughn, D. W., Green, S., Kalayanarooj, S., Innis, B. L. *et al.*, Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *J. Infect. Dis.* 2000, *181*, 2–9.

[52] Thisyakorn, U., Nimmannitya, S., Nutritional status of children with dengue hemorrhagic fever. *Clin. Infect. Dis.* 1993, *16*, 295–297.

[53] Remoue, F., Cisse, B., Ba, F., Sokhna, C. *et al.*, Evaluation of the antibody response to *Anopheles* salivary antigens as a potential marker of risk of malaria. *Trans. R. Soc. Trop. Med. Hyg.* 2006, *100*, 363–370.

[54] Poinsignon, A., Cornelie, S., Mestres-Simon, M., Lanfrancotti, A., Novel peptide marker corresponding to salivary

protein gSG6 potentially identifies exposure to *Anopheles* bites. *PLoS One* 2008, *3*, e2472.

[55] Poinsignon, A., Cornelie, S., Remoue, F., Grébaut, P. *et al.*, Human/vector relationships during human African trypanosomiasis: Initial screening of immunogenic salivary proteins of *Glossina* species. *Am. J. Trop. Med. Hyg.* 2007, *76*, 327–333.

[56] Morris, R. V., Shoemaker, C. B., David, J. R., Lanzaro, G. C., Titus, R. G., *Sandfly maxadilan* exacerbates infection with *Leishmania major* and vaccinating against it protects against *L. major* infection. *Insect Biochem. Mol. Biol.* 2001, *167*, 5226–5230.

[57] Valenzuela, J. G., Belkaid, Y., Garfield, M. K., Mendez, S. *et al.*, Toward a defined anti-*Leishmania* vaccine targeting vector antigens. Characterization of a protective salivary protein. *J. Exp. Med.* 2001, *194*, 331–342.

[58] Rabilloud, T., Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains. *Proteomics* 2002, *2*, 3–10.

