Quantitative Proteomic Analysis of the *Anopheles gambiae* (Diptera: Culicidae) Midgut Infected with O'nyong—Nyong Virus

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Quantitative Proteomic Analysis of the *Anopheles gambiae* (Diptera: Culicidae) Midgut Infected With O’nyong–Nyong Virus

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ABSTRACT Alphaviruses are arthropod-borne pathogens that infect a range of hosts. In humans and other mammals, alphavirus infection can cause severe disease. In mosquito hosts, however, there are generally few symptoms. Little is known about the cellular responses of mosquitoes that allow them to cope with infection. In this investigation, a six-plex tandem mass tagging proteomic approach was used to study protein accumulation changes in the midgut of *Anopheles gambiae* (Giles) (Diptera: Culicidae) mosquitoes infected with o’nyong-nyong virus (*Togaviridae, Alphavirus*). Five hundred thirty-six nonredundant proteins were identified. Twenty-two were found in significantly different quantities in infected midguts compared with controls. Of interest, analysis revealed molecular pathways possibly targeted by virus proteins, such as those involving TAF4 and DNA polymerase phi proteins. Also identified was an FK506-binding protein. FK506-binding protein orthologs have been described as conserved host resistance factors, which suppress dengue and West Nile virus infection in human HeLa cells. This investigation constitutes the first study of the midgut-specific proteome of *An. gambiae* in relation to alphavirus infection. Our findings offer insight into mosquito immunity, including factors that possibly contribute to the different pathological outcomes observed in vertebrate and insect hosts.

KEY WORDS o’nyong-nyong virus, *Anopheles gambiae*, tandem mass tag, FK506-binding protein, midgut
There are many tools currently available for investigating whole-organism or tissue-specific changes in cellular processes induced by pathogens. Microarray technology and mRNA detection techniques effectively characterize gene expression changes, but often do not accurately reflect abundances of downstream effector proteins (Jansen and Schalkwijk 2003). Therefore, other strategies focus directly on the quantification of protein. Several of these methods have been compared (Jansen and Schalkwijk 2003, Wu et al. 2006, Brewis and Brennan 2010). The separation of proteins by liquid chromatography (LC) and one- and two-dimensional gel electrophoresis, followed by mass spectrometry (MS), is generally considered the standard (Wu et al. 2006). These techniques have evolved into two-dimensional differential gel electrophoresis and tandem mass spectrometry (MS/MS) methods like isobaric tags for relative and absolute quantification (iTRAQ) and tandem mass tagging (TMT) (Wu et al. 2006, Brewis and Brennan 2010). MS-based methods such as iTRAQ and TMT offer greater detection sensitivity, especially concerning the detection of proteins of extreme molecular weight, of multispansing integral membrane proteins, and of those that are basic (Brewis and Brennan 2010). In the current study, we used the six-plex TMT proteomics platform to quantify and compare protein expression changes induced in the midgut of Anopheles gambiae (Giles) resulting from infection with o’nyong-nyong virus (ONNV).

ONNV (family Togaviridae) is a positive-sense single-stranded RNA virus of the genus Alphavirus. Alphaviruses are known to cause debilitating disease in humans and animals, usually associated with a significant amount of cell death. In mosquitoes, however, infection typically persists for life and results in little cytopathology. ONNV is closely related to chikungunya virus, with which it shares ~89% genetic sequence homology (Vanlandingham et al. 2006). ONNV, however, causes milder disease than chikungunya (in vertebrates), and is unique among arboviruses in its anopheline host specificity. In addition to the aforementioned characteristics of ONNV, a reasonably well-annotated genome of An. gambiae makes ONNV and its natural vector mosquito a particularly attractive model system for studying arbovirus influences on protein accumulation in the mosquito midgut. Our findings suggest several interesting candidate proteins for future characterization. These include previously described resistance and host factors, cellular targets of virus proteins, and putative pathogenesis factors. Our results also represent the first look at the midgut proteome of An. gambiae, a disease vector of great public health importance.

Materials and Methods

Mosquito Rearing and Infection. An. gambiae (G3 strain) mosquitoes were reared as previously described (Vanlandingham et al. 2005). Half of the adult females (150 of the 300 total) were deprived of a 10% sucrose solution at 48 h and water at 24 h before presentation with an infectious bloodmeal in a Hemotek membrane feeder (Discovery workshops, Accrington, Lanc, United Kingdom). The bloodmeal consisted of a 1:1 mixture of defibrinated sheep blood (Colorado Serum Co, Denver, CO) and −80°C frozen tissue culture fluid from BHK-21s cells that had been electroporated with a full-length ONNV infectious clone. This clone was derived from the SG650 virus strain, and contained a green fluorescent protein (GFP) reporter (pONN-5-eGFP) (Sim et al. 2007). Post electroporation, tissue culture was incubated at 37°C with 5% CO2 for 48 h. The median tissue culture infectious dose was assessed in Vero cells, and the resulting virus titer was 3.31 million infectious particles per milliliter. For the control, mosquitoes were fed a bloodmeal consisting of a 1:1 mixture of defibrinated sheep blood and tissue culture media from BHK-21s cells. Fully engorged mosquitoes were aspirated and transferred to new containers and provided with a 10% sucrose solution on cotton pads. At 6 days postinfection (dpi), samples were collected, cold-anesthetized, briefly placed in 70% ethanol for surface sterilization, and promptly transferred to ice-cold Dulbecco’s phosphate-buffered saline (Cellgro, Manassas, VA) with 1X Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA). Midguts were removed and briefly examined for GFP expression to enrich the sample population for positive ONNV-infected mosquitoes. The presence of GFP in the midgut indicated ONNV replication in the midgut epithelium (Sim et al. 2007). Positive midguts were immediately transferred to a solution of ice-cold insect cell protein extraction reagent (Thermo Scientific, Waltham, MA) with 1X Halt. Samples were then pooled in groups of 50, three replicates per group, and stored at −80°C until needed for further analysis.

Mosquito Midgut Tissue Lysis. Pools of midguts were lysed in radio-immunoprecipitation assay lysis buffer with 50 mM Tris-HCl at pH 7.6, 150 mM NaCl, 2 mM ethylene diamine tetraacetic acid (EDTA), 1% nonyl phenoxypolyethoxylethanol (NP-40), 1% sodium deoxycholate, and 0.1% sodium dodecylsulfate (SDS) followed by sonication and centrifugation at 20,000 × g for 10 min to remove insoluble material from the crude lysate. The supernatant was collected and protein concentration was determined with a bicinchoninic acid assay (BCA) protein assay (Noble and Bailey 2009). The protein concentration was measured to be in the range of 1–2 mg/ml.

Reduction, Alkylation, and Trypsin Digestion. Aliquots of 100 µg of proteins from each sample were added into 100 µl of 200 mM triethyl ammonium bicarbonate (Sigma, St. Louis, MO). Reduction was performed by adding 5 µl of 200 mM Tris (2-carboxyethyl) phosphine (Sigma, St. Louis, MO) to each replicate and incubating for 1 h at 55°C. Alkylation was carried out by adding 5 µl of 375 mM iodoacetamide (Bio-Rad Laboratories, Hercules, CA) to each sample and incubating for 30 min at room temperature. After alkylation, ~1 ml of prechilled acetone was added and precipitation was allowed to proceed for 3 h at −20°C. The acetone-precipitated protein pellets were sus-
pended with 100 µl of 200 mM triethyl ammonium bicarbonate. According to manufacturer’s instructions, 2.5 µg of sequencing grade modified trypsin (Promega Corp., Madison, WI) was added to digest each sample overnight at 37°C.

Isobaric Labeling With TMT. TMT with varying molecular weights (126–131) were applied as isobaric labels for the relative quantification of mosquito proteins by using the six-plex set TMT® system (Thermo Scientific, Waltham, MA). According to the manufacturer’s protocols, the six digested samples were individually labeled with TMT® reagents as follows. Three control mosquito midgut protein samples: TMT-126 (batch 1), TMT-127 (batch 2), and TMT-128 (batch 3); and three infected mosquito samples: TMT-129 (batch 1), TMT-130 (batch 2), and TMT-131 (batch 3). The labeled peptide mixtures were combined at equal ratios. The peptide mixtures were then fractionated, to improve MS/MS peptide resolution.

Fractionation of Labeled Peptide Mixture by Using a Strong Cation Exchange Column. Proteins were fractionated with a SCX strong cation exchange column (Thermo Scientific, Waltham, MA) on a Shimadzu 2010 high performance liquid chromatography (HPLC) equipped with a ultraviolet detector (Shimadzu, Columbia, MD). Mobile phase consisted of buffer A (5 mM KH2PO4, 25% acetonitrile, and pH 2.8) and buffer B (buffer A plus 350 mM KCl). The column was equilibrated with buffer A for 30 min before sample injection. The mobile phase gradient was set as follows, at a flow rate of 1.0 ml/min: 1) 0–10 min: 0% buffer B; 2) 10–40 min: 0–25% buffer B; 3) 40–45 min: 25–100% buffer B; 4) 45–50 min: 100% buffer B; 5) 50–60 min: 100–0% buffer B; and 6) 60–90 min: 0% buffer B. In all, 60 fractions were initially collected, lyophilized, and combined into 14 final fractions based on SCX chromatogram peaks.

Desalination of Fractionated Samples. A C18 solid-phase extraction (SPE) column (Thermo Scientific, Waltham, MA) was used to desalt all collected fractions. The combined 14 fractions were each adjusted to a final volume of 1 ml, containing 0.25% (vol/vol in water) trifluoroacetic acid (TFA). The C18 SPE column was conditioned before use by filling with 1 ml acetonitrile and allowing the solvent to pass through the media slowly (~3 min). The column was then rinsed three times with 1 ml 0.25% (vol/vol in water) TFA solution. The fractions were loaded on to the top of the SPE cartridge column slowly and loaded once on to the column to decrease lost peptide during the column binding. Columns were washed four times with 1 ml 0.25% TFA aliquots before the peptides were eluted three times, each with 400 µl of 80% acetonitrile/0.1% formic acid (aqueous). All of the eluted samples were lyophilized for the LC-MS/MS.

LC-MS/MS Analysis on LTQ-Orbitrap. Peptides were analyzed on an LTQ-Orbitrap XL (Thermo Scientific, Waltham, MA) instrument interfaced with an Ultimate 3000 Dionex LC system (Dionex, Sunnyvale, CA) by using high mass resolution for peptide identification and high energy collision dissociation (HCD) for reporter ion quantification. The reverse-phase HPLC system consisted of a peptide Cap-Trap cartridge (0.5 by 2 mm) (Michrom BioResources, Auburn, CA) and a prepacked BioBasic C18 PicoFrit analytical column (75 µm inner diameter × 15 cm length; New Objective, Woburn, MA) fitted with a FortisTip emitter tip. Samples were loaded onto the trap cartridge and washed with mobile phase A (98% H2O, 2% acetonitrile, and 0.1% formic acid) for concentration and desalting. Subsequently, peptides were eluted over 180 min from the analytical column via the trap cartridge by using a linear gradient of 6–100% mobile phase B (20% H2O, 50% acetonitrile, and 0.1% formic acid) at a flow rate of 0.3 µl/min by using the following gradient: 6% B for 5 min; 6–60% B for 125 min; 60–100% B for 5 min; keep at 100% B for 5 min; 100–6% B for 2 min; and keep at 6% B for 38 min.

The Orbitrap mass spectrometer was operated in a data-dependent mode in which each full MS scan (60,000 resolving power) was followed by six MS/MS scans where the three most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation by using a normalized collision energy of 35%, and the same three molecular ions were also scanned by HCD-MS2 with collision energy of 45%. MS scans were acquired in profile mode and MS/MS scans in centroid mode.

LTQ-Orbitrap settings were as follows: spray voltage 2.0 kV, one microscan for MS1 scans at 60,000 resolution (fwhm at m/z 400), microscans for MS2 at 7,500 resolution (fwhm at m/z 400); full MS mass range, m/z 400–1,400; MS/MS MS mass range, m/z 100–2,000. The “FT master scan preview mode,” “Charge state screening,” “Monoisotopic precursor selection,” and “Charge state rejection” were enabled so that only the 2+, 3+, and 4+ ions were selected and fragmented by collision-induced dissociation and HCD.

Database Search and TMT Quantification. The protein search engine Sequest (Thermo Scientific) was used for peptide matching and protein identification. The minimum number of peptides used for identification and quantification of proteins was one unique peptide. Sequest format files were generated by the Proteome Discoverer (v.1.2) data processing software (Thermo Scientific) by using the following criteria: database, agambiae.PEPTIDES-AgamP3.5.fa (www.vectorbase.org)(Megy et al. 2012); enzyme, trypsin; maximum missed cleavages, 2; static modifications, carbamidomethylation (+57 d), N-terminal terminal TMT 6-plex (+229 d), lysyl-TMT 6-plex (+229 d). Dynamic modifications, N-terminal Cln- pyro-Glu (+17 d); methionine oxidation (+16 d); serine, threonine, tyrosine (STY) phosphorylation (+80 d). MS peptide tolerance was set at 15 ppm; MS/MS tolerance at 0.05 d. Peptides reported by the search engine were accepted only if they met the false discovery rate of P < 0.05 (target decoy database). For TMT quantification, the ratios of TMT reporter ion intensities in MS/MS spectra (up to six reporter ions ranging from m/z 126.12 to m/z 131.14) from raw data sets were used to calculate fold changes in proteins between control and treatment.
Statistical Analysis and Gene Ontology. The Bioconductor package limma was used to model changes in protein accumulation associated with infection (Smyth 2005). Average fold changes were estimated and evaluated for each peptide by using $t$-tests, with $P$ values adjusted for multiple comparisons by using the Benjamini–Hochberg correction. The threshold for statistical significance used was 0.10, as mass-balanced labeling methods such as those used in TMT tend to underestimate abundance differences (Brewis and Brennan 2010). To categorize the midgut proteins identified in the study, each protein was searched against the eukaryotic clusters of orthologous groups of proteins (KOGs) at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/COG/grace/shokog.cgi). The KOG terms with the best e-values of at least 1.0 by $10^{-4}$ were selected for the gene ontology grouping of the midgut proteome (Tatusov et al. 2003, Megy et al. 2012).

Results and Discussion

Midgut infection with ONNV was monitored by examining 5–10 midguts at 2, 3, and 6 dpi for GFP expression. As virus infection was most prevalent and intense at 6 dpi (Fig. 1), and viral dispersion patterns were typical of those observed resulting from wild-type virus infection (Brault et al. 2004), ONNV-infected An. gambiae midguts at 6 dpi were collected and used for the study. These data represent the ONNV-infected proteome of the An. gambiae midgut at peak observed tissue infection. In total, 536 nonredundant proteins that were expressed in both control and infected samples were identified and quantified (Supp Table 1 [online only]). Among these midgut proteins identified, 22 were significantly modulated in ONNV-infected midguts compared with control. The results of this investigation along with descriptions of some of the most promising candidates for future characterization studies in the context of host–virus interactions have been mentioned later in the text. Proteins with unknown function were described by their domain characteristics or the function of their orthologs. The domain analysis was done by using the Conserved Domain Database of NCBI (Marchler-Bauer et al. 2011) and the Kyoto Encyclopedia of Genes and Genomes (Kanehisa and Goto 2000, Kanehisa et al. 2012), unless noted otherwise. E values associated with the domain analysis were generated by Kyoto Encyclopedia of Genes and Genomes. Proteins were classified by KOG terms (Fig. 2; Table 1) (Tatusov et al. 2003).

Proteins Involved in Energy Production and Conversion. Two proteins associated with cellular energy production and conversion were identified in this study. The first, AGAP010792-PA, increased in abundance by 41% in virus-infected mosquitoes compared
with the uninfected control. This protein is an ortholog of human nicotinamide adenine dinucleotide (NADH) dehydrogenase (ubiquinone) one alpha subcomplex nine (NDUFA9), an atypical short-chain dehydrogenase/reductase. NDUFA9 is a conserved host factor important for dengue virus infection in both humans and *Aedes aegypti* (L.) mosquitoes (Sessions et al. 2009). It is part of the mitochondrial electron transport chain that mediates the transfer of electrons from NADH to ubiquinone. NADH dehydrogenase plays an integral role in adenosine triphosphate (ATP) generation. As structural and biochemical processes are altered during alphavirus infection, modulation of metabolic pathways involved in energy consumption is logical (Laakkonen et al. 1998, El-Bacha et al. 2004). NDUFA9 gene expression in *Drosophila melanogaster* (Meigen) reduces in response to persistent sigma virus (a negative-sense RNA virus of the *Rhabdoviridae* family) infection (Carpenter et al. 2009). This mRNA decrease contrasts with the increase in protein abundance we observed. This may reflect inherent inconsistencies between transcript and protein accumulation. It may also reflect differences in host factors, tissue specificities of the different viruses, or temporal factors.

The second protein identified in this category was AGAP007121-PA. Protein accumulation diminished in infected mosquitoes by 38%. It is an ortholog of *Drosophila* cytochrome b5 (Cyt-b5), with predicted electron carrier function. Cyt-b5 is a member of the large P450 superfamily of proteins characterized by their heme and steroid-binding domains. P450 proteins have been implicated as viral host factors in mosquitoes (Sessions et al. 2009). In insects, some P450 proteins are involved in biosynthetic pathways that regulate growth, development, reproduction, and detoxification (Tijet et al. 2001).

**Proteins Involved in Amino Acid Transport and Metabolism.** AGAP010383-PA of the major facilitator superfamily of secondary transporters was identified in 40% greater abundance in virus-infected mosquitoes. It is an ortholog of the phagosome-associated fruit fly transporter Yn (Marygold et al. 2013) and also the human SLC15 transporters PepT1 and PepT2. These transmembrane proteins transport a variety of substrates, including those of immunological relevance. For example, bacterial peptidoglycan products from phagosomes are transported via PepT1 or PepT2 to cytosolic pattern recognition sensors in intestinal epithelia cells or to phagocytes, respectively (Zucchielli et al. 2009, Charrière et al. 2010, Dalmaso et al. 2010). In mammals, this activates NF-κB via the NOD2 (nucleotide-binding oligomerization domain containing 2) pathway and expression of pro-inflammatory...
cytokines (Charrière et al. 2010). Although the immunological function of Yin in dipterans has not been elucidated, a role analogous to that in mammals has been suggested (Charrière et al. 2010). Further research aimed at deciphering the role such oligopeptide transporters play in virus infection is warranted. Other endosomal major facilitator superfamily peptidyl peptide transporters play a role in virus infection (Marchler-Bauer et al. 2011). The peritrophic matrix is a chemically heterogenous sheath that coats the luminal side of the gut epithelium in many insects, and can act as a barrier to infection by pathogens. The peritrophic matrix is the primary defense of the gut epithelium in many insects (Marchler-Bauer et al. 2011). The peritrophic matrix proteins in insects (Marchler-Bauer et al. 2011) that may actively bind and hydrolyze chitin (Marchler-Bauer et al. 2011). The AGAP013260-PA CH18 domain is characteristic of group II chitinases. These proteins are typically secreted and contain multiple catalytic and binding domains (Zhu et al. 2008). The AGAP006414-PA CH18 domain suggests it may be a chitinase-like lectin (chitolectins) or chitotriosidase (Marchler-Bauer et al. 2011). Both invertebrates and vertebrates use lectins to recognize and defend against pathogens (Franc and White 2000). In humans, the chitolectin YKL-40 is considered a cytokine, and mediates inflammatory processes (Badariotti et al. 2007, Lee et al. 2011). Chitin-hydrolyzing chitotriosidases are expressed in neutrophils and maturing macrophages. Their modulation by toll-like receptors and the NOD2 pathway suggests a role in microbial defense (van Eijk et al. 2007, Marchler-Bauer et al. 2011).

Proteins Involved in Carbohydrate Transport and Metabolism. Two chitinases were identified in this study: AGAP013260-PA underwent a 42% decrease in protein accumulation, and AGAP006414-PA increased by 48% relative to the control. Hydrolytic chitinases are found in many phyla. Their pervasiveness reflects the nature of their substrate, chitin, the second most ubiquitous natural polymer (Lee et al. 2011). Chitinases play a key role in insect growth and development, as the chitin-containing exoskeleton and peritrophic matrix is periodically degraded and replaced (Zhu et al. 2008). Chitinases are also a component of innate immunity (Lee et al. 2011), and their regulation in response to ONNV suggests they may play a role in virus infection. Chitinases are used by both parasites and hosts in invasion and deterrence processes (Lee et al. 2011). In An. gambiae, some chitinase family proteins accumulate in hemolymph rapidly, and specifically in response to bacterial infections (Shi and Paskevitz 2004). Yan et al. (2002) showed that they may protect against chitin-containing pathogens in tsetse flies (Glossina morsitans morsitans) (Yan et al. 2002). Both proteins identified here have glycosyl hydrolase 18 family (or family-like) domains (E value ≤ 1.3e-77) that may actively bind and hydrolyze chitin (Marchler-Bauer et al. 2011). The AGAP013260-PA CH18 domain is characteristic of group II chitinases. These proteins are typically secreted and contain multiple catalytic and binding domains (Zhu et al. 2008). The AGAP006414-PA CH18 domain suggests it may be a chitinase-like lectin (chitolectins) or chitotriosidase (Marchler-Bauer et al. 2011). Both invertebrates and vertebrates use lectins to recognize and defend against pathogens (Franc and White 2000). In humans, the chitolectin YKL-40 is considered a cytokine, and mediates inflammatory processes (Badariotti et al. 2007, Lee et al. 2011). Chitin-hydrolyzing chitotriosidases are expressed in neutrophils and maturing macrophages. Their modulation by toll-like receptors and the NOD2 pathway suggests a role in microbial defense (van Eijk et al. 2007, Marchler-Bauer et al. 2011).

AGAP006414-PA in addition has a chitin-binding peritrophin-A domain (E value ≤ 2.8e-14) associated with peritrophic matrix proteins in insects (Marchler-Bauer et al. 2011). The peritrophic matrix is a chemically heterogenous sheath that coats the luminal side of the gut epithelium in many insects, and can act as a barrier to infection by pathogens. The peritrophic matrix is the primary defense of An. gambiae mosquitoes against infecting malaria parasites (Sieber et al. 1991). Proteins with peritrophic-A domains can have antimicrobial characteristics, such as the arthropod protein tachycitin (Tachypleus tridentatus) (Kawabata et al. 1996). Sanders et al. (2005) showed that mRNA expression of four genes with peritrophic-A domains increased after 4 d of infection with Sindbis virus (SINV, Togaviridae, Alphavirus) in A. aegypti (Sanders et al. 2005). Likewise, in the current study, after 6 d of ONNV infection, AGAP006414-PA accumulation increased.

### Table 1. Protein expression altered by ONNV infection

<table>
<thead>
<tr>
<th>Description</th>
<th>Identification</th>
<th>Ratio of expression (infected/control)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone)</td>
<td>AGAP010792-PA</td>
<td>1.4</td>
<td>0.089</td>
</tr>
<tr>
<td>Cysteine b5-like heme/steroid-binding domain</td>
<td>AGAP007121-PA</td>
<td>0.6</td>
<td>0.084</td>
</tr>
<tr>
<td>MFS superfamily</td>
<td>AGAP010353-PA</td>
<td>1.4</td>
<td>0.100</td>
</tr>
<tr>
<td>Chitinase</td>
<td>AGAP013260-PA</td>
<td>0.6</td>
<td>0.047</td>
</tr>
<tr>
<td>Chitinase</td>
<td>AGAP006414-PA</td>
<td>1.5</td>
<td>0.079</td>
</tr>
<tr>
<td>Sodium/hydrogen exchanger 3</td>
<td>AGAP009036-PA</td>
<td>0.5</td>
<td>0.047</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>AGAP006400-PA</td>
<td>1.4</td>
<td>0.100</td>
</tr>
<tr>
<td>Information storage and processing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA polymerase phi</td>
<td>AGAP003218-PA</td>
<td>0.7</td>
<td>0.095</td>
</tr>
<tr>
<td>Transcription initiation factor TFIIID subunit</td>
<td>AGAP005719-PA</td>
<td>0.6</td>
<td>0.047</td>
</tr>
<tr>
<td>Linker histone 1 and 5</td>
<td>AGAP011193-PA</td>
<td>0.6</td>
<td>0.044</td>
</tr>
<tr>
<td>Cellular processes and signaling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FKBP-type peptidyl-prolyl cis-trans isomerase</td>
<td>AGAP007473-PA</td>
<td>1.3</td>
<td>0.095</td>
</tr>
<tr>
<td>E3 ubiquitin-protein ligase listerin</td>
<td>AGAP007143-PA</td>
<td>0.6</td>
<td>0.090</td>
</tr>
<tr>
<td>Serine/threonine protein phosphatase</td>
<td>AGAP006750-PA</td>
<td>0.6</td>
<td>0.047</td>
</tr>
<tr>
<td>LIM domain; domain of unknown function</td>
<td>AGAP000225-PB</td>
<td>0.6</td>
<td>0.044</td>
</tr>
<tr>
<td>Bab5-like GTPase</td>
<td>AGAP007290-PA</td>
<td>1.4</td>
<td>0.092</td>
</tr>
<tr>
<td>Drp1, Sec92, translocation ER complex proteins</td>
<td>AGAP010792-PA</td>
<td>1.4</td>
<td>0.099</td>
</tr>
<tr>
<td>General function prediction only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ras-like GTPase</td>
<td>AGAP005161-PA</td>
<td>0.5</td>
<td>0.089</td>
</tr>
<tr>
<td>Poorly characterized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cadherin repeat domain</td>
<td>AGAP004166-PA</td>
<td>2.2</td>
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<tr>
<td>Trypsin-like serine protease</td>
<td>AGAP006070-PA</td>
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<tr>
<td>Sallimus, Titin, Ket, CC18242, D-titin</td>
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<td>0.7</td>
<td>0.047</td>
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<tr>
<td>DUF1397t; protein of unknown function</td>
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<td>1.7</td>
<td>0.079</td>
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<tr>
<td>AGAP003864-PA</td>
<td>AGAP003864-PA</td>
<td>1.7</td>
<td>0.044</td>
</tr>
</tbody>
</table>

### Table Notes

- **Description:** The identification of the protein.
- **Identification:** The protein's accession number.
- **Ratio of expression (infected/control):** The ratio of expression under infected and control conditions.
- **P value:** The significance level of the expression change.
These data, and domain analyses, suggest that proteins bearing certain chitin-binding and chitinase domains may be part of a host’s general antimicrobial and inflammation response triggered by virus infection.

Proteins Involved in Inorganic Ion Transport and Metabolism. Inorganic ion transport is of fundamental importance to the maintenance of membrane potentials, pH, ion composition, and fluid dynamics of cellular systems (Brett et al. 2005). The membrane protein AGAP009036-PA identified in this study reduced in abundance by 54%. This protein is a sodium/hydrogen exchanger (NHE) closely related to Ae. aegypti NHE3, where it is localized primarily in the basolateral membrane of epithelia cells in the midgut, Malpighian tubules, and gastric caeca (Pullikuth et al. 2006). In some insects (e.g., Aedes and Drosophila spp.), NHE and other cation proton antiporter superfamily proteins appear to function in association with vacuolar ATPases that together drive the electro motive force critical for ion exchange (Pullikuth et al. 2006, Day et al. 2008).

Of these ion-regulating proteins, Sanders et al. (2005) previously reported a twofold increase in the expression of vacuolar ATPases at 4 dpi, in their investigation of the effects of SINV on Ae. aegypti (Sanders et al. 2005). In the current study, we found that abundance of the associated NHE3 proteins halved at 6 dpi. Although the accumulation differences observed of these associated ion-regulating proteins are difficult to resolve, these data suggest an important role for such regulatory mechanisms in virus infection. One potential explanation for the modulation of NHE3 may involve the alphavirus viraoprotein protein 6K. 6K has been reported to increase membrane permeability, especially to Na+ ions but also K+, Ca2+, and, to a lesser extent, Cl− (Melton et al. 2002). NHE3 modulation may reflect a cellular response to virus-induced membrane permeability to these ions.

Although it is unknown what part sodium or hydrogen exchangers play in alphavirus infection of mammals, it has been suggested that early alteration to ion transport may rapidly induce apoptosis (Ulug et al. 1996). This contrasts with entomological systems where persistent infections develop. As there are notable differences in ion transport mechanisms between mammals and insects (Djaggoz et al. 1998), future investigation may not only reveal the role of NHE3 in controlling or fostering virus infection, but also the mechanisms responsible for orchestrating events that lead to cell death versus persistent infection.

Another inorganic ion transport and metabolism protein identified was an alkaline phosphatase (ALKP). AGAP006400-PA was identified in quantities 43% greater in infected mosquitoes compared with controls. In insects, ALKP expression is associated with the apical epithelial brush border and lumen of the gut (Houk and Hardy 1984). ALKP activity is age-dependent, peaking during late larval stages, and in adults, during gamete maturation (Nath and Butler 1972). In this regard, it has been suggested to be associated with nutrition and metabolism, particularly of fats and carbohydrates.

In other biological systems, intestinal ALKPs are well-known indicators of disease states, particularly of the liver and bone (Coleman 1992, Lalles 2010). They play a major role in maintaining intestinal homeostasis, including lipid metabolism and pH regulation. Intestinal ALKPs also play a role in innate immunity, which may be interesting in light of the increased ALKP accumulation observed in ONNV-infected mosquitoes here. ALKP mitigates the inflammatory response to common gut flora by detoxification (dephosphorylation of gram-negative bacteria lipopolysaccharides [LPS]), and also by preventing microbial penetration of midgut epithelia. In response to LPS, intestinal ALKP is upregulated, and subsequently, activates the TLR4/ NF-κB immunomodulatory pathway (Lalles 2010). In zebrafish, neutrophil recruitment to gut epithelia is dependent on ALKP activation (Lalles 2010). Neutrophils carry ALKP in their antimicrobial granules, and expression is enhanced in neutrophils in response to LPS (Lalles 2010). It is reasonable to speculate the ALKP may play a homeostasis role, or perform general immunomodulatory functions in An. gambiae gut tissue in response to the physical impact of virus infection.

Proteins Involved in Transcription. Two proteins identified were associated with transcription processes. Both were less abundant in the virus-infected mosquitoes. They were DNA polymerase phi (AGAP003218-PA), the abundance of which decreased by 35%, and TAF4 (TATA-box binding protein-associated factor 4, AGAP005719-PA), which decreased by 41%. DNA polymerase phi (Pol5p), initially described in yeast in 2002, synthesizes ribosomal RNA (Shimizu et al. 2002). TAFs and the associated transcription factor TATA-box binding protein are components of the transcription factor II D (TFIID) RNA polymerase II transcription initiation complex (Lewin 1994). TAF and TATA-box binding protein interactions may allow the TFIID transcription complex to recognize promoters of different genes, thereby differentially regulating expression of proteins. Some cellular pathways associated with TAF4 and polymerase phi may be targeted by viral proteins, resulting in suppression of a subset of host protein expression. This process may be critical for virus fitness, ultimately affecting the production of effector molecules.

TAF4 is a component of the Wnt signaling pathway. It activates expression of the gene naked cuticle (nkdc) via interaction with the transcription cofactor Pygopus, in Drosophila (Wright and Tjian 2009). Interestingly, in mice, the Wnt signaling pathway has been shown to be important in the generation of memory CD8 T cells that recognize viral and bacterial infections, a process that facilitates a prompt immune response on subsequent encounters with the same pathogens (Zhao et al. 2009, Jeannet et al. 2010). It remains to be determined what effect TAF4 and Wnt signaling have on virus infections of mosquitoes.

Some Old-World alphaviruses suppress host transcription and translation (Frolov et al. 2009). Evidence from infected mammalian cell cultures suggests this process is mediated by virus nonstructural protein two (nsP2), which induces cytopathogenic effect (CPE)
and cell death (Garmashova et al. 2006, Frolov et al. 2009). Viral nsP2 has been shown to inhibit both the production of ribosomal and cellular messenger RNA by mechanisms that do not involve its helicase and protease activity (Garmashova et al. 2006). In the nucleolus, alphavirus nsP2 closely associates with ribosomes (Ranki et al. 1979), suggesting that translation suppression may be mediated by viral proteins directly targeting (as of yet unknown) cellular host factor(s). Future studies characterizing the roles of TAF4 and Pol5p during virus infection are warranted. As less CPE resulting from infection is observed in mosquitoes than is seen in vertebrates, understanding differences in these systems may expose cytopathogenesis mechanisms, and lead to novel therapies for alphavirus-related disease.

**Proteins Involved in Chromatin Structure and Dynamics.** The abundance of AGAP011193-PA decreased by 41% in infected mosquitoes. AGAP011193-PA is a H15 superfamily protein with a nucleosome subunit linker histone one-fifths domain (E value: 7.9e-31). Such H1/H5 histones facilitate the condensation of chromatin, stabilizing and effectively blocking access to nucleosomal DNA by other cellular proteins (Kasinsky et al. 2001). However, H1/H5 histones can both positively or negatively affect transcription (Brown et al. 1996). The reduced H1/H5 observed in our study suggests a positive effect is more likely, as less protein may allow transcription complexes to more easily access chromatin. Although virus-infected cells may experience some specific transcription suppression mediated by TAF4 and Pol5p described previously, cells may also experience transcription enhancement of some genes. As ONNV replication requires ample access to many host factors, specific transcription enhancement may also be essential for virus fitness.

**Proteins Involved in Posttranslational Modification, Protein Turnover, Chaperones.** The FK506-binding protein (FKBP)-type peptidyl-prolyl cis-trans isomerase, FKBP1 (AGAP007473-PA), was detected in ONNV-infected mosquitoes in abundances 32% greater than in controls. FKBP1s are immunophilins, which, when bound to the drug FK506 or rapamycin, cause suppression of T-cell activation in vertebrates (Kang et al. 2000). They also regulate apoptosis and cell growth via the target of rapamycin (TOR) nutrient sensing pathway (Kang et al. 2008, Ma et al. 2008). The immunological role of these proteins in response to pathogens, and in the absence of FK506 or rapamycin, is poorly defined. However, mRNA transcription of Ae. aegypti FKBP1s altered by alphavirus infection of the midgut (Sanders et al. 2005). In addition, FKBP1B, a paralog of FKBP1, acts as a resistance factor to both dengue and West Nile virus (Flaviviridae) in human cells (Krishnan et al. 2008). This suggests FKBP1s have antiviral functions. The few FKBP1 orthologs that have been studied are functionally diverse and highly specific to the tissue and organism. For example, the Arabidopsis thaliana ortholog (AtFKBP53) is a histone chaperone that represses expression of 18S rRNA at the chromatin level (Li and Luan 2010). An. gambiae FKBP1 may also interact directly with chromatin. It has a potential nucleolus domain (E value: 1.7e-4) and centromere kinetochore component (CENP-T) domain (E value: 0.19), which are both associated with chromatin organization functions. Neye et al. (2004) demonstrated that the human ortholog, FKBP2 (also known as FKBP13), binds complement protein C1q in liver cDNA binding screens (Neye and Verspohl 2004). In human placental screens, FKBP2 was shown to play a role in vesicular trafficking (Padilla et al. 2003). Given the dearth of research characterizing AGAP007473-PA and its orthologs, and considering the diversity of described immunophilin activities, functional characterization, and silencing studies in mosquito disease vectors will be particularly intriguing.

Also identified in this category was AGAP007143-PA, found 39% less abundant in virus-infected mosquitoes than in controls. It is an ortholog of vertebrate listerin E3 ubiquitin protein ligase 1 (Ltn1), which plays a critical role in targeting peptides for ubiquitylation and subsequent protein degradation. This activity is central to the regulation of a vast array of cellular processes, including immune responses (Varshavsky 1997, Malynn and Ma 2010). In SINV infections of Ae. aegypti, Sanders and colleagues suggested decreases in ubiquitin ligase levels may inhibit the Toll pathway, and possibly activate the immune deficiency (IMD) pathway (Sanders et al. 2005).

Ltn1 is conserved among eukaryotes (Bengtson and Joazeiro 2010). In yeast, Ltn1 mediates the targeting of nascent nonstop peptides for proteasomal degradation (Bengtson and Joazeiro 2010). In the absence of this quality-control activity, mRNA deficient in stop codons may induce ribosomal arrest. Thus, Ltn1 plays a role in maintaining translation efficiency, confers resistance to stress induced by stop codon-deficient mRNA accumulation, and prevents the generation of aberrant proteins (Bengtson and Joazeiro 2010).

**Proteins Involved in Signal Transduction Mechanisms.** Two proteins in this category were identified that were downregulated in ONNV-infected mosquitoes. AGAP006750-PA is an ortholog of Culex quinquefasciatus protein phosphatase 4 (Ppp4) and mammalian Ppp4 regulatory subunit 1 (Kanchehisa and Goto 2000, Kanchehisa et al. 2012, Megy et al. 2012). Ppp4 is a ubiquitous serine/threonine protein phosphatase. It was downregulated by 41% in ONNV-infected mosquitoes. The second protein, AGAP000222-PB, was downregulated by 42%. It contains a calponin homology domain (E value: 5.1e-7) associated with actin binding and signal transduction.

Although difficult to attribute specific roles for these proteins in viral infection, serine/threonine protein phosphatases may be important to ONNV-specific regulation. For example, alphavirus nsP3 is phosphorylated at >70 serine/threonine sites, which may play a role in the regulation of its RNA polymerase activity (Vihinen and Saarinen 2000). Serine/threonine protein phosphatases also regulate fundamental cellular processes (Cohen et al. 2005). In mammals, bacterial infections activate Ppp4 via tumor necrosis factor (TNFα). Ppp4 activation increases the activity of the...
NF-κB pathway and the release of immune mediators. Ppp4 may also affect transcription at the chromatin level. It negatively regulates histone deacetylases, which repress transcription by removing acetyl groups on histones. Consequently, less Ppp4 protein may both activate the transcription of antimicrobial effector molecules (via the NF-κB pathway) and, at the same time, suppress the transcription of virus host factors.

**Proteins Involved in Intracellular Trafficking, Secretion, and Vesicular Transport.** The intracellular trafficking, secretion, and vesicular transport-associated proteins identified in this study are highly conserved and relatively well described. AGAP007901-PA was found in 38% greater abundance in virus-infected mosquitoes. It is an ortholog of Rab5, a key component of the clathrin-mediated endocytosis pathway, which is perhaps the most exploited mechanism of cell entry by enveloped viruses, including the alphaviruses (Cosset and Lavillette 2011). Rab5 is necessary for infection of mosquito cells by the alphavirus, Venezuelan equine encephalitis virus (Colpitts et al. 2007). In addition, it is necessary for mammalian cell infection of chikungunya virus, one of the closest relatives of ONNV (Bernard et al. 2010).

Also upregulated (39%) was the mammalian Sec62 ortholog (AGAP009788-PA). Sec62 is associated with the Sec61 translocon, responsible for the translocation of nascent proteins across, or into, the endoplasmic reticulum (ER) membrane. In eukaryotes, this is a highly conserved and essential process that occurs either cotranslationally or posttranslationally. By the former route, transport is coupled with translation and is mediated by a signal recognition particle. By the latter route, Hsp70 and Hsp40 chaperone family proteins escort nascent proteins to the translocon where membrane-bound Sec62 and Sec63 together act in lieu of signal recognition particle (Rapoport 2007). Despite its implied role as a host susceptibility factor here, a Hsp70 family protein, hsc70B, has previously been found to be upregulated in response to ONNV infection in *An. gambiae* mosquitoes, and was shown to suppress ONNV replication. Therefore, hsc70B seems to represent a protective cellular response (Sim et al. 2005, 2007). The apparent conflict merits future investigation into the roles of protein translocation pathways in virus infection.

**Poorly Characterized Proteins.** Six poorly characterized proteins were identified that did not cluster well into orthogonal groupings. They were described by domain analysis or with respect to a near ortholog. These include a protein with a Ras family GTPase motif that spans multiple domains (AGAP005161-PA; 49% accumulation in infected versus controls) (Marchler-Bauer et al. 2011), a protein with multiple cadherin domains (AGAP004166-PA; 123% accumulation; E value: ≤4.3e-07), a trypsin-like serine protease (AGAP006707-PA; 108% accumulation; E value: ≤5.9e-04), a *Drosophila* Sallimus ortholog (AGAP007563-PC; 31% accumulation), and two proteins with uncharacterized domains (AGAP003864-PA, downregulated 73%; and AGAP007663-PA, downregulated 72%). The Ras superfamily consists of several subfamilies such as Rab, Rho, and Ras, which are involved in a wide variety of cellular functions. They orchestrate cytoskeletal modifications, gene expression, vesicle trafficking, and others (Marchler-Bauer et al. 2011). The serine proteases are also of central importance to many molecular processes, including the maturation of the alphavirus particle itself (Strauss and Strauss 1994). Their role in mitigating parasite infection of insect gut tissue is well described (Siden-Kiamos et al. 1996, Gorman and Paskewitz 2001). They are involved in a suite of immune responses, including melanotic encapsulation, antimicrobial peptide synthesis, and hemolymph coagulation (Gorman and Paskewitz 2001). Less well described is the importance of these defenses to virus immunity (Woodring et al. 1996).

Little is also known about the role of Sallimus-related proteins or cadherins in virus immunity. Sallimus has several immunoglobulin-like and fibronectin-like domains. This large 2.3-MDa protein is known to be critical to flight muscle function and development (Burkart et al. 2007). Cadherins are calcium-dependent adhesion molecules required for cellular cohesion of multicellular organisms, organization of tissues, and morphogenesis (Hynes and Zhao 2000). They may play a role in flavivirus cell entry (Colpitts et al. 2011). Cadherin expression has also been shown to be suppressed in association with cellular permeability observed during dengue virus infection (Dewi et al. 2008).

In conclusion, these data suggested that ONNV induces the expression modulation of many proteins in *An. gambiae* midgut tissue. The identified proteins represent a wide variety of functional classes of molecules involved in metabolism, information storage, cellular processes, and signaling, and also include novel proteins that bear little similarity to previously described proteins or domains. These results speak to the complex and multifaceted nature of the infection, and the infection mitigation process. Eleven proteins were found in greater abundance in response to ONNV infection, and 11 proteins were found in lesser abundance. The orthologous cluster group with the greatest number of proteins was the group of “poorly characterized” proteins. This suggests that much of the molecular underpinnings of the virus infection process are yet to be uncovered. However, protein pathways such as those involving Rab5, identified here, have long been known to play a role in alphavirus infection. The identification of Rab5 and other orthologs previously shown to be modulated by alphaviruses in mosquitoes validates the TMT proteomics methodology. It also strengthens the arguments for roles of the identified proteins in cellular processes induced by virus infection.

Some considerations must be made when making inferences about the underlying biological processes at work. The use of the genetically encoded reporter molecule, GFP, facilitated the monitoring of ONNV infection in the mosquito midgut. Although GFP-expressing viruses behave like wild-type viruses in vitro and in vivo (Braut et al. 2004, Sanders et al. 2005, Vanlandingham et al. 2005, Tsutsarkin et al. 2006), it
nevertheless cannot be ruled out from these experiments that GFP induced some cellular response. In addition, the sensitive detection of virus-induced cellular changes may be limited by the characteristic focal ONNV infection of the midgut (Fig. 1). Robust and diffuse infection patterns characteristic of other arbovirus infections facilitate the identification of regulated proteins. Although a cell culture system may produce homogeneous cellular responses that are readily detectable, the aim of this investigation was to identify protein changes closely reflecting those occurring in nature. The identification of proteins previously found to be associated with virus infection supports the functionality of this methodology. Although more proteins may have been identified by using cell culture methods, the amount identified in this study was consistent with similar reports (Tchankouo–Nguecheu et al. 2010). Among the 22 proteins identified, the degree of modulation observed was consistently modest. This has been noted elsewhere as being characteristic of MS-based methods such as iTRAQ and TMT (Brewis and Brennan 2010). This may also be the result of the focal, rather than enriched, or diffuse infection. As different proteomics techniques have been shown to complement each other, rather than highlight redundancy (Wu et al. 2006), other studies and approaches will likely benefit the understanding of this system.

Future investigation will characterize these proteins by using RNAi gene silencing in An. gambiae and will describe the role of these proteins in virus infection from the perspective of the pathogen and host. Of the 22 differentially modulated proteins identified here, many offer promising leads toward uncovering mechanisms that govern infection outcomes. Interestingly, our results suggest that more proteins may be at work than those currently known to be involved in classical immune-response pathways like IMD, Toll, RNAi, and Jack-STAT. Modulations in, for example, the TOR/nutrient sensing pathway (via FKBP1, H1/H5, and Ppp4), may significantly contribute to the mechanisms that control virus infections. The subtle and complex nature of arbovirus infection processes emphasizes the importance of studying these systems from many approaches and perspectives, including those involving the arthropod phase of the pathogen lifecycle, to identify new strategies of control and treatment of these diseases.

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