

Global gene expression analysis of *Anopheles gambiae* responses to microbial challenge

Ruth Aguilar, Anne E. Jedlicka, Margaret Mintz, Vassiliki Mahairaki,
Alan L. Scott, George Dimopoulos*

*W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health,
Johns Hopkins University, 615 North Wolfe Street, Baltimore, MD 21205-2179, USA*

Accepted 11 February 2005

Abstract

Anopheles gambiae transcript responses to experimental challenge with heat inactivated *Salmonella typhimurium*, *Staphylococcus aureus* and *Beauveria bassiana* have been analyzed with an Affymetrix GeneChip comprising the entire predicted mosquito transcriptome. Significant up- or down-regulation (greater than 2-fold) can be assayed for approximately 2% of the mosquito transcriptome and affected genes represent a variety of functional classes that include immunity, apoptosis, stress response, detoxification, metabolism, blood digestion, olfaction and others. Transcript responses to the 3 microbial elicitors exhibit an exceptionally high degree of specificity and only a few genes are significantly regulated by more than 1 of the tested elicitors. This study identifies several transcripts that have not been linked directly to immune response in *A. gambiae* previously; their infection responsiveness and sequence features do however suggest implication in defence reactions; examples are genes encoding leucine-rich repeat domain proteins, cuticle domain proteins and proteins containing immunoglobulin and fibronectin domains.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: Anopheles; Transcriptome; Immune response

1. Introduction

Anopheles gambiae, the major vector of malaria, has a sophisticated and multifaceted immune system consisting of a pathogen recognition system that comprises a variety of proteins such as lectins, fibrinogen domain proteins, Gram-negative bacteria binding proteins (GNBPs) and other components capable of binding to pathogen surface patterns. A signal modulation and amplification system, consisting of serine proteases and serine protease inhibitors, transduces the pathogen recognition signal to activate defence mechanisms directly or indirectly through intracellular signal transduction pathways that control transcription of immune

effector genes. The insect innate immune system is specific and capable of activating different defence mechanisms upon challenge with different pathogens. This specificity is mainly determined by the pattern recognition repertoire and downstream signaling pathways (Dimopoulos, 2003; Osta et al., 2004). Activation of the immune defence components and mechanisms in *A. gambiae* is to a large extent controlled at the transcriptional level and can therefore be studied with gene arrays that assay differences in mRNA abundance for the entire transcriptome between challenged and non-challenged mosquito samples (Dimopoulos et al., 2002). An Affymetrix *Plasmodium/Anopheles* Genome Array: GeneChip™ comprising the entire *A. gambiae* transcriptome has been utilized to assay the adult female mosquito responses to independent injections with a Gram-negative and a Gram-positive bacterium, and a fungus. These analyses establish the microbial challenge

*Corresponding author. Tel.: +1 443 287 0128;
fax: +1 410 95 50105.

E-mail address: gdimopou@jhsph.edu (G. Dimopoulos).

responsive transcript repertoires for each elicitor and the degree of specificity of these response patterns, which are only minimally overlapping.

2. Materials and methods

2.1. Mosquito rearing and challenge

A. gambiae Keele strain mosquitoes were maintained on sugar solution at 27°C and 70% humidity. Approximately 20,000 heat inactivated *Salmonella typhimurium* and *Staphylococcus aureus*, and 1000 heat inactivated *Beauveria bassiana* spores mixed with hyphae, were injected separately into the hemolymph of 20 cold anaesthetized 4-day-old female mosquitoes, in a 50 nl PBS suspension using a microcapillary Nanoject II injector (Drummond). Concentrations of bacteria and fungi were estimated based on OD and counting with light microscopy. Control mosquitoes were injected with 50 nl sterile PBS. RNA was extracted 4 h after challenge from whole mosquitoes using the RNeasy kit (QIAGEN).

2.2. GeneChip assays

Quantification of RNA was performed using a Beckman DU640 spectrophotometer, and quality assessment was determined by RNA Nano LabChip analysis on an Agilent Bioanalyzer 2100. Processing of templates for GeneChip™ analysis was done in accordance with methods described in the Affymetrix GeneChip™ Expression Analysis Technical Manual, Revision Three, including the following specifications. Double stranded cDNA was synthesized from 7.5 µg of total RNA and was purified by phenol/chloroform extraction followed by ethanol precipitation. Linearly amplified complementary mRNA (cmRNA) was synthesized from one half of the double-stranded cDNA by in vitro transcription (IVT) using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Life Sciences). Resultant cmRNAs were purified by column purification with the GeneChip™ Sample Cleanup Module (Affymetrix), and quantified. The cmRNA (15 µg) was fragmented by metal-induced hydrolysis. Aliquots of pre- and post-fragmentation cmRNAs were quality assessed by RNA Nano LabChip analysis on an Agilent Bioanalyzer 2100. Hybridization cocktails were prepared as recommended for arrays of “Standard” format including incubation at 94°C for 5 min and 45°C for 5 min, and centrifugation at maximum speed for 5 min prior to hybridization to the GeneChips™ (Affymetrix *Plasmodium/Anopheles* Genome Array: www.affymetrix.com/support/technical/other/genechip_system_brochure.pdf). Hybridization was performed at 45°C for 16 h at 60 rpm in the Affymetrix rotisserie hybridization oven. The signal amplification protocol for washing and

staining of eukaryotic targets was performed in an automated fluidics station (Affymetrix FS450) as described in the Affymetrix Technical Manual, Revision Three. The arrays were then scanned with the GCS3000 laser scanner (Affymetrix) at an emission wavelength of 570 nm at 2.5 µm resolution. The intensity of hybridization for each probe pair was computed by GCOS 1.1 software. For more detailed methods, please refer to the websites of the Malaria Research Institute Gene Array Core Facility (MRI-GACF) at the Johns Hopkins Bloomberg School of Public Health (<http://jhmmi.jhsph.edu/>), or the Malaria Research and Reference Reagent Resource Center (MR4) (<http://www.malaria.mr4.org>).

2.3. Data analysis

Primary analysis consisted of a quality assessment of the hybridization of each sample. Ratios of signal for probe sets at 5' and 3' regions of housekeeping genes were calculated and monitored as an indication of transcript quality for each sample. The hybridization intensity for each probe array was analyzed through an expression algorithm which designates each transcript as present (P), absent (A) or marginal (M).

All assays were biologically replicated 3 times and global mean normalization was performed for all sets (elicitor challenged and PBS injected) independently prior to calculation of the elicitor challenge/PBS injected intensity ratio. Only transcripts with at least 2 replica signal values were considered for the analysis. The 3 replica ratios (elicitor challenged/PBS injected) for each transcript were averaged after removing inconsistent replicas (those differing by greater than 0.7 from the median of replicate ratios) to generate the final dataset. Normalizations were done using the SNOMAD (Standardization and Normalization of MicroArray Data) gene expression data analysis tools (<http://pevsnerlaboratorykennedykrieger.org/snomadinput.html>). The 3 replica ratios for each transcript were averaged using the GEPAS (Gene Expression Pattern Analysis Suite v 1.1) tools (<http://gepas.bioinfo.cnio.es/tools.html>).

3. Results and discussion

Global transcript responses of adult female *A. gambiae* were assayed using a full genome Affymetrix GeneChip at 4 h after injection of heat inactivated *S. typhimurium*, *S. aureus* and *B. bassiana*. Challenge with the 3 microbial species resulted in distinct and only partially overlapping transcript responses of greater than a 2-fold up- or down-regulation of genes compared to PBS injected control mosquitoes (Fig. 1). Injection with *S. typhimurium*, *S. aureus* and *B. bassiana* resulted

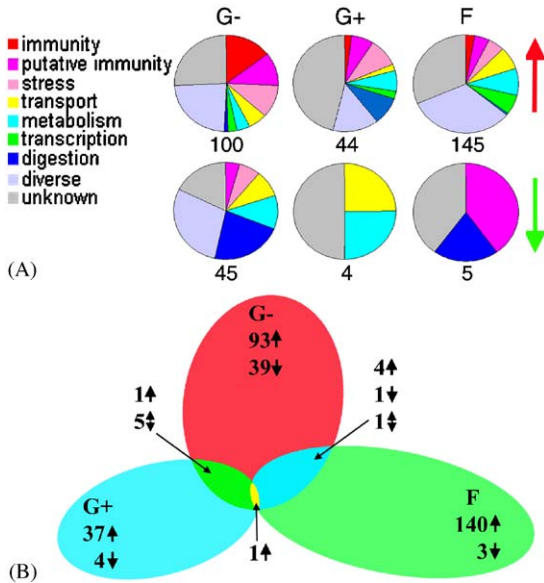


Fig. 1. (A) Pie charts indicate relative proportions of up-regulated (upper pies) and down-regulated (lower pies) functional gene groups in mosquitoes challenged with the Gram-negative bacterium *S. typhimurium* (G⁻), the Gram-positive bacterium *S. aureus* (G⁺) and the fungus *B. bassiana* (F). The total number of genes represented by each pie chart is indicated below each pie. Functional groups and their corresponding color in the pie charts are indicated. (B) Proportions of regulated genes upon challenge with the Gram-negative bacterium *S. typhimurium* (G⁻), the Gram-positive bacterium *S. aureus* (G⁺) and the fungus *B. bassiana* (F). Numbers of up- and down-regulated genes are indicated with arrows pointing up and down, respectively. Significantly regulated genes by 2 or more elicitors are indicated in the overlapping sections of each ellipse.

in significant up- or down-regulation (≥ 2 -fold) of 145, 48 and 150 genes, respectively. As much as 84.5% of the significantly regulated genes were induced; injection with *S. typhimurium* induced 69% of the total regulated genes, while challenge with *S. aureus* and *B. bassiana* resulted in the up-regulation of 91% and 96% of genes, respectively (Fig. 1). The Gram-negative bacterium *S. typhimurium* induced the largest number of immune genes and genes with likely implication in immunity that corresponded to approximately 25% of the total number of induced genes, while immune genes induced by *S. aureus* and *B. bassiana* represented only 11.4% and 7.6% of the total, respectively. Interestingly, only 1 gene (unknown function) was significantly induced (above a 2-fold) by all 3 elicitors. The small overlap reflects a high degree of response specificity. Previous microarray studies of *A. gambiae* cell line transcript responses to challenge with *S. typhimurium* and *S. aureus* showed a significantly greater overlap (Dimopoulos et al., 2002). In addition, a greater than 2-fold up- or down-regulation was detected for 329 genes, or approximately 2% of the *A. gambiae* transcriptome, upon challenge with at least 1 of the 3 tested elicitors. This is a significantly smaller number of transcripts

regulated by immune challenge of female mosquitoes compared to previous studies with *A. gambiae* immune competent cell lines. The high degree of specificity and relatively small number of regulated genes is most probably attributed to a lower dose of elicitors, their attenuated nature, the short duration of challenge and the use of whole mosquitoes. Regulation of many genes in specific cell types and organs is likely to be masked by the overall high constitutive expression levels in other tissues when transcription is assayed in the whole mosquito. Furthermore, the elicitors may not fully induce the entire repertoire of immune responsive genes at only 4 h after challenge; earlier studies have shown a gradual increase of up-regulated genes up to 12 h after microbial challenge of adult mosquitoes (Dimopoulos et al., 2002). Transcription analyses of multiple time points after challenge can provide information on the temporal kinetics of gene regulation. Microarray transcription analyses of infection responses in *Drosophila melanogaster* have shown high dependence of response patterns on the actual route of infection (i.e. whether the elicitor is injected into the hemocoel or fed to the insect) (De Gregorio et al., 2001; Roxstrom-Lindquist et al., 2004). Infection-responsive genes, identified in this study, belong to the following major functional classes: immunity, stress response, transport, metabolism, transcription, digestion and other diverse classes (Fig. 1).

3.1. Elicitor specificity

The *S. typhimurium* challenge induces the largest number of immune genes and stress responsive genes that included 7 putative pattern recognition proteins represented by a GNBP, 4 fibrinogen domain immunolactins (FBN), a peptidoglycan recognition protein S1 (PGRPS1) and a thioester-containing protein 1 (TEP1). Several components of serine protease cascades are induced and include 5 immunity-related serine proteases and a serine protease inhibitor (serpin). Lysozyme, cecropin and a hemomucin-like transcript are also induced by *S. typhimurium*, and 2 components with likely implication in apoptotic processes are down-regulated (discussed below). Among the induced genes with likely implication in immune response are 5 cuticle domain encoding transcripts. The *S. typhimurium* challenge repressed expression of 10 midgut genes encoding for enzymes implicated in blood digestion and components of the peritrophic matrix. Nine putative stress responsive proteins and enzymes are induced by challenge and 3 are repressed. Other components that are regulated by *S. typhimurium* challenge belong to diverse functional classes (Table 1).

The *S. aureus* challenge induces only 1 immunity gene that encodes a serine protease transcript (ISPR10). Other induced genes that encode for components with possible function in defence include 2 putative

Table 1

Genes discussed in the text are indicated with their fold up- or down-regulation as a measure of normalized challenge/control intensity ratio for each challenge: the Gram-negative bacterium *S. typhimurium* (*S.t.*), the Gram-positive bacterium *S. aureus* (*S.a.*) and the fungus *B. bassiana* (*B.b.*)

Gene ID	Gene name	<i>S.t.</i>	<i>S.a.</i>	<i>B.b.</i>	References	Method
<i>Immunity</i>						
ENSANGT00000011248	Fibrinogen domain immunoelectin (FBN9)	5.4			Christophides et al. (2002)	Microarray
ENSANGT00000022867	Fibrinogen domain immunoelectin (FBN _L -1)	2.9				
ENSANGT00000022610	Fibrinogen domain immunoelectin (FBN _L -2)	2.5				
ENSANGT00000026193	Fibrinogen domain immunoelectin (FBN _L -3)	2				
ENSANGT00000017320	Peptidoglycan recognition protein S1 (PGRPS1)	2.1			Christophides et al. (2002)	Microarray
ENSANGT00000016857	Thioester containing protein 1 (TEP1)	2.2			Levashina et al. (2001)	RT-PCR
ENSANGT00000020260	Gram-negative bacteria binding-protein A1(GNBPA1)	2.3			Christophides et al. (2002)	Microarray
ENSANGT00000017017	Gram-negative bacteria-binding protein 2 (GNBPB2)			3.2		
ENSANGT00000029388	C-type lectin domain (LECD)			2.8		
ENSANGT00000021259	Serine protease ISPR5 (ISPR5)	2.7			Oduol et al. (2000)	Northern blot
AF203338.1	Serine protease ISPR10 (ISPR10)	4.6	2		Oduol et al. (2000)	cDNA library
ENSANGT00000012930	CLIPB6: serine protease (CLIPB6)	2				
ENSANGT00000022596	CLIPB8: serine protease (CLIPB8)	2				
ENSANGT00000015815	CLIPB15: serine protease (CLIPB15)	2.1			Christophides et al. (2002); Kumar et al. (2003)	Microarray
ENSANGT00000021651	Serpin 1 (SPN1)	2.2				
ENSANGT00000025070	Lysozyme (LYS)	3.1			Kang et al. (1996)	RT-PCR
ENSANGT00000011957	Cecropin A (CEC)	2.9		2.3	Dimopoulos et al. (2000); Vizioli et al. (2000)	RT-PCR
ENSANGT00000026771	Transferrin (TRF)			2.2		
<i>Putative immunity</i>						
ENSANGT00000013220	Cuticle protein (CUT-1)	3.2		-2.5		
ENSANGT00000014314	Cuticle protein (CUT-2)	2.7				
ENSANGT00000014823	Cuticle protein (CUT-3)	2				
BM575515	Cuticle-like protein (CUT _L)	3.2				
ENSANGT00000027189	Cuticle protein domain (CUTd)	2.2		2.4		
ENSANGT00000008497	Hemomucin-like (HEM _L)	2.4			Dimopoulos et al. (2000)	RT-PCR
ENSANGT00000016208	Ig-like and fibronectin domains (IG&FNd)		2.3			
ENSANGT00000019113	Leucine-rich repeat domain protein (LRRd-1)	-2.2				
ENSANGT00000015763	Leucine-rich repeat domain protein (LRRd-2)			2.2		
ENSANGT00000022325	Angiotensin-converting enzyme (ACE-1)	2.4				
ENSANGT00000010993	Angiotensin-converting enzyme (ACE-2)		3.5			
ENSANGT00000028617	Angiotensin-converting enzyme (ACE-3)			-2.4		
ENSANGT00000012556	Phospholipase A2 domain (PLAA2d)			2.2		
ENSANGT00000000545	Phospholipase D1 (PLAD1)			2.1		
ENSANGT00000013457	Ubiquitin linking IAP with cytoskeleton (PLIC)			2.5		
ENSANGT00000020517	Serine protease, gastrulation defective (SPGD)			2		
ENSANGT00000015870	Serine protease (SP-1)	2.4				
ENSANGT00000015619	Serine protease (SP-2)	2				
ENSANGT00000028603	Serine protease (SP-3)	-2.3				
ENSANGT00000025969	Putative serpin (SPN _L)			2.1		
ENSANGT00000013093	Saposin B domain (SAPBd)	2.6				
ENSANGT00000028106	Apolipoprotein D (APOD)		3.3			
ENSANGT00000018613	MAP kinase (MAPK)		2.3			

<i>Apoptosis</i>					
ENSANGT00000021367	Caspase 6 (CASPS6)	–2			
ENSANGT00000019358	Acid sphingomyelinase phosphodiesterase (ASP)	–10	2.7		
<i>Stress</i>					
ENSANGT00000004895	Putative esterase (EST _L -1)	–12.5			
ENSANGT00000018571	Putative esterase (EST _L -2)		2.5		
ENSANGT00000013097	Glutathione S-transferase D11 (GSTD11-1)	2.5		Kumar et al. (2003)	Microarray
ENSANGT00000013141	Glutathione S Transferase D11 (GSTD11-2)	–2.2			
ENSANGT00000017077	GST domains (GSTd)	2.4			
BM583595	Thioredoxin-like protein (TRX _L -1)			2	
ENSANGT00000019750	Thioredoxin domain (TRXd-1)			2.2	
ENSANGT00000019185	Thioredoxin domain (TRXd-2)			2.5	
ENSANGT00000020140	Thioredoxin domain (TRXd-3)	2.5			
ENSANGT00000010558	Cytochrome P450 (CYP450-1)	2.1			
ENSANGT00000001701	Cytochrome P450 (CYP450-2)			2	
ENSANGT00000019843	Cytochrome P450 (CYP450-3)	–2.4			
ENSANGT00000012893	Heat shock protein Hsp70 (HSP70)	2.5			
ENSANGT00000015826	Heat Shock protein (HSP-1)	4.2		Dimopoulos et al. (2002)	Microarray
ENSANGT00000022059	Heat shock protein (HSP-2)	2.8			
ENSANGT00000018254	Heat shock protein (HSP-3)	2.4			
ENSANGT00000011260	Heat shock protein (HSP-4)		2.1		
ENSANGT00000020201	Peroxiredoxin (PRX)	2.4			
BM629523	Cu/Zn-superoxide dismutase (SOD)			2.3	
<i>Digestion</i>					
ENSANGT00000013945	Serine protease (SP-4)	–6.7	2.4		
ENSANGT00000024468	Serine protease (SP-5)		2.1		
ENSANGT00000006721	Trypsin 3 precursor (TRY-3)	–3.3			
ENSANGT00000018384	Trypsin 4 precursor (TRY-4)	–2.3			
ENSANGT00000018317	Trypsin 5 precursor (TRY-5)	–2	2.4		
ENSANGT00000018354	Trypsin 6 precursor (TRY-6)	–2.3			
ENSANGT00000018316	Trypsin 7 precursor (TRY-7)	–7.7	2.9		
BM649829	Chymotrypsin 2 (CHYM2)	–3.6			
ENSANGT00000021092	Chymotrypsin serine protease (CHYMd)	–2.5			
ENSANGT00000014953	Triacylglycerol lipase gastric precursor (TGL)	–2.2			
ENSANGT00000014496	Chitin-binding domain, peritrophin-A (PERd-1)	2.7			
ENSANGT00000015766	Chitin-binding domain, peritrophin-A (PERd-2)	–2.6			
ENSANGT00000018877	Chitin-binding domain, peritrophin-A (PERd-3)			2.7	
BM587345	Mucin-like peritrophin (MUC)			–2	
ENSANGT00000012761	Lipase (LIP)		2		
<i>Chemosensory</i>					
ENSANGT00000015595	Odorant-binding protein-4 (OBP4)			2.2	
ENSANGT00000012251	Odorant-binding protein-7 (OBP7)			2.1	
ENSANGT00000018364	OBP domain (OBPd-1)	–2.3			
ENSANGT00000018441	OBP domain (OBPd-2)			2.2	

Only genes that were up- or down-regulated greater than 2-fold are shown; fold up-regulation is indicated by positive numbers and fold down-regulation is indicated by negative numbers. Abbreviations have been assigned to genes that lacked specific identifiers in the mosquito genome database ENSEMBL. Putative functions have been assigned to genes according to gene annotation data from the mosquito genome database ENSEMBL. Genes have been grouped in the following functional groups: immunity, putative immunity, apoptosis, stress, digestion, chemosensory. References indicate published documentation of infection responsive regulation of genes through Northern, microarray or RT-PCR analyses, or isolation from infection specific subtraction libraries.

immune-related serine proteases, an angiotensin converting enzyme, a protein with an immunoglobulin-like domain and a fibronectin domain, a member of the apolipoprotein family that has been implicated in phagocytosis and a MAP kinase (discussed below). A transcript encoding for an acidic sphingomyeline phosphodiesterase with possible implication in immune and apoptotic processes is induced. Induction of an esterase domain protein and a heat shock protein indicate stress and detoxification response. *S. aureus* challenge also induces 4 blood digestive enzymes of which 3 are repressed by the *S. typhimurium* challenge (Table 1) (discussed below). Only 4 genes are down-regulated by *S. aureus* and encode for proteins of diverse functions.

B. bassiana challenge induces 4 immune genes that include GNBPB2, a C-type lectin, the antimicrobial peptide cecropin and a transferrin. Seven genes with likely implication in immune response are also induced and include a cuticle domain protein, a leucine-rich repeat domain protein, 2 phospholipases and a ubiquitin linking integrin associated protein (IAP) with cytoskeleton, a putative immune serine protease, SPGD, and a putative serpin (discussed below). The *B. bassiana* challenge induces 3 odorant binding protein transcripts and significantly down-regulates 5 genes that include 2 putative immune components; a cuticle protein transcript, an angiotensin-converting enzyme-related carboxypeptidase transcript, and a putative peritrophic matrix component; a mucin-like peritrophin (discussed below).

S. typhimurium and *S. aureus* induce 2 common transcripts of which 1 is the immunity-related serine protease ISPR10. *S. typhimurium* and *B. bassiana* induce 5 common genes of which 2 are likely to be implicated in immune defence and encode a cuticle protein and the antimicrobial peptide cecropin. *S. aureus* and *B. bassiana* induce only common transcript that is of unknown function (Table 1). The lack of regulatory overlap between *S. aureus* and *B. bassiana* strongly suggest that these 2 elicitors activate distinct immune gene activation pathways and the mosquito is largely utilizing different defence components and mechanisms to defend against them (discussed below).

3.2. Specific gene classes

3.2.1. Pathogen recognition proteins

Infection-responsive pattern recognition receptors include PGRPS1, TEP1, GNBPA1, GNBPB2, a C-type lectin, LECd, 4 FBNs, FBN9, FBN_L-1, FBN_L-2 and FBN_L-3, and a hemomucin-like protein, HEM_L (Table 1). The differential induction of GNBPA1 and GNBPB2 upon challenge with *S. typhimurium* and *B. bassiana* suggest them being implicated in different immune gene activation pathways. The *A. gambiae* genome harbors 6 GNBPs of which 1 has been

characterized and shown to be up-regulated by *Plasmodium* infection in the midgut, fat body and salivary glands (Dimopoulos et al., 1997). A GGBP has been linked to the activation of the Toll pathway together with a PGRP in *D. melanogaster* (Kim et al., 2000; Gobert et al., 2003). TEP1 is implicated in phagocytosis of bacteria and antiplasmodial defence at the stage of ookinete invasion of the mosquito midgut (Levashina et al., 2001; Blandin et al., 2004). The *A. gambiae* genome encodes 7 C-type lectins that include CTLMA4 and CTL2 which are utilized by *Plasmodium* to evade the mosquito's immune system (Osta et al., 2004). C-type lectins play various roles in both vertebrate and invertebrate immune system. The *A. gambiae* genome harbors as many as 57 putative FBNs that share similar domain organization and sequences with the horseshoe crab Tachylectins 5A and 5B that have been implicated in enhancement of antimicrobial activity (Adema et al., 1997; Gokudan et al., 1999). The vertebrate ficolins, which are implicated in phagocytosis and complement activation, also contain fibrinogen domains in their carboxy terminus. The *D. melanogaster* genome harbors only 13 FBN lectins; the massive expansion of this gene family in *A. gambiae* suggests a significant role in the mosquito's immune system (Christophides et al., 2002; Zdobnov et al., 2002). A recent study in the mosquito *Armigeres subalbatus* describes a novel fibrinogen domain-containing lectin (aslectin or AL-1) that is up-regulated following *Escherichia coli* and *Micrococcus luteus* challenge; AL-1 binds to both microbes suggesting a function as a pattern recognition receptor (Wang et al., 2004). All 3 putative FBN immunoelectins are specifically induced by the *S. typhimurium* challenge, suggesting implication in the same response process. The FBN gene family is currently under intense analysis towards the elucidation of its function in *A. gambiae* (Dong and Dimopoulos, in preparation). Hemomucin has a modular domain organization consisting of a mucin domain and strictocidin domain, and has been shown to be implicated in immune response activation upon interaction with a snail lectin (Theopold et al., 1996).

3.2.2. Serine protease cascade components

Five immune-related serine proteases with clip domains, ISPR5, ISPR10, CLIPB6, CLIPB8 and CLIPB15, and a serpin are up-regulated upon challenge with *S. typhimurium*; suggesting a robust activation of immune response amplification signals (Jiang and Kanost, 2000). A putative serpin, SPN_L, is also induced by challenge with *B. bassiana*. The induction of serine protease inhibitors upon immune challenge suggests a role in protecting the mosquito from the detrimental effects of an uncontrolled spread of defence reactions (Dimopoulos, 2003). AgISPR5 expression is also induced upon ookinete invasion of the midgut (Oduol et al., 2000). One serine protease gene, SPGD, with

significant similarity to the *D. melanogaster* developmental enzyme gastrulation defective, is induced upon *B. bassiana* challenge; immune pathways and developmental pathways frequently share components in invertebrates and immune-related serine proteases share structural features to those implicated in development. Four putative immune serine proteases, SP-1, SP-2, SP-3 and SP-4, are differentially induced and repressed by *S. typhimurium* and *S. aureus* challenges. The SP-4 protease has been described in *A. gambiae* as a gut-specific serine protease, it is down-regulated after blood ingestion and its similarity to the infection-responsive serine protease IRSP13 suggests a possible role in immunity (Dimopoulos et al., 1996; Shen et al., 2000). The other 3 proteases, SP-1, SP-2 and SP-3 have not been described as immune-related, but their infection responsiveness and sequence similarity to known immunity-related serine proteases suggest that they function in antimicrobial defence (Table 1).

3.2.3. Antimicrobial components

A lysozyme gene is specifically induced by the Gram-negative bacterium *S. typhimurium* challenge, while a cecropin is induced by *S. typhimurium* and *B. bassiana* challenge (Table 1). The *A. gambiae* lysozyme gene is expressed at high levels in sugar fed adult females and its upstream genomic DNA sequence contain several NF-kappa B-like motifs that are characteristic for immune inducible genes. Lysozyme genes from both *Aedes aegypti* and *Aedes albopictus* are inducible by microbial challenge (Kang et al., 1996; Hernandez et al., 2003). Cecropins are small (3–5 kDa), basic, helical peptides that have been isolated from both insects and mammals. *A. gambiae* cecropin is active against numerous Gram-negative and Gram-positive bacteria, as well as several species of filamentous fungi and yeasts. The gene is expressed at high levels in the midgut and other tissues (Vizioli et al., 2000). Other proteins with likely antimicrobial activities are discussed below.

3.2.4. Apoptosis related components

Transcription of the *A. gambiae* caspase 6 orthologue is repressed in response to challenge with *S. typhimurium*. The apoptotic machinery consists of pro-apoptotic and antiapoptotic regulators. Caspase 6 is a S-prodomain pro-apoptotic effector caspase involved in triggering cell death (Christophides et al., 2002). An acidic sphingomyelinase phosphodiesterase is induced and repressed by the 2 bacterial elicitors (Table 1). Acidic sphingomyelinase phosphodiesterases (ASP) are implicated in ceramide metabolism and the regulation of apoptotic pathways and defence against bacterial pathogens in vertebrates. Several ASP homologues in *A. stephensi* are induced in the midgut epithelium at the time of ookinete invasion, when epithelial cells undergo apoptosis. Their infection-responsive expression in *A. gambiae* suggests a role in

immune response and possibly also apoptosis (Han et al., 2000; Cuvillier, 2002; Xu et al., 2005).

3.2.5. Cuticle domain proteins

Induction of 5 transcripts encoding for cuticle proteins, CUT-1, CUT-2 and CUT-3, and cuticle domain-containing proteins, CUT_L, and CUT_d, suggest that these proteins are a part of the mosquito defence response (table 1). An obvious role is wound healing and it is likely that the presence of certain bacterial species enhances this response; hence the stronger up-regulation of these genes in bacteria-challenged mosquitoes compared to PBS injected control mosquitoes (Lundstrom et al., 2002; Jiang et al., 2004). The cuticular exoskeleton of the giant silkworm moth, *Hyalophora cecropia*, has been shown to actively participate in defence. Abrasions and challenge of the ectodermally derived integument will lead to transcriptional activation of the antimicrobial cecropin gene (Brey et al., 1993). An in vitro study showed that certain cuticular proteins are implicated in non-self-recognition of *E. coli* through binding to its surface (Marmaras et al., 1993). Cuticle proteins are also expressed by hemocytes; one of the major immune competent cell types in invertebrates (Muñoz et al., 2002; Bartholomay et al., 2004).

3.2.6. Stress and detoxification components

Two putative esterases, EST_L-1 and EST_L-2, 3 glutathione S-transferases, GSTD11-1, GSTD11-2 and GST_d, 3 cytochromes P450, CYP450-1, CYP450-2 and CYP450-3, and 4 thioredoxin domain proteins, TRX_L-1, TRX_d-1, TRX_d-2 and TRX_d-3, are most likely essential detoxification components, and some are induced and other are repressed by challenge with the 3 elicitors. Molecular components of microbes may have toxic effects and can interfere with cell functions in the mosquito and thereby cause these stress responses which have been previously documented in a microbial challenged *A. gambiae* cell line (Table 1) (Dimopoulos et al., 2002).

3.2.7. Blood digestion enzymes and peritrophic matrix components

Several genes encoding for enzymes implicated in blood digestion, including 2 putative chymotrypsins, CHYM2 and CHYM_d, 5 trypsins, TRY-3, TRY-4, TRY-5, TRY-6 and TRY-7, a triacylglycerol lipase and a serine protease, SP-4, are down-regulated by challenge with the Gram-negative bacterium *S. typhimurium* while 5 of these genes, TRY-5, TRY-7, SP-4, SP-5 and a lipase, are up-regulated by challenge with *S. aureus* (Table 1) (Muller et al., 1993; Vizioli et al., 2001). Infection-responsive regulation of blood digestive enzyme genes is intriguing and the biological significance not clear. A plausible explanation may be that *S. typhimurium* and *S. aureus* challenge induced pathways

may interfere with the transcription regulatory networks of blood digestive enzymes, or that these genes play additional roles in immunity. Three chitin-binding domain-containing peritrophin genes, PERd-1, PERd-2 and PERd-3, and a mucin-like peritrophin, MUC, are differentially induced and repressed by the different elicitors (Table 1). These proteins are components of the peritrophic matrix in the midgut lumen, which forms upon blood feeding and protects the epithelium from direct contact with the blood that could cause abrasions. The peritrophic matrix also protects the epithelium from pathogens (Shao et al., 2001). Transcriptional regulation of these peritrophic matrix proteins may suggest additional roles in immunity. Chitin-binding domains have been found in plant and arthropod defence molecules and are believed to be involved in binding to microbial carbohydrate surfaces. Invertebrate proteins containing mucin domains have been linked to defence mechanisms in previous studies (Pearson et al., 1995; Kawabata et al., 1996; Theopold et al., 1996; Nielsen et al., 1997; Dimopoulos et al., 1998; Muñoz et al., 2002).

3.2.8. Chemosensory components

Three components of the mosquito's olfactory system: 2 odorant-binding proteins, OBP4 and OBP7, and an OBP domain-containing protein, OBPd-2, are significantly up-regulated upon challenge with *B. bassiana*. One OBP domain protein, OBPd-1, is down-regulated by the *S. typhimurium* challenge (Table 1). Odorant-binding proteins, similar to pattern recognition receptors in immunity, are also involved in recognition of non-self molecular patterns and the triggering of signal cascades (Zhou et al., 2004). Infection response of these components may indicate a link between the olfactory system and the immune system in invertebrates. Alternatively, bacterial components can be sensed as odorants and stimulate the mosquito olfactory system. A recent gene discovery project, based on *Aedes aegypti* and *Armigeres subalbatus* immune response-activated hemocyte cDNA libraries, identified a sensory appendage protein (Bartholomay et al., 2004).

3.2.9. Other genes with likely implication in immune responsive processes

An infection-responsive saposin-like transcript is likely to represent an antimicrobial factor. This idea is supported by previous studies that describe other saposin-like polypeptides as immune effector molecules (Bruhn et al., 2003; Nicholas and Hodgkin, 2004). Another infection-responsive gene encodes a protein that contains immunoglobulin and fibronectin domains. These types of domains are also found in other invertebrate immunity-related protein genes and are likely to be implicated in pathogen binding (Brown et al., 2001; Zhang et al., 2004). Two leucine-rich repeat

domain proteins, LRRd-1 and LRRd-2, are down- and up-regulated, respectively, by challenge with *S. typhimurium* and *B. bassiana*. Leucine-rich repeat (LRRs) domains are 20–29-residue sequence motifs present in tandem arrays in a variety of proteins with diverse functions, such as hormone–receptor interactions, enzyme inhibition, cell adhesion, apoptosis signaling and immune response. The ectodomains of Toll-like receptors (TLRs) contain LRRs (Christophides et al., 2002). An *A. gambiae* leucine-rich domain protein, LRIM1, was recently linked to antiplasmodial defence at the stage of ookinete invasion of the midgut (Osta et al., 2004). Two putative phospholipase transcripts, PLAA2d and PLAD1, are up-regulated upon challenge with *B. bassiana*. Phospholipases have been implicated in phagocytosis of bacteria in earlier studies (Downer et al., 1997; Lord et al., 2002). Two transcripts encoding angiotensin converting enzyme genes, ACE-1 and ACE-2, are induced upon challenge with *S. typhimurium* and *S. aureus*, and one ACE-3, is repressed upon challenge with *B. bassiana*. Homologues in *Locusta migratoria* are highly induced in hemocytes upon challenge with bacterial lipopolysaccharides, suggesting a role in immunity (Macours et al., 2003). An apolipoprotein transcript is induced upon challenge with *S. aureus*. Insect apolipoprotein III has diverse roles in immunity including pattern recognition, encapsulation reactions, stimulation of antimicrobial peptide production and phagocytosis (Whitten et al., 2004). An ubiquitin linking IAP with the cytoskeleton is induced upon challenge with *B. bassiana*. This and other proteins that link IAPs with the cytoskeleton are implicated in cellular adhesion and phagocytic processes in vertebrates (Prieto et al., 1989; Wu et al., 1999). Another gene induced by *B. bassiana* challenge is transferrin. Immune challenge of *Aedes aegypti* and *Aedes albopictus* cell lines strongly induces a transferrin gene which therefore was suggested to play a role as an acute phase protein implicated in limiting bacterial growth by sequestering iron (Yoshiga et al., 1997). Infection-responsive transcription of a MAP kinase suggests implication in immune signal transduction pathways (Table 1) (Naitza and Ligoxygakis, 2004).

4. Conclusions

The small overlap in gene transcriptional regulation between the Gram-positive bacterium *S. aureus* and the fungus *B. bassiana* is surprising considering studies in *D. melanogaster* that link the challenge with Gram-positive bacteria and fungi to the activation of the Toll pathway, while Gram-negative bacteria are more specific for the activation of the Imd pathway (Hoffmann and Reichhart, 2002). Comparative genome sequence analysis between *A. gambiae* and *D. melanogaster* strongly

suggest that these pathways are highly conserved between the 2 species and functional in the mosquito. However, the pattern recognition repertoires, that discriminate between the different microbial classes and activate specific downstream signaling pathways, have diverged significantly between the 2 insects and may therefore be responsible for altered pathway activation specificity. It is also possible that different microbial species within a certain class can differentially activate pathways of the innate immune system (Christophides et al., 2002).

The majority of regulated genes does not belong to the known immunity or stress responsive classes and most likely represent adjustments of other physiological systems to the altered biochemical environment of the mosquito hemolymph caused by microbial challenge (Fig. 1). Mosquitoes were challenged for a relatively short time with a low dose of dead microorganisms to increase the degree of elicitor specific transcriptional regulation of immune genes. A longer exposure with a higher dose of live microbes may have resulted in activation of a larger number of genes and a less specific response for each elicitor. Previous microarray studies of *A. gambiae* transcript responses to septic injury showed a gradual increase in the number of up- and down-regulated genes during the first 12 h after challenge (Dimopoulos et al., 2002).

Immune gene regulation can differ significantly between different tissues and cell types and it will therefore be important to assess transcriptomic regulation in the mosquito's major immune competent tissues such as the fat body, midgut and hemocytes, in order to assign specific defence components and mechanisms to specific tissues and determine the complete infection-responsive repertoire of *A. gambiae* (Dimopoulos et al., 1998; Tzou et al., 2000). Finally, analyses of *A. gambiae* responses to *Plasmodium* infection will provide essential information on anti-*Plasmodium* defence mechanisms and reveal their relations to antimicrobial immune pathways and defence mechanisms.

Acknowledgements

This work has been supported by a Malaria Research Institute Pilot Grant and a New Scholars Award on Global Infectious Disease from the Ellison medical Foundation. The expertise, facilities, and instrumentation for Affymetrix GeneChip experimentation and analyses are provided and supported by the Johns Hopkins University Malaria Research Institute. We thank Dr. Jose' Ribeiro for help with gene annotation, the members of Dimopoulos lab for fruitful discussions.

Appendix A. Supplementary Materials

The online version of this article contains additional supplementary data. Please visit [doi:10.1016/j.ibmb.2005.02.019](https://doi.org/10.1016/j.ibmb.2005.02.019).

References

- Adema, C.M., Hertel, L.A., Miller, R.D., Loker, E.S., 1997. A family of fibrinogen-related proteins that precipitates parasite-derived molecules is produced by an invertebrate after infection. *Proc. Natl. Acad. Sci. USA* 94, 8691–8696.
- Bartholomay, L.C., Cho, W.L., Rocheleau, T.A., Boyle, J.P., Beck, E.T., Fuchs, J.F., Liss, P., Rusch, M., Butler, K.M., Wu, R.C., Lin, S.P., Kuo, H.Y., Tsao, I.Y., Huang, C.Y., Liu, T.T., Hsiao, K.J., Tsai, S.F., Yang, U.C., Nappi, A.J., Perna, N.T., Chen, C.C., Christensen, B.M., 2004. Description of the transcriptomes of immune response-activated hemocytes from the mosquito vectors *Aedes aegypti* and *Armigeres subalbatus*. *Infect. Immun.* 72 (7), 4114–4126.
- Blandin, S., Shiao, S.H., Moita, L.F., Janse, C.J., Water, A.P., Kafatos, F.C., Levashina, E.A., 2004. Complement-like protein TEPI is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. *Cell* 116, 661–670.
- Brey, P.T., Lee, W.J., Yamakawa, M., Koizumi, Y., Perrot, S., Francois, M., Ashida, M., 1993. Role of the integument in insect immunity: epicuticular abrasion and induction of cecropin synthesis in cuticular epithelial cells. *Proc. Natl. Acad. Sci. USA* 90, 6275–6279.
- Brown, S., Hu, N., Hombria, J.C., 2001. Identification of the first invertebrate interleukin JAK/STAT receptor, the *Drosophila* gene domeless. *Curr. Biol.* 11, 1700–1705.
- Bruhn, H., Riekens, B., Berninghausen, O., Leippe, M., 2003. Amoebapores and NK-lysin, members of a class of structurally distinct antimicrobial and cytolytic peptides from protozoa and mammals: a comparative functional analysis. *Biochem. J.* 375, 737–744.
- Christophides, G., Zdobnov, E.M., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C., et al., 2002. Immunity-related genes and gene families in *Anopheles gambiae*. *Science* 298, 159–165.
- Cuvillier, O., 2002. Sphingosine in apoptosis signaling. *Biochim. Biophys. Acta* 585, 153–162.
- De Gregorio, E., Spellman, P.T., Rubin, G.M., Lemaitre, B., 2001. Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. *Proc. Natl. Acad. Sci. USA* 98, 12590–12595.
- Dimopoulos, G., 2003. Insect immunity and its implication in mosquito–malaria interactions. *Cell. Microbiol.* 5, 3–14.
- Dimopoulos, G., Richman, A., della Torre, A., Kafatos, F.C., Louis, C., 1996. Identification and characterization of differentially expressed cDNAs of the vector mosquito, *Anopheles gambiae*. *Proc. Natl. Acad. Sci. USA* 93 (23), 13066–13071.
- Dimopoulos, G., Richman, A., Müller, H.-M., Kafatos, F.C., 1997. Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proc. Natl. Acad. Sci. USA* 94, 11508–11513.
- Dimopoulos, G., Seeley, D., Wolf, A., Kafatos, F.C., 1998. Malaria infection of the mosquito *Anopheles gambiae* activates immune-responsive genes during critical transition stages of the parasite life cycle. *EMBO J.* 17, 6115–6123.
- Dimopoulos, G., Casavant, T.L., Chang, S., Scheetz, T., Roberts, C., Donohue, M., Schultz, J., Benes, V., Bork, P., Ansong, W., Soares, M.B., Kafatos, F.C., 2000. *Anopheles gambiae* pilot gene discovery project: identification of mosquito innate immunity genes

- from expressed sequence tags generated from immune-competent cell lines. *Proc. Natl. Acad. Sci. USA* 97 (12), 6619–6624.
- Dimopoulos, G., Christophides, G.K., Meister, S., Schultz, J., White, K.P., Kafatos, F.C., 2002. Genome expression analysis of *Anopheles gambiae*: responses to injury, bacterial challenge and malaria infection. *Proc. Natl. Acad. Sci. USA* 99, 8814–8819.
- Dong, Y., Dimopoulos, G., Molecular characterization of the fibrinogen domain pattern recognition receptor family in *Anopheles gambiae*, in preparation.
- Downer, R.G., Moore, S.J., L. Diehl-Jones, W., Mandato, C.A., 1997. The effects of eicosanoid biosynthesis inhibitors on prophenoloxidase activation, phagocytosis and cell spreading in *Galleria mellonella*. *J. Insect Physiol.* 19 (431), 1–8.
- Gobert, V., Gottar, M., Matskevich, A.A., Rutschmann, S., Royet, J., Belvin, M., Hoffmann, J.A., Ferrandon, D., 2003. Dual activation of the *Drosophila* toll pathway by two pattern recognition receptors. *Science* 302, 2126–2130.
- Gokudan, S., Muta, T., Tsuda, R., Koori, K., Kawahara, T., Seki, N., Mizunoe, Y., Wai, S.N., Iwanaga, S., Kawabata, S.-I., 1999. Horseshoe crab acetyl group-recognizing lectins involved in innate immunity are structurally related to fibrinogen. *Proc. Natl. Acad. Sci. USA* 96, 10086–10091.
- Han, Y.S., Thompson, J., Kafatos, F.C., Barillas-Mury, C., 2000. Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinete invasion of mosquitoes. *EMBO J.* 19, 6030–6040.
- Hernandez, V.P., Higgins, L., Fallon, A.M., 2003. Characterization and cDNA cloning of an immune-induced lysozyme from cultured *Aedes albopictus* mosquito cells. *Dev. Comp. Immunol.* 27, 11–20.
- Hoffmann, J.A., Reichhart, J.M., 2002. *Drosophila* innate immunity: an evolutionary perspective. *Nat. Immunol.* 3, 121–126.
- Jiang, H., Kanost, M.R., 2000. The clip-domain family of serine proteinases in arthropods. *Insect Biochem. Mol. Biol.* 30, 95–105.
- Jiang, H., Ma, C., Lu, Z.Q., Kanost, M.R., 2004. Beta-1,3-glucan recognition protein-2 (betaGRP-2) from *Manduca sexta*, an acute-phase protein that binds beta-1,3-glucan and lipoteichoic acid to aggregate fungi and bacteria and stimulate prophenoloxidase activation. *Insect Biochem. Mol. Biol.* 34 (1), 89–100.
- Kang, D., Romans, P., Lee, J.Y., 1996. Analysis of a lysozyme gene from the malaria vector mosquito, *Anopheles gambiae*. *Gene* 174, 239–244.
- Kawabata, S., et al., 1996. Tachycitin, a small granular component in horseshoe crab hemocytes, is an antimicrobial protein with chitin binding activity. *J. Biochem.* 120, 1253–1260.
- Kim, Y.S., Ryu, J.H., Han, S.J., Choi, K.H., Nam, K.B., Jang, I.H., Lemaitre, B., Brey, P.T., Lee, W.J., 2000. Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and beta-1,3-glucan that mediates the signaling for the induction of innate immune genes in *Drosophila melanogaster* cells. *J. Biol. Chem.* 275, 32721–32727.
- Kumar, S., Christophides, G.K., Cantera, R., Charles, B., Han, Y.S., Meister, S., Dimopoulos, G., Kafatos, F.C., Barillas-Mury, C., 2003. The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*. *Proc. Natl. Acad. Sci. USA* 100 (24), 14139–14144.
- Levashina, E.A., Moita, L.F., Blandin, S., Vriend, G., Lagueux, M., Kafatos, F.C., 2001. Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. *Cell* 104, 709–718.
- Lord, J.C., Anderson, S., Stanley, D.W., 2002. Eicosanoids mediate *Manduca sexta* cellular response to the fungal pathogen *Beauveria bassiana*: a role for the lipoxygenase pathway. *Arch. Insect Biochem. Physiol.* 511, 46–54.
- Lundstrom, A., Kang, D., Liu, G., Fernandez, C., Warren, J.T., Gilbert, L.I., Steiner, H., 2002. A protein from the cabbage looper, *Trichoplusia ni*, regulated by a bacterial infection is homologous to 3-dehydroecdysone 3beta-reductase. *Insect Biochem. Mol. Biol.* 32 (8), 829–837.
- Macours, N., Hens, K., Francis, C., De Loof, A., Huybrechts, R., 2003. Molecular evidence for the expression of angiotensin converting enzyme in hemocytes of *Locusta migratoria*: stimulation by bacterial lipopolysaccharide challenge. *J. Insect Physiol.* 498, 739–746.
- Marmaras, V.J., Bournazos, S.N., Katsoris, P.G., Lambropoulou, M., 1993. Defense mechanisms in insects: certain integumental proteins and tyrosinase are responsible for nonself-recognition and immobilization of *E. coli* in the cuticle of developing *Ceratitis capitata*. *Arch. Insect Biochem. Physiol.* 23 (4), 169–180.
- Muñoz, M., Vandenbulcke, F., Saulnier, D., Bachère, E., 2002. Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial challenged shrimp. *Eur. J. Biochem.* 269 (11), 2678–2689.
- Muller, H.M., Crampton, J.M., della Torre, A., Sinden, R., Crisanti, A., 1993. Members of a trypsin gene family in *Anopheles gambiae* are induced in the gut by blood meal. *EMBO J.* 12, 2891–2900.
- Naitza, S., Ligoxygakis, P., 2004. Antimicrobial defences in *Drosophila*: the story so far. *Mol. Immunol.* 40, 887–896.
- Nicholas, H.R., Hodgkin, J., 2004. Responses to infection and possible recognition strategies in the innate immune system of *Caenorhabditis elegans*. *Mol. Immunol.* 41 (5), 479–493.
- Nielsen, K.N., Nielsen, J.E., Madrid, S.M., Mikkelsen, J.D., 1997. Characterization of a new antifungal chitin-binding peptide from sugar beet leaves. *Plant Physiol.* 113, 83–91.
- Oduol, F., Xu, J., Niare, O., Natarajan, R., Vernick, K.D., 2000. Genes identified by an expression screen of the vector mosquito *Anopheles gambiae* display differential molecular immune response to malaria parasites and bacteria. *Proc. Natl. Acad. Sci. USA* 97, 11397–11402.
- Osta, M.A., Christophides, G.K., Kafatos, F.C., 2004. Effects of mosquito genes on *Plasmodium* development. *Science* 303, 2030–2032.
- Pearson, A., Lux, A., Krieger, M., 1995. Expression cloning of dSRCI, a class C macrophage-specific scavenger receptor from *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 92, 4056–4060.
- Prieto, J., Subira, M.L., Castilla, A., Arroyo, J.L., Serrano, M., 1989. Opioid peptides modulate the organization of vimentin filaments, phagocytic activity, and expression of surface molecules in monocytes. *Scand. J. Immunol.* 29 (4), 391–398.
- Roxstrom-Lindquist, K., Terenius, O., Faye, I., 2004. Parasite-specific immune response in adult *Drosophila melanogaster*: a genomic study. *EMBO Rep.* 5, 207–212.
- Shao, L., Devenport, M., Jacobs-Lorena, M., 2001. The peritrophic matrix of hematophagous insects. *Arch. Insect Biochem. Physiol.* 47, 119–125.
- Shen, Z., Edwards, M.J., Jacobs-Lorena, M., 2000. A gut-specific serine protease from the malaria vector *Anopheles gambiae* is down-regulated after blood ingestion. *Insect Mol. Biol.* 9, 223–229.
- Theopold, U., Samakovlis, C., Erdjument-Bromage, H., Dillon, N., Axelsson, B., Schmidt, O., Tempst, P., Hultmark, D., 1996. *Helix pomatia* lectin, an inducer of *Drosophila* immune response, binds to hemocytin, a novel surface mucin. *J. Biol. Chem.* 271, 12708–12715.
- Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., Reichhart, J.M., Lemaitre, B., Hoffmann, J.A., Imler, J.L., 2000. Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia. *Immunity* 13, 737–748.
- Vizioli, J., Bulet, P., Lowenberger, C., Blass, C., Müller, H.-M., Dimopoulos, G., Hoffmann, J., Kafatos, F.C., Richman, A., 2000. Cloning and analysis of a cecropin gene from the malaria vector mosquito *Anopheles gambiae*. *Insect Mol. Biol.* 9, 75–84.
- Vizioli, J., Catteruccia, F., della Torre, A., Reckmann, I., Muller, H.M., 2001. Blood digestion in the malaria mosquito *Anopheles*

- gambiae*: molecular cloning and biochemical characterization of two inducible chymotrypsins. Eur. J. Biochem. 268, 4027–4035.
- Wang, X., Rocheleau, T.A., Fuchs, J.F., Hillyer, J.F., Chen, C.C., Christensen, B.M., 2004. A novel lectin with a fibrinogen-like domain and its potential involvement in the innate immune response of *Armigeres subalbatus* against bacteria. Insect Mol. Biol. 13 (3), 273–282.
- Whitten, M.M., Tew, I.F., Lee, B.L., Ratcliffe, N.A., 2004. A novel role for an insect apolipoprotein (apolipoprotein III) in beta-1,3-glucan pattern recognition and cellular encapsulation reactions. J. Immunol. 15 172 (4), 2177–2185.
- Wu, A.L., Wang, J., Zheleznyak, A., Brown, E.J., 1999. Ubiquitin-related proteins regulate interaction of vimentin intermediate filaments with the plasma membrane. Mol. Cell. 4 (4), 619–625.
- Xu, X., Dong, Y., Abraham, E.G., Kocan, A., Srinivasan, P., Ghosh, A.K., Sinden, R.E., Ribeiro, J.M.C., Jacobs-Lorena, M., Kafatos, F.C., Dimopoulos, G., 2005. Transcriptome analysis of *Anopheles stephensi* – *Plasmodium berghei* interactions. Mol. Biochem. Parasitol., in press.
- Yoshiga, T., Hernandez, V.P., Fallon, A.M., Law, J.H., 1997. Mosquito transferrin, an acute-phase protein that is up-regulated upon infection. Proc. Natl. Acad. Sci. USA 94, 12337–12342.
- Zdobnov, E.M., Letunic, I., von Mering, V., Torrents, D., Suyama, M., Copley, R., et al., 2002. Comparative genome and proteome analysis of *Anopheles gambiae* and *Drosophila melanogaster*. Science 298, 149–159.
- Zhang, S.M., Adema, C.M., Kepler, T.B., Loker, E.S., 2004. Diversification of Ig superfamily genes in an invertebrate. Science 305, 251–254.
- Zhou, J.J., Huang, W., Zhang, G.A., Pickett, J.A., Field, L.M., 2004. “Plus-C” odorant-binding protein genes in two *Drosophila* species and the malaria mosquito *Anopheles gambiae*. Gene 327, 117–129.