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Microarray analysis of genes showing variable expression following a blood meal in *Anopheles gambiae*

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Abstract

A microarray analysis of 14 900 genes of the malaria vector mosquito, *Anopheles gambiae*, shows that as many as 33% (4924) of their corresponding transcription products vary in abundance within 24 h after a blood meal. Approximately half (2388) of these products increase in their accumulation and the remainder (2536) decrease. Expression dynamics of 80% of the genes analysed by expressed sequence tag (EST) projects reported previously are consistent with the observations from this microarray analysis. Furthermore, the microarray analysis is more sensitive in detecting variation in abundance of gene products expressed at low levels and is more sensitive overall in that a greater number of regulated genes are detected. Major changes in transcript abundance were seen in genes encoding proteins involved in digestion, oogenesis and locomotion. The microarray data and an electronic hyperlinked version of all tables are available to the research community at http://www.angagepuci.bio.uci.edu/1/.

Keywords: gene expression, blood feeding, vector mosquito, *Anopheles*.

Introduction

Malaria is responsible for 2–3 million deaths and more than 500 million cases per year worldwide (Land, 2003), emphasizing the urgency of developing new strategies to combat the transmission of this disease. Anti-parasite drugs and vector population reduction with chemical insecticides have been the most effective ways to control malaria transmission (Whitty *et al*., 2002). However, continuous application of insecticides leads to the selection of resistant mosquitoes (Brogdon & McAllister, 1998), making urgent the need for novel ways to target vector populations. *Anopheles gambiae* is the major malaria vector in the African continent. Blood feeding by adult female mosquitoes is essential for its reproduction and offers a series of physiological processes that could be targeted by novel mosquito control strategies. These processes can be observed in the modulation of host seeking activity (Takken *et al*., 2001), increased diuretic activity by the Malphigian tubules (Beyenbach, 2003), and enhanced secretory activity of the midgut cells which results in the formation of the peritrophic membrane (Shen & Jacobs-Lorena, 1998) and accumulation of digestive enzymes in the lumen of the midgut (Muller *et al*., 1993). Furthermore, fat body cells synthesize yolk proteins after a blood meal (Ahmed *et al*., 2001), and oogenesis is activated in the ovaries, concluding with egg formation (Fiil, 1976). Hormonal regulation of cellular functions is at the centre of these orchestrated processes that occur during each gonotrophic cycle (Gade & Goldsworthy, 2003).

A genome-wide analysis of gene expression in blood fed and non-blood fed adult female *An. gambiae* was conducted taking advantage of a commercial microarray platform (Affymetrix GeneChip® Plasmodium/Anopheles Genome Array). The results complement and extend the analyses of EST databases derived from mosquitoes in similar physiological conditions (Holt *et al*., 2002; Ribeiro, 2003). Here we provide a comprehensive catalogue of genes whose transcription products increase or decrease in abundance at 24 h following blood feeding, and discuss the roles of their products in mosquito physiology and their potential use for the development of novel malaria control strategies.

Results and discussion

Changes in *An. gambiae* gene expression 24 h after a blood meal were assessed by microarray analyses using the Affymetrix GeneChip Plasmodium/Anopheles genome array, which includes probe sets to 4300 *Plasmodium*

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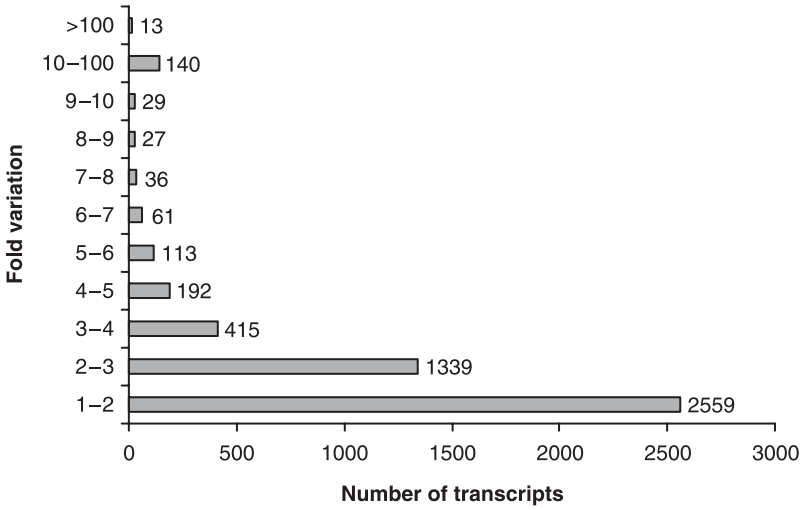

Figure 1. Distribution of transcripts showing significant difference in expression ($P < 0.001$) following a blood meal. Absolute values were considered and the transcripts grouped according to their levels of fold variation.

falciparum and 14 900 *Anopheles gambiae* genes. A total of 5192 of the probe sets showed variations in signal following hybridization with samples prepared from animals with and without a blood meal. Only five of these (*AF039280.1_RC_at*, *AF368940.1_RC*_at, *AJ420386.1_RC_at*, *Pf.11.260.0 °CDS_at*, *Pf.7.226.0 °CDS_s_at*), correspond to *P. falciparum* transcripts and represent parasite probe sets that cross hybridize with mosquito RNAs. The remaining 5187 probe sets represent 4924 transcription products from *An. gambiae* genes responding significantly to the blood meal ($P < 0.001$). A total of 2388 transcripts show an increase in signal and 2536 transcripts show a decrease. The majority of the 4924 transcripts have low levels of variation after a blood meal, with 79% of them changing less than three-fold. Only 3% of the genes vary more than ten-fold and only 0.2% vary more than 100-fold (Fig. 1).

Genes showing at least three-fold variation in abundance following blood feeding were selected for further analysis. According to this criterion, 1072 probe sets were selected and compared to data generated in a larger experiment looking at gene expression profiles throughout development (O. Marinotti, unpubl. data). The comparison revealed 662 probe sets, corresponding to 646 transcripts (~4% of the total number of genes in *An. gambiae*) showing consistent variation ($P < 0.001$ and at least three-fold variation) in both experiments. Of these, 282 (44%) were increased in abundance 24 h after a blood meal, while 364 (56%) were decreased. The locations of regulated genes in *An. gambiae* chromosomes showed that those that increased and decreased in abundance are equally distributed throughout the genome (Fig. 2). A statistical analysis of the distribution of the genes along the chromosomes also showed no significant linkage clustering (results not shown).

Comparisons of the results produced by two methods, microarray (this report) and EST analysis (Ribeiro, 2003), demonstrated that 408 of the 435 genes identified as regulated in the EST sequencing also showed variation in abundance on the microarray method. Additionally, a group of 194 regulated transcripts identified by the microarray had no corresponding indication of change in the EST analysis. These additional genes represent those present at low levels and therefore were not detected in the sequenced EST population. Forty-four of the microarray-selected, differentially accumulated transcripts correspond to mRNAs included but equally represented in the EST databases of non-blood fed and blood-fed mosquitoes. Eighty-five transcripts showed contrasting variation in abundance in the two data sets, with an increase in one, and a decrease in the other. The vast majority of transcripts comprising this group had only one or two ESTs sequenced and therefore did not have a sufficient representation in that database to provide confidence in the calculated variation. The few exceptions include ENSANGT00000022257, encoding a heat shock 70 protein, showing a total of ninety sequences and an increase in accumulation (+1.5-fold) according to ESTs and a decrease (–14-fold) according to the microarray analysis, ENSANGT00000021680, encoding a product of unknown function, with thirty-six sequences (+1.3 ESTs/–7 microarray), ENSANGT00000022733, encoding an elongation factor 1-alpha with 109 sequences (+1.1 ESTs/–4 microarray), and ENSANGT00000017824, encoding a sugar transporter with thirty-one sequences (+2.1 ESTs/–3.7 microarray).

Three hundred and thirty-nine transcripts showed a consistent increase or decrease by the two methods, corresponding to an 80% agreement of the transcripts analysed. The percentage of agreement increases as the number of ESTs increases, reaching 95% when considering only transcripts with more than thirty-two EST sequences. The microarray selected genes with significant ($P < 0.001$) transcription variation and at least three-fold variation in two independent microarray experiments, were analysed

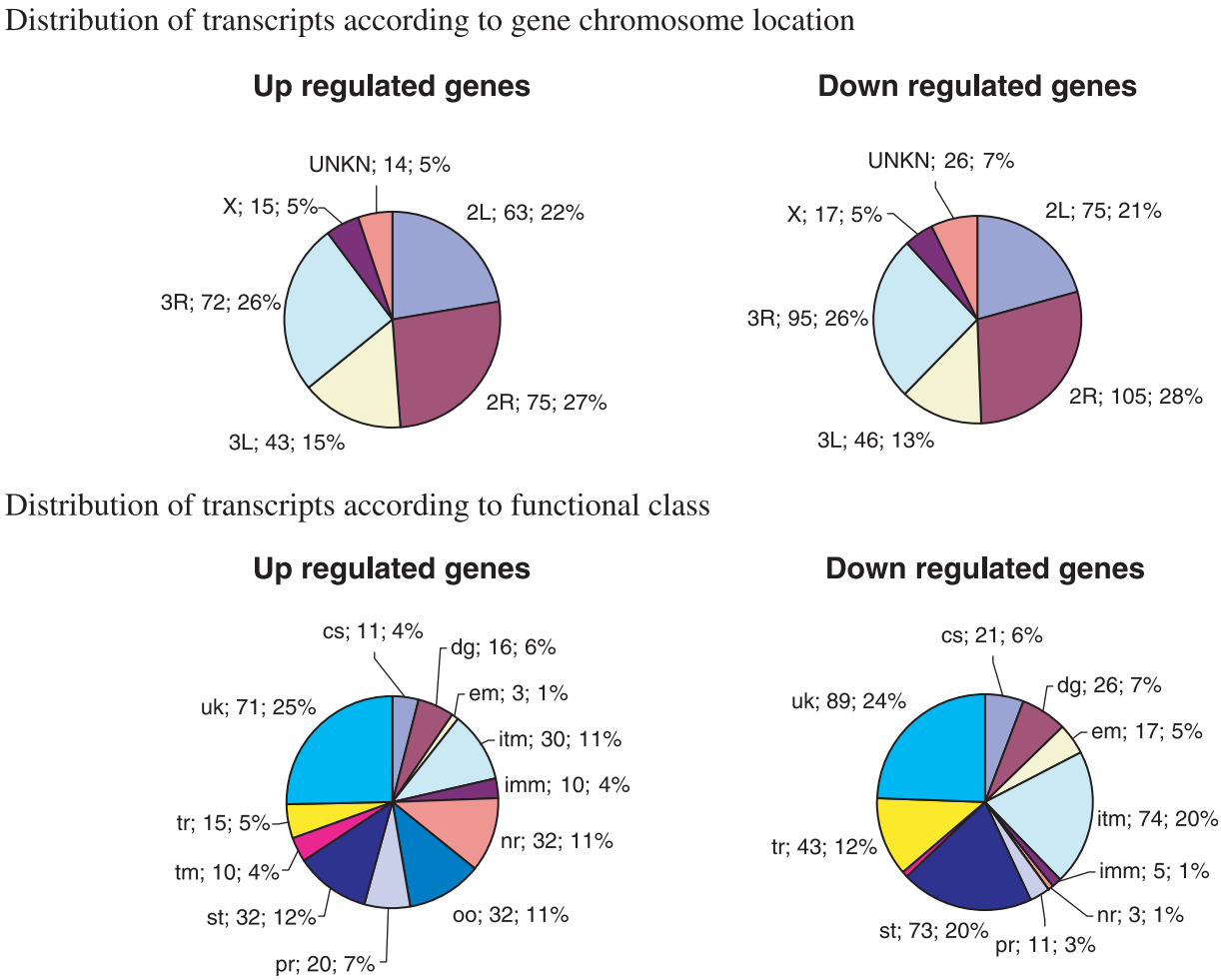

Figure 2. Distribution of *Anopheles gambiae* transcripts up- and down-regulated upon blood-feeding, in accordance with their chromosomal location (top panels) or functional class (bottom panels). The sequences were classified according to their functions related to: cs, cytoskeleton (including muscle related proteins); dg, digestion; em, extracellular matrix; itm, intermediate metabolism; imm, immunity; nr, nuclear regulation; oo, oogenesis; pr, protein synthesis and processing; st, signal transduction; tm, transcriptional machinery; tr, transporters; uk, unknown. Legends indicate classification, total number of transcripts, and percentages they represent in each category, respectively.

and grouped according to the functions of their encoded proteins (supplementary Tables S1–S12).

Cytoskeletal and muscle proteins

The majority of transcripts (twenty-one) in this category decrease in quantity at 24 h after blood feeding. Among them are those encoding an articulin (ENSANGT00000026097, –33-fold), implicated in the maintenance of cell shape (Huttenlauch & Stick, 2003), and a kettin (BM652962, –16-fold), component of the insect indirect flight muscle that contribute to the high passive stiffness of this tissue (Bullard *et al.*, 2002). All other transcripts showing decreased accumulation vary less than ten-fold, including muscular and cytoskeletal proteins such as myosin, actin, troponin, flightin and tetraspanin. The general decrease of these transcripts likely reflects a change in mosquito flying behaviour after a blood meal. Blood-seeking behaviour is inhibited at 24 h after a blood meal, although reports indicate that *An. gambiae* can take multiple blood meals during each gonotrophic cycle (Briegel & Horler, 1993). Trends of variations in gene expression, supporting a change in host or blood seeking behaviour of mosquitoes also were observed in other groups of transcripts, as discussed below.

Eleven transcripts showed increased accumulation, and one, encoding a regulator of cytokinesis (ENSANGT-00000018995), increases 28.3-fold after a blood meal. Several transcripts related to members of the kinesin family also increased, as well as those encoding microtubule and actin binding proteins (ENSANGT00000017471, ENSANGT00000010518) and a restin (ENSANGT-00000015685). This group of genes may be expressed in the fast growing ovaries. For example, AJ281048 increases 22-fold in abundance and encodes a protein similar to the *Drosophila melanogaster scribble* that is associated with

morphogenesis of the ovarian follicular cells and embryos (Huang *et al*., 2003). They could also correspond to genes expressed in other tissues such as the midgut were morphological changes (stretching) occur after a blood meal.

Extracellular matrix

This group is composed of twenty transcripts of which seventeen decrease in abundance following the blood meal. The largest decrease, –227-fold, is in a transcript (ENSANGT00000028128) that encodes a protein related to melanization processes, and this is accompanied by the decrease of two other transcripts with similar nucleotide sequences most likely encoding proteins of the same function. Other transcripts associated with cuticular proteins, collagen, cell adhesion and mucin-like proteins also show decreases to various levels. Only three transcripts of this group increase in abundance. They encode a yellow protein (ENSANGT00000013095) associated with cuticle tanning (Johnson *et al*., 2001), a putative peritrophic membrane protein (ENSANGT00000012073), which is probably associated with the processes of blood digestion, and a glycine-rich protein (ENSANGT00000016101), which may be involved in egg formation as it contains an amino acid sequence similar to those of chorion proteins.

Transport

This group comprises fifty-eight transcripts encoding proteins related to a variety of biological functions. Among them, large increases in accumulation were detected for transcripts encoding a putative phosphatidylinositol-transfer protein (ENSANGT00000018338), followed by multicopper oxidase (ENSANGT00000017050), and hexamerin (ENSANGT00000004408).

The demand for lipid mobilization is increased in mosquitoes after a blood meal, and this requires the transfer of dietary lipids from the midgut to other tissues including the developing ovaries (Capurro *et al*., 1994). Consistent with this physiological requirement, we observed an increase in accumulation of four lipid transporter transcripts. Phosphatidylinositol transfer proteins, which correspond to the most up-regulated transcripts in this group, are found in both unicellular and multicellular organisms, bind phosphatidylinositol and phosphatidylcholine, and transfer them from one membrane compartment to another (Allen-Baume *et al*., 2002).

Mosquitoes have to mitigate the toxic high concentration of iron in a blood meal. Mosquitoes manage some of this iron load by expressing iron-binding proteins (Geiser *et al*., 2003). Ferritin is up-regulated following a blood meal in *Aedes aegypti*, but this was not observed in *An. gambiae*. In contrast, ferritins are expressed constitutively and at high levels suggesting that these two mosquitoes respond differently to excess iron. Alternatively, the up-regulation of *An. gambiae ferritin* genes could occur earlier after a blood meal and not be detected in our experiments. The transcript of a multicopper oxidase, which also may be involved in iron metabolism, increases in abundance following blood feeding.

Hexamerins are high molecular weight proteins found mostly in the larval haemolymph of insects and are proposed to function as storage proteins for later usage (Telfer & Kunkel, 1991). However, the expression of hexamerins during the adult stage of several insects, including *An. gambiae*, is also observed, although no induction after a blood meal has been described (Zakharkin *et al*., 1997). An increase in expression of a hexamerin during egg development was described in *Musca domestica* and its involvement with egg formation was suggested (Capurro *et al*., 2000). A similar function could be performed by hexamerins induced during the gonotrophic cycle of the mosquito.

Several transcripts of proteins related to mitochondrial functioning, such as an ABC transporter (ENSANGT00000010177), carnitine carriers (ENSANGT00000020378, ENSANGT00000020305), and others decrease in abundance, and this is consistent with the previously proposed diminished muscular activity in blood-fed mosquitoes. Sugar transporters transcripts (ENSANGT00000002479 and ENSANGT00000022745) are among those that decrease the most in this group, which could again be related to the decrease of flight muscle activity.

Signal transduction

A large number of transcripts related to signal transduction (108) vary in abundance at 24 h after blood ingestion. These transcripts encode proteins involved in apoptosis, circadian rhythms, vision, gustatory and olfactory reception, hormonal regulation and several other biological functions. Five transcripts related to genes encoding proteins involved in the circadian rhythm, seven encoding putative antennal proteins involved in olfaction, and twelve transcripts encoding proteins related to vision decrease in abundance. Together with the previously described reduction in muscle transcripts, these data are consistent with the general interpretation that following a blood meal there is a reduction in response of the mosquitoes to environmental stimuli, including those generated by vertebrate hosts. Furthermore, transcripts encoding a glutamate receptor (ENSANGT00000029705), a glutamate decarboxylase (ENSANGT00000013909) and three transcripts encoding putative glutamate transporters (ENSANGT00000028803, ENSANGT00000024383, ENSANGT00000021612) decrease. Ribeiro (2003) has proposed that the reduced production and action of the neuromediator, γ-aminobutyric acid, may reflect a diminished need for locomotion and flight after a blood meal.

Two transcripts encoding odorant binding proteins (ENSANGT00000017561, ENSANGT00000017924) decrease, while one (ENSANGT00000027726) increases

in abundance after blood feeding. These variations could lead to a change in food preference, for example from blood to nectar, or more effective location of oviposition sites.

Sixteen of the transcripts in this group are related to hormone synthesis, processing and action. 20-hydroxyecdysone levels in mosquitoes increase after a blood meal while juvenile hormone levels drop (Clements, 1992). Consistent with these observations, transcripts encoding proteins related to ecdysteroid metabolism and receptors increase in abundance after blood feeding (ENSANGT00000011344, ENSANGT00000017216 and ENSANGT00000009112), and transcripts related to juvenile hormone biosynthesis, farnesyltransferase (BX061080.1), and allatotropin (ENSANGT00000016805), a neuropeptide involved in the control of juvenile hormone synthesis (Yiping *et al*., 2004), decrease. Transcripts encoding proteins expected to be involved in programmed polypeptide processing by cleavage, such as convertases (ENSANGT00000011718) and several others containing kinase domains possibly involved in the phosphorylation of proteins related to the signal transduction cascades, vary significantly in abundance after the blood meal. Their expression profile and links to biological processes still are to be determined.

Nuclear regulation and transcriptional machinery

Consistent with their assigned functions, transcripts associated with nuclear regulation and transcription are detected at low levels. Components of this group encoding proteins involved in cell cycle and cell division (kinetochrome components, ENSANGT00000014595) and mitotic spindle assembly checkpoint proteins (ENSANGT00000017449 and ENSANGT00000008371) increase in abundance, most likely reflecting the cytokinesis that occurs during oogenesis. Also increased are the transcripts encoding histones and histone methyltransferases (ENSANGT00000027931, ENSANGT0000001681926372, ENSANGT00000016276) involved in chromatin structure and organization. The transcripts related to proteins involved in DNA replication (DNA polymerases, ENSANGT00000016819, ENSANGT-00000014184; DNA replication factors ENSANGT-00000020952, ENSANGT00000010410) also increase in abundance. In addition to cell division and its requirements for DNA synthesis, these nuclear components could be involved in gene-specific amplification such as that observed for the chorion genes in the follicular cells of developing ovaries of *Drosophila melanogaster* and *Bombyx mori* (Tower, 2004). New synthesis of digestive enzymes as well as synthesis of abundant proteins for egg production requires the activation of the transcriptional machinery in the blood-fed mosquitoes, and this is represented in the increased accumulation of gene products that encode proteins involved in transcription (helicases ENSANGT-00000026646, ENSANGT00000010202, RNA polymerase BX019798.1, transcription regulators BM653011, pre-RNA splicing and polyadenylation factors ENSANGT00000009824 ENSANGT00000015892 ENSANGT00000010577).

Protein synthesis, modification, transport and degradation

The digestion and absorption of nutrients from the blood meal makes available amino acids for the maintenance of basic metabolism as well as the synthesis of vitellogenic proteins during each gonotrophic cycle (Briegel, 1985; Zhou *et al*., 2004). The peak of protein synthesis occurs at ~24 h after feeding, and this is achieved by the activation of the mechanisms for translation, and protein modification and transport to their intracellular or secretory compartments. The expression profile reflects this activation with the increase in accumulation of several transcripts encoding elongation factors (ENSANGT00000027857, ENSANGT00000027333) and components of post-translational protein processing such as glycosylation (ENSANGT00000021398 ENSANGT-00000011382) and secretion (ENSANGT00000015077). Some of the transcripts of this group (ENSANGT-00000021226, ENSANGT00000010386, ENSANGT-00000022733) decrease in abundance and these correspond most likely to genes with reduced expression in organs that are not essential for reproduction.

Intermediate metabolism

A total of 171 transcripts related to intermediate metabolism show variations in abundance. Among the transcripts that increase are a number involved in amino acid metabolism including three encoding ornithine decarboxylases (ENSANGT00000008815, ENSANGT00000020301, ENSANGT00000009019). Ornithine decarboxylase is an essential enzyme in the synthesis of polyamines, which are ubiquitous polycations with multiple functions. In mosquitoes, mRNA levels of vitellogenin, digestive trypsin, and the vitelline membrane proteins are decreased by inhibition of polyamine synthesis (Kogan & Hagedorn, 2000). Pyrroline-5-carboxylate reductase (ENSANGT00000011470) transcripts, whose homologous gene in *Ae. aegypti* is involved in the utilization of proline as an energy substrate during flight (Scaraffia & Wells, 2003), decreases in abundance following a blood meal. This is consistent with the proposed inhibition of locomotion in blood-fed mosquitoes. The transcript abundance of those genes whose products are involved in carbohydrate metabolism are reduced after blood feeding. All transcripts that are involved with glycolysis, Krebs cycle and glycogen metabolism that showed variation were decreased after feeding. Twenty-eight transcripts associated with oxidative phosphorylation that had been detected as slightly down regulated (−1.2- to −4.6-fold) by EST sequencing (Ribeiro, 2003) were not selected as decreased by blood feeding in the microarray based experiments. The majority of transcripts related to lipid metabolism also decrease, however, eleven transcripts encoding proteins related to nucleotides metabolism increase.

It has been suggested that the decrease in abundance following the blood meal of mitochondrial and nuclear transcripts associated with oxidative phosphorylation and the tricarboxylic acid (TCA) cycle could indicate less expression of mRNAs associated with striated muscles (Ribeiro, 2003). The flight muscle alone comprises about 10% of the insect's weight and contain mitochondria representing ~40% of the muscle volume (Wigglesworth, 1972). However, transcript levels of glycogen synthase and genes involved in glycolysis also decreased in the midguts of blood-fed *Ae. aegypti* (Sanders *et al.*, 2003), a tissue expected to be active. The biological meaning of this apparent down-regulation has yet to be determined.

P450 enzymes are ubiquitous proteins involved in many processes of detoxification of xenobiotic compounds, control of reproduction, development and homeostasis (Feyereisen, 1999). P450s are involved in insecticide metabolism and an increased activity of members of this family has been associated with pyrethroid resistance in different insect species, including mosquitoes. Analysis of the *An. gambiae* genome revealed 104 cytochrome *P450* genes (Ranson *et al.*, 2002) and we now show that twenty-two of them decrease in transcript abundance at 24 h postblood meal. This result is in agreement with the *An. gambiae* EST studies, and is in contrast with the results obtained for *Culex quinquefasciatus* fat bodies and *Ae. aegypti* midguts, where cytochrome *P450* genes increase in abundance in response to blood feeding (Baldridge & Feyereisen, 1986; Sanders *et al.*, 2003).

Immunity and melanization pathways

Both increased and decreased accumulation of mRNAs associated with genes encoding immune functions are found following blood feeding. Increased transcript abundance includes those encoding phenoloxidases (ENSANGT00000002437, ENSANGT00000010740) and a pro-phenoloxidase activating enzyme (ENSANGT00000010496). Phenoloxidases are components of the invertebrate immune system, however, these enzymes also are involved in eggshell melanization (Kim *et al.*, 2005). Several other transcripts related to innate immunity and lipid recognition also increase (especially ENSANGT00000016907, which is up-regulated 144-fold), however, their function needs to be determined. Other transcripts related to the immune system that show significant variation after blood feeding are two encoding lysozymes (ENSANGT000000018395, BX035040.1), a TNF-receptor of the toll pathway (ENSANGT00000022348) and a peptidoglycan recognition protein (ENSANGT00000026500). The variations in transcript abundance obtained from the EST sequencing project are in many cases incongruent with the microarray results. Because these experiments did not control for the exposure of mosquitoes to environmental and blood-borne bacteria and fungi, the observed differences could be due to variable immune status of the experimental groups of mosquitoes.

Digestion

The up-regulation of enzymes and proteins involved in blood digestion is well-documented (Muller *et al.*, 1995; Shen & Jacobs-Lorena, 1998, Shen *et al.*, 1999; Vizioli *et al.*, 2001). Accordingly, the transcription products of several serine protease and carbohydrase genes were identified in our study as increasing in abundance following blood feeding. In addition, transcripts encoding a microvillar protein of unknown function (ENSANGT00000017713), mucins and peritrophic membrane proteins (extracellular matrix, Table S2), also increase.

Several of the transcripts in this group decrease in abundance. These likely represent genes expressed in tissues that are not involved in digestive functions. Alternatively, these genes could encode products such as early trypsins and chymotrypsins, necessary at the initial steps of the digestion, but dispensable later (Shen *et al.*, 2000; Giannoni *et al.*, 2001). Among the transcripts that decrease in abundance are twelve that represent genes expressed in the salivary glands of the mosquitoes and encode secreted proteins (Francischetti *et al.*, 2002). Only one salivary gland expressed gene encoding a D7-related protein (ENSANGT00000018280), increases in abundance in blood-fed mosquitoes.

Oogenesis

Genes encoding vitellogenins, other yolk components and chorion-related proteins (ENSANGT00000012892, ENSANGT00000027001) were identified by the microarray experiments as having large increases in transcript abundance confirming the EST-based data and the previously described increase in vitellogenin expression in blood-fed mosquitoes (Ahmed *et al.*, 2001). Chorion proteins are components of the eggshell and are expressed abundantly in the ovaries of vitellogenic insects. These proteins and related gene products have been studied extensively in model organisms such as *D. melanogaster* and *B. mori* (Margaritis, 1985), but no information has been reported on the chorion proteins of *An. gambiae*. Here we identified by sequence similarity some of the transcripts encoding these proteins. However, their positive characterization as chorion components requires confirmation. Several other transcripts associated with meiosis, egg formation and embryo development were also included in this group.

Genes of unknown function

The products of 161 (25%) of the transcripts included in our analysis have no significant similarity to proteins with known functions. Several of them represent families of genes or proteins conserved among mosquitoes or insects (ENSANGT00000027284, ENSANGT00000016832,

ENSANGT00000023042, ENSANGT00000020908). Functional studies are of primary importance to link the large amount of sequence information generated by the mosquito genome sequencing with biochemical and physiological events and, as a result, increase our knowledge of mosquito biology. Further studies on these novel or uncharacterized proteins may reveal important features of mosquito biology and eventually provide new targets for mosquito population and parasite transmission control.

Final remarks

The identification of genes expressed abundantly, as well as the characterization of gene expression variation induced by blood feeding, are important steps in understanding mosquito biology and their interactions with the vertebrate hosts and the pathogens they transmit. For example, we have identified odorant-binding proteins that appear to be under differential transcriptional regulation in non-blood-fed and blood-fed mosquitoes. These molecules are the objective of studies (e.g. Justice *et al*., 2003) and are potential targets for the development of new repellents and/or female mosquito attractants useful for mosquito traps. Several transcripts encoding proteins related to the innate immune system decrease in accumulation after blood feeding. Could this apparent down-regulation open opportunities for the successful establishment of parasite infection and development in the mosquito? Could the immune system of mosquitoes be modulated in a way that parasites do not develop to their infective stages? A comprehensive analysis of immune responses in bacteria and *Plasmodium*-infected mosquitoes is being conducted (Christophides *et al*., 2002; Dimopoulos *et al*., 2002; Kumar *et al*., 2003) and will bring answers to these questions. Our results also confirmed previous observations that several transcripts associated with vision, olfaction and gustatory sensitivity decrease in abundance after feeding.

A list of transcripts increasing in abundance after feeding and involved with blood digestion, oogenesis and reproduction was generated. Disruption of the normal expression of these genes could lead to sterility and this is potentially useful for the development of mosquito population control methods such as sterile insect techniques (Handler, 2004). Studies of insecticide resistance-related genes and their products are also of primary importance and we observed that twenty-two cytochrome *P450* genes decrease in abundance following a blood meal. Could this repression modulate the insecticide resistance status of female mosquitoes?

This study confirmed the regulation of 339 *An. gambiae* genes previously determined in an EST-based study (Ribeiro, 2003) to have their expression regulated upon blood feeding. However, other genes regulated during the adult life of mosquitoes were not detected by this single-time point analysis. For example, the *An. gambiae* digestive carboxypeptidase is up-regulated immediately after mosquitoes feed on blood (Edwards *et al*., 1997) and the variation in the amount of these transcripts is not detected at the time we conducted our studies. Similarly, genes regulated at the later phases of oogenesis, for example, those involved in the formation of the exochorion would not be detected by this study.

Finally the limitations of the EST sequencing and microarray-based analysis are that the determined variations in transcript abundance may not correlate with a similar variation in the amount of the encoded protein. Furthermore, enzyme activity may be subject to regulation by feedback inhibition by the corresponding pathway product, allosteric interactions, reversible covalent modifications and programmed proteolytic cleavage. Further studies encompassed by functional genomics to generate a comprehensive picture of gene expression, proteins synthesis and enzymatic activities throughout the mosquito development are needed and may help in the efforts to control malaria transmission.

Experimental procedures

Mosquitoes

The Pink eyes strain of *An. gambiae* (Githeko *et al*., 1992) was maintained at 25 °C, 75–85% relative humidity and 18/6 h light/dark cycles. Larvae were fed on finely powdered fish food (Tetramin) mixed 1 : 1 with yeast powder. Adults (males and females) were kept in cages with access to raisins and water *ad libitum*. The two groups of mosquitoes compared in this study consisted of four-day-old adult females fed on mice at 24 h prior to RNA extraction or adult females of the same age that were not allowed to feed on blood. Males and females were kept together in cages until blood feeding.

Computational analysis and gene annotation

The consensus nucleotide sequences used to generate the primer sets included in the Affymetrix GeneChip® Plasmodium/Anopheles Genome array (https://www.affymetrix.com/support/file_download.affx?onloadforward = /analysis/downloads/data/Plasmodium_Anopheles_consensus.zip) were compared by BLASTX to the *Anopheles gambiae* protein database, release four. The similarities, putative functions and number of sequenced ESTs, corresponding to each identified protein (ENSANGP######) were derived from the AnoXcel database accessed online at ANOBASE (http://www.anobase.org/) (Ribeiro *et al*., 2004).

Microarray analysis

Total RNA of five adult mosquitoes was extracted to prepare each sample and a total of six samples, three of non-blood-fed (NBF) and three of blood-fed (BF) mosquitoes, were analysed. Isolated total RNAs were processed as recommended by Affymetrix, Inc. (Affymetrix GeneChip Expression Analysis Technical Manual, Affymetric, Inc., Santa Clara, CA). In brief, total RNA was isolated using TRIzol Reagent (Gibco BRL Life Technologies, Rockville, MD), and passed through an RNeasy spin column (Qiagen, Chatsworth, CA). All starting RNA samples were quality assessed prior to use by processing an aliquot with a RNA Laboratory-On-A-Chip (Caliper Technologies Corp., Mountain View, CA) that was evaluated on

an Agilent Bioanalyser 2100 (Agilent Technologies, Palo Alto, CA). cDNA was synthesized from the poly(A) + mRNA present in the isolated total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen Corp., Carlsbad, CA). A portion of the resulting ds-cDNA was used as a template to generate biotin-tagged cRNA from an *in vitro* transcription reaction (IVT), using the BioArray High-Yield RNA Transcript Labeling Kit (T7) (Enzo Diagnostics, Inc., Farmingdale, NY). The biotin-tagged cRNA was fragmented to strands of 35–200 bases in length following prescribed protocols (Affymetrix GeneChip Expression Analysis Technical Manual) and subsequently hybridized with rotation at 45 °C for 16 h (Affymetrix GeneChip Hybridization Oven 640) to probe sets present on an Affymetrix GeneChip® Plasmodium/Anopheles Genome Array. The GeneChip arrays were washed and then stained (SAPE, streptavidin-phycoerythrin) on an Affymetrix Fluidics Station 450, followed by scanning on a GeneChip Scanner 3000. The results were quantified and analysed using GCOS 1.1.1 software (Affymetrix, Inc.) using default values (Scaling, Target Signal Intensity = 500; Normalization, All Probe Sets; Parameters, all set at default values). The microarray data are available at http://www.angagepuci.bio.uci.edu/1/.

Statistical tests

The signal values generated by GCOS 1.1.1 software were used to perform a Bayesian *t*-test using a web-based statistical analysis package, Cyber-T (Long *et al*., 2001) and to generate a list of probe sets with a *P*-value of less than 0.001. Expression profiles were further analysed by using the software package GeneSpring (Silicon Genetics, Inc., Redwood City, CA.).

To determine possible spatial aggregation of up- or down-regulated genes in each of the mosquito chromosomes or chromosome arms, we obtained the gene chromosome location and coordinates for each selected gene from the AnoXcel database. We computed the mean distance from each of these genes to their nearest neighbour, and compared these distances to that obtained from 1000 Monte Carlo simulations of the same number of genes randomly dispersed in the same chromosome or chromosome arm, considering the coordinates of all known genes for each chromosome, or chromosome arm. The standard deviations of the Monte Carlo simulations were used to determine the significance level of the comparisons between the observed set and the randomly expected set. Programs were written in Visual Basic.

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Supplementary material

The following material is available online:

Appendix S1. Tables showing transcripts regulated by a blood meal associated with cytoskeletal proteins (Table S1), the extracellular matrix (Table S2), transport (Table S3), signal transduction (Table S4), nuclear regulation (Table S5), transcriptional machinery (Table S6), protein synthesis and modification (Table S7), intermediate metabolism (Table S8), immunity (Table S9), digestion (Table S10), oogenesis (Table S11) and transcripts with unknown function (Table S12).