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Genome-wide analysis of gene expression in adult *Anopheles gambiae*

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Abstract

With their genome sequenced, *Anopheles gambiae* mosquitoes now serve as a powerful tool for basic research in comparative, evolutionary and developmental biology. The knowledge generated by these studies is expected to reveal molecular targets for novel vector control and pathogen transmission blocking strategies. Comparisons of gene-expression profiles between adult male and nonblood-fed female *Anopheles gambiae* mosquitoes revealed that roughly 22% of the genes showed sex-dependent regulation. Blood-fed females switch the majority of their metabolism to blood digestion and egg formation within 3 h after the meal is ingested, in detriment to other activities such as flight and response to environment stimuli. Changes in gene expression are most evident during the first, second and third days after a blood meal, when as many as 50% of all genes showed significant variation in transcript accumulation. After laying the first cluster of eggs (between 72 and 96 h after the blood meal), mosquitoes return to a nongonotrophic stage, similar but not identical to that of 3-day-old nonblood-fed females. Ageing and/or the nutritional state of mosquitoes at 15 days after a blood meal is reflected by the down-regulation of ~5% of all genes. A full description of the large number of genes regulated

at each analysed time point and each biochemical pathway or biological processes in which they are involved is not possible within the scope of this contribution. Therefore, we present descriptions of groups of genes displaying major differences in transcript accumulation during the adult mosquito life. However, a publicly available searchable database (http://www. angagepuci.bio.uci.edu/) has been made available so that detailed analyses of specific groups of genes based on their descriptions, functions or levels of gene expression variation can be performed by interested investigators according to their needs.

Introduction

The fruit fly *Drosophila melanogaster* is a proven model for studies of physiology, biochemistry, behaviour, and other aspects of insect biology. However, the knowledge obtained is not always directly applicable to other species, due mainly to the enormous diversification of insects and their adaptations to different niches. Hematophagy with its multiple determining factors and consequences (host-seeking, blood ingestion, digestion and the unavoidable challenge of dealing with a heavy load of iron molecules ingested at each meal, and its direct implication in mosquito reproduction and pathogen transmission) is just one example of a mosquito evolutionary adaptation that cannot be addressed by studying *Drosophila* or other model organisms. Based on these differences, insect vectors of diseases, comprising species of mosquitoes, tsetse flies, sand flies, black flies, fleas, kissing bugs and bedbugs, that transmit viruses and parasites to humans, have been the objective of extensive investigation with the main purpose of finding novel and efficient ways to disrupt or diminish pathogen transmission. The concluded venture to sequence the genome of the human malaria vector, *Anopheles gambiae* (Holt *et al.*, 2002), and the ongoing projects to sequence the genomes of two other mosquito species, *Aedes aegypti* (Severson *et al.*, 2004) and *Culex quinquefasciatus*, represent major advances in this direction. They

are expected to present opportunities to improve vector control by revealing new gene targets for insecticides and insect repellents, and to advance novel strategies to control pathogen transmission (Toure *et al.*, 2004). However, a key question to be answered by vector biologists is how to explore this sequence information in pursuit of these goals. The tools of functional genomics represent a direct way to obtain answers to this question. Determination of spatial and temporal expression patterns and the linking of genes to their biological functions are basic and fundamental steps to progress toward using sequence data for controlling disease transmission. In addition, with their genomes sequenced, mosquitoes can now serve as powerful tools for basic research, comparative, evolutionary and developmental biological studies that should lead to the understanding of the processes that shape insect genomes and their phenotypic expressions. We have looked at global changes in gene expression in adult, female *An. gambiae* before and after a blood meal, and at differences between males and females and young and old mosquitoes. It is impossible to describe here the large number of genes regulated at each analysed time point and each biochemical pathway or biological processes in which they are involved. Thus, descriptions are based on groups of genes displaying major differences in transcript accumulation during the adult mosquito life. However, by using a publicly available searchable database (http://www.angagepuci.bio.uci.edu/), detailed analyses of specific groups of genes based on their descriptions, functions or levels of gene expression variation can be performed by any investigator according to need.

Results and discussion

Identification of An. gambiae-*regulated transcripts and assignment of biological functions*

The Gene Chip Plasmodium/Anopheles genome array (Affymetrix), which includes probe sets to 14 900 *An. gambiae* genes, was used to evaluate sex-biased gene expression in adult mosquitoes, changes in transcript accumulation following blood meals in females, and changes associated with ageing. The *An. gambiae* genome is estimated to have approximately 15 000 genes (Holt *et al.*, 2002), therefore this microarray platform represents a comprehensive tool for genome-wide enquiries of variations in gene expression in this species.

We developed a searchable database to facilitate the analyses and add biological meaning to the large amount of microarray-derived data. Consensus DNA sequences used for the microchip development (http://www.affymetrix.com/support/file_download.affx?onloadforward=/analysis/downloads/data/Plasmodium_Anopheles_consensus.zip) were used as input for BLAST searches performed locally from executables obtained at the NCBI FTP site (Altschul *et al.*, 1997). The electronic version of the

database complete with hyperlinks to web-based information and to BLAST results is available for download at http://www.angagepuci.bio.uci.edu/. Online searches also are available through this site. The annotation of the *An. gambiae* genome and transcriptomes, and consequently of the array elements, is an ongoing process, mostly due to low or lack of significant similarity of some *An. gambiae*-derived cDNA sequences to known genes and transcripts of other organisms. Approximately 35% of all probe sets correspond to transcripts that have no identified biological function and are without BLAST matches in gene ontology and protein families (PFAM) databases. Furthermore, in contrast to what is available for *D. melanogaster*, where a high proportion of sequenced genes are linked to a verified biochemical function or mutant phenotype, the putative functions assigned to *An. gambiae* gene products are based almost exclusively on comparative sequence similarity and conserved domains. Although comparative genomic analysis improves the *An. gambiae* gene annotation, functional genomics, proteomics and high-throughput gene expression studies are required to contribute and validate these predicted functions.

Sex-biased gene expression

We first compared in a global perspective the expression of genes in nonblood-fed (NBF) adult female mosquitoes (third day after emergence and fed only on raisins) with adult males (third day after emergence and fed on raisins). For this work, genes differentially expressed were defined by statistical analysis as those showing variations with *P*-values lower than 0.001.

A total of 3275 transcripts was identified as having gender-specific or preferential expression (Fig. 1 and Supplementary Material Table S1). Sex-biased gene expression also is significant in *Drosophila*, where 30–50% of the genes were found to be differentially expressed (Jin *et al.*, 2001; Arbeitman *et al.*, 2002; Ranz *et al.*, 2003). A similar analysis performed recently, comparing virgin males and females at 36 h posteclosion (Hahn & Lanzaro, 2005) found 2901 genes differentially expressed (*P* < 0.05). Our experiments used older mosquitoes that were allowed to mate, and this could account for the difference in number of sex-biased genes found in each study. Of a list of 167 highly significant sex-biased genes provided by Hahn & Lanzaro (2005), we confirmed all but one, Ag.3R.3557.0, which was more expressed in females (1.2-fold) in our experiment and less expressed in females (1.7-fold) in that Hahn & Lanzaro (2005).

Approximately 47% of 3275 genes that exhibit sex-differential expression accumulate transcripts at higher levels in males, while the balance are found preferentially or specifically in females. Five hundred of the transcripts identified as expressed preferentially or exclusively in
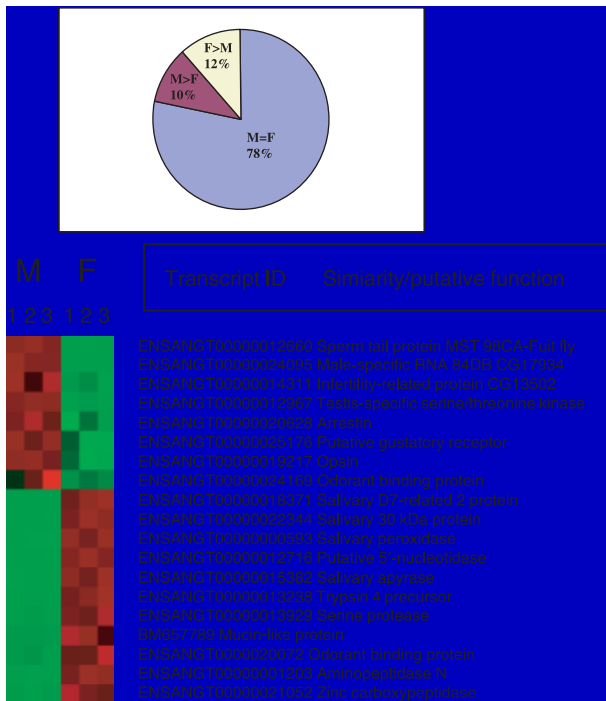
Figure 1. Sex-biased gene expression in adult *Anopheles gambiae.* (Top) Percentile representation of sex-biased gene expression of 14 900 *An. gambiae* transcripts. A total of 3194 probe sets, representing 3002 transcripts, are expressed at different levels (*P*-values < 0.001) in males and females (Supplementary Material Table S1). (Bottom) Selected genes displaying stronger accumulation in males (M 1–3) or females (F 1–3). The numbers 1, 2 and 3 represent triplicate samples for each sex. Transcript ID and annotation were obtained from the *An. gambiae* microarray database (http://www.angagepuci.bio.uci.edu/). Expression data for each probe set were normalized and organized according to preferential accumulation in males or females. Tree View software was used and red and green represent higher and lower relative expression, respectively.

males correspond to transcripts that were not present in a set of 80 000 *An. gambiae* adult, female-derived expressed sequence tags (ESTs) (Ribeiro, 2003), providing additional support for them being male-specific. The majority of genes identified as expressed preferentially in males but with some representation in the female EST database had low numbers of sequenced ESTs. For example, 225 ESTs were represented only once and 1187 had less than five sequenced cDNAs. Genes represented in the male-enhanced transcript list (Fig. 1 and Supplementary Material Table S1) include several putative testis and sperm components such as those similar to the *D. melanogaster* male-specific RNA 84DB, a spermiogenesis-associated testis specific serine/threonine protein kinases expressed in *Homo sapiens* and *D. melanogaster*, an infertility-related sperm protein, a protein with sequence similarity to sperm-specific thioredoxin 2 of *H. sapiens* and other sperm- and spermatogenesis-associated transcripts.

Several sensory reception-related transcripts such as an arrestin, opsin and a gustatory receptor also were identified

as having male-enhanced expression (Fig. 1). Genes encoding olfaction-related proteins expressed preferentially in males are probably involved in locating nectar sources for feeding or females for mating (Biessmann *et al.*, 2005). Thirty-four transcripts with gene ontology annotation related to translation and transcription regulation also were enhanced in males, and these may be involved in controlling the gender-related differential gene expression patterns.

Female-enhanced transcripts found prior to blood feeding (Fig. 1 and Supplementary Material Table S1) correspond to a series of salivary gland components, digestive enzymes and ovary-expressed genes. A total of 48 salivary gland-expressed transcripts showed female preferential expression. Among these, several were known previously to be expressed preferentially in females such as members of the *D7-related* group, an apyrase, and a salivary peroxidase (Arca *et al.*, 1999). This gender- and tissue-specific expression has been interpreted to indicate that the products of these genes represent adaptations of mosquitoes to blood feeding. Accordingly genes equally expressed in the salivary glands of both sexes, such as *Maltase like I* and *AMY I* of *Aedes aegypti* and their homologues in anopheline mosquitoes are probably involved in sugar or nectar ingestion and digestion (Francischetti *et al.*, 2002; Calvo *et al.*, 2004).

Midgut-expressed enzymes involved with blood digestion generally are synthesized after a meal is ingested. However, some of them are present in the midgut before the blood meal and participate in the initial steps of digestion, presumably providing signals for the midgut cells to produce the large bulk of digestive enzymes needed during the process. Several proteins, including digestive trypsin and peritrophin, are known to be stored prior to a blood meal in secretory vesicles of midgut epithelial cells (Devenport *et al.*, 2004). Accordingly, we find several transcripts encoding serine proteases, trypsin and chymotrypsins, aminopeptidases, and carboxypeptidases in NBF females. Transcripts corresponding to components of the peritrophic matrix (a chitin/protein-containing acellular sheath that surrounds the blood meal and separates the food bolus from the midgut epithelium) are also found in the NBF mosquitoes.

The behaviour of mature adult males is dictated mostly by the requirements of mating. They feed solely on sugar sources (nectar and fruit sources), and their digestive system is not adapted to respond to a blood meal. Their reproductive system continuously produces sperm and does not undergo substantial changes after adult emergence, and their flight-activity periodicities and responses to environmental stimuli do not change until the effects of ageing are noticeable (Clements, 1999). While the physiology of males does not change much during adult life, females go through extensive changes after taking a blood meal (Holt *et al.*, 2002; Ribeiro, 2003; Dana *et al.*, 2005).
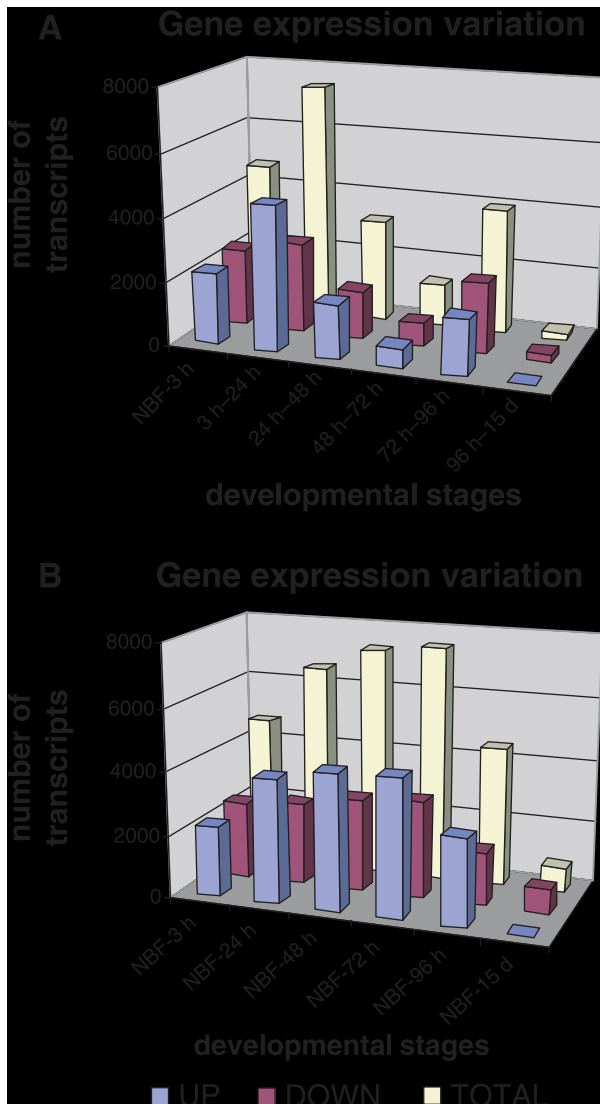
Figure 2. Numbers of *Anopheles gambiae* transcripts up- or down-regulated significantly (*P*-values < 0.001) in females with and without a blood meal. The numbers of varying transcripts were calculated by comparison of each sample with its immediate subsequent experimental sample [nonblood-fed (NBF)] (A) or by comparing each time point with three-day-old NBF females (B). Numbers of regulated transcripts were extracted from Supplementary Material Tables S1–25.

Figure 3. Global patterns of gene expression during oogenesis and ageing in *Anopheles gambiae* adult females. Each row represents data for one probe set and each column corresponds to a developmental time point, referred on the top as hours (h) or days (d) post blood meal (average of three determinations, and the data for each experiment and standard deviations are available as Supplemental Material Tables S2, S3, S14 and S15–23). Expression values are relative to the reference, 3-day post emergence nonblood-fed female. Red indicates higher and green indicates lower levels of transcript accumulation. Data were displayed using Tree View software. Details of the expression patterns of selected genes mentioned in the text are presented in Supplementary Material Fig. S1.

Global changes in transcript accumulation during a gonotrophic cycle

We examined variations of gene expression at several time points post blood meal (PBM) during a complete gonotrophic cycle, and also determined changes in ageing mosquitoes 18 days after adult emergence (Figs 2, 3 and 4). Gene expression at each developmental stage was compared with experimental time points immediately prior to and after it (Supplementary Material Tables S2–13),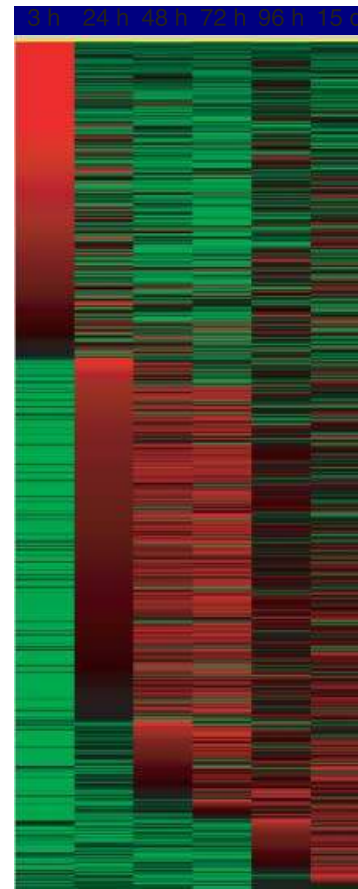 and all time points were compared to NBF females (Supplementary Material Tables S2, S3 and S14, S15–23), which we considered as the standard nonvitellogenic stage. As well as comparisons of transcript accumulation based on *P*-values (< 0.001) (Figs 2, 3 and Supplementary Material Fig. S1), genes expressed differentially at each stage of the gonotrophic cycle also were identified by ANOVA tests (Fig. 4 and Supplementary Material Tables S24–62). The following descriptions are based on groups of genes displaying major differences in transcript accumulation during the adult female mosquito life. The searchable database developed here (http://www.angagepuci.bio.uci.edu/) allows detailed analyses of specific groups of genes based on their descriptions, functions or levels of gene expression variation to be performed by any investigator in the research community.
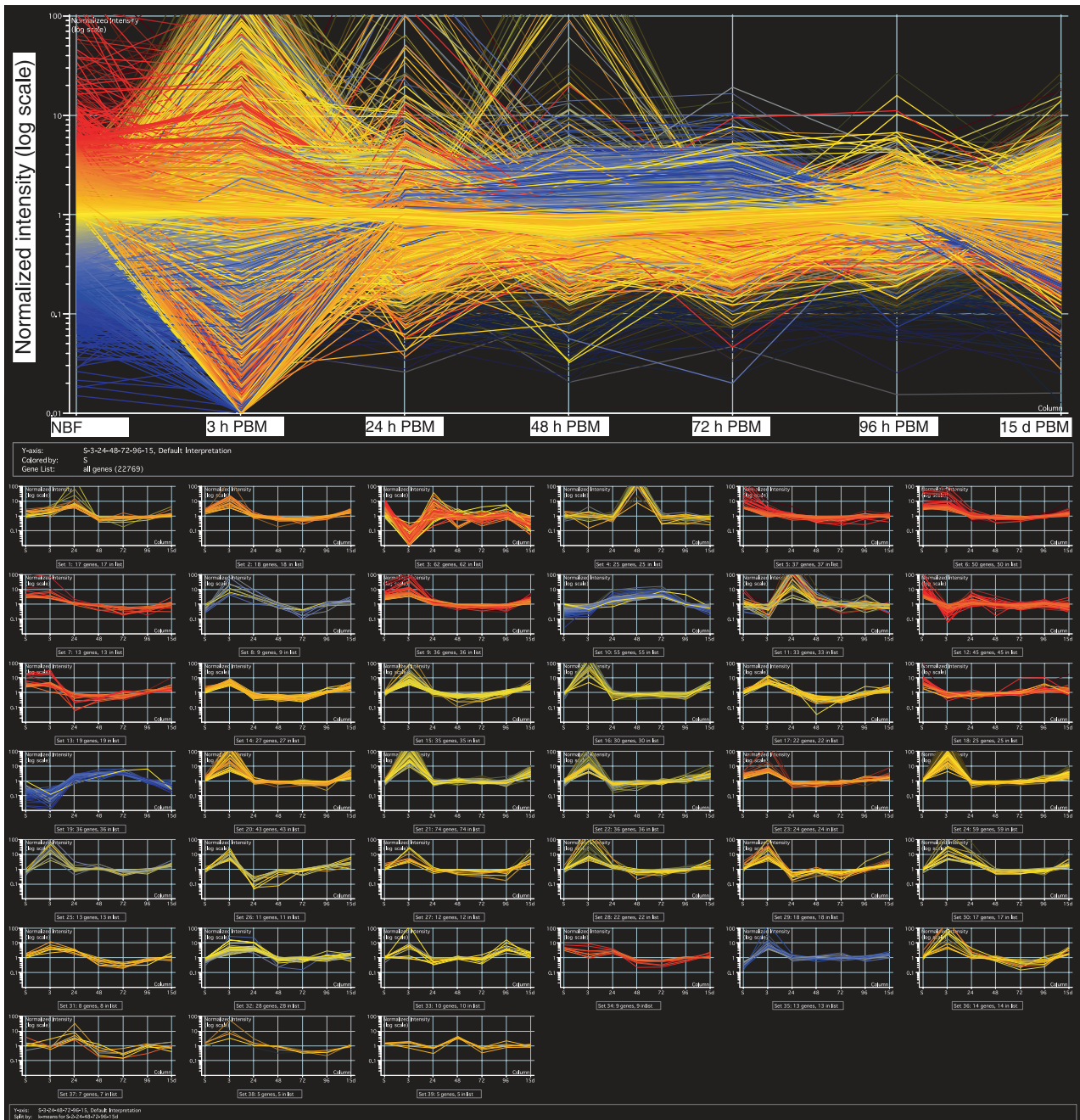
Figure 4. A Welch ANOVA-based statistical analysis of gene expression during mosquito development with multiple testing correction, Benjamini and Hochberg False Discovery Rate, with a 0.001 *P*-value cutoff. Probe sets corresponding to mosquito genes regulated upon blood feeding and ageing were identified and grouped for similarity patterns using the K-Means clustering method, generating 39 sets (hyperlinked lists of transcripts included in each set are in Supplementary Material Tables S24–63).

Extensive variation in gene expression was observed in blood-fed (BF) females (Figs 2−4). A total of 10 631 probe sets corresponding to 9724 transcripts were found to vary (*P*-values < 0.001) in accumulation during at least one of the analysed experimental time points. Of these, 5423 varied at least two-fold, and 2625 varied more than three-

fold (Supplementary Material Fig. S2). While the majority of transcripts varied less than three-fold, 126 transcripts were accumulated at levels at least 100-fold different from those detected in NBF females.

Thirty per cent (4697/14 900) of all analysed transcripts were regulated immediately after the females fed on blood

(3 h PBM), with 2254 up-regulated and 2443 down-regulated as compared with NBF females of the same age. The total number of genes regulated in BF females as compared with NBF females continued to increase during the three-day period that followed the meal, reaching a maximum of 7494 at the third day (72 h PBM), which corresponds to ~50% of all genes (Fig. 2B). The number of genes regulated decreased thereafter, coincident with the end of the two major processes occurring in mosquitoes, blood digestion and egg formation. Changes in transcript accumulation, particularly noticeable during the initial steps (NBF: 3 h PBM and 3–24 h PBM, Fig. 2B) of the gonotrophic cycle, were not as large after egg laying (which occurs between 72 and 96 h PBM), both in number of transcripts and fold-variation. These data represent the pattern of gene expression characteristic of mosquitoes that do not take a second blood meal during the gonotrophic cycle. *Anopheles gambiae* females may feed on blood several times during each gonotrophic cycle both in laboratory and field conditions, and each meal triggers again the expression and regulation processes of several genes involved in digestion and egg formation (Briegel & Horler, 1993; Koella *et al.*, 1998; Nirmala *et al.*, 2005). Therefore, the patterns of gene expression described here may not reflect what happens in nature.

Immediate responses to a blood meal

Detailed analyses of variation in gene expression at 3 h PBM revealed a large number of up-regulated digestive enzymes including those involved in proteolysis (trypsins, chymotrypsins, aminopeptidase, carboxypeptidase) and those related to lipid (lipases) and carbohydrate (α-glucosidases and α-amylases) digestion. These data are consistent with a prompt reaction of the midgut cells to a blood meal, resulting in the secretion of enzymes responsible for the digestive process. Other transcripts encoding molecules involved with the digestive process such as peritrophins and intestinal mucins, also are up-regulated at that time point.

Several of the salivary gland-expressed genes are regulated at higher or lower levels 3 h PBM. During probing the vertebrate host skin in search of a blood source, mosquitoes secrete saliva containing a variety of molecules with antihaemostatic activity. Following a blood meal, the secreted salivary components are reduced significantly (Marinotti *et al.*, 1990; Golenda *et al.*, 1995). All of the salivary components are synthesized *de novo,* however, the rates of salivary gland replenishment varies for different components (Marinotti *et al.*, 1990). Possibly some of the salivary components need to be restored quickly to pre blood-fed levels to allow mosquitoes to efficiently obtain a next meal, while others could recover at a slower rate without affecting the ability of the females to obtain further blood or nectar meals necessary for proper egg development.

Hormone-related transcripts change in abundance at 3 h PBM. The changes in expression of these genes are consistent with the known hormone accumulation patterns observed during mosquito adult life and particularly during production of the first cluster of eggs (Redfern, 1982; Lu & Hagedorn, 1986; Hagedorn, 2005). Ecdysone receptor transcripts are up-regulated in concert with the increasing concentration of 20-hydroxyecdysone that follows a blood meal. No experimental identification of juvenile hormone esterases from mosquitoes has yet been performed, however, transcripts encoding proteins similar to juvenile hormone esterases of other insects also are up-regulated, and their products putatively lead to a decrease in concentration of circulating juvenile hormone (Kamita *et al.*, 2003).

Down-regulation of transcripts related to vision, gustatory and olfactory reception and flight activity is already initiated at 3 h PBM. This is consistent with what was described in mosquitoes at 24 h PBM (Ribeiro, 2003) (Fig. 3). Opsins, arrestins and odourant-binding proteins involved with environment perception, and muscle structural components, flightin and troponin, as well as pyrroline-5-carboxylate reductase and proline oxidase involved in the utilization of the amino acid proline as an energy substrate during flight, are expressed at lower levels in BF mosquitoes. This is consistent with the inhibition of flight activity in BF mosquitoes (Jones & Gubbins, 1978). Some ovary-expressed genes, such as *oskar* and several immune-related transcripts, are also down-regulated during these initial steps of the gonotrophic cycle. We interpret this to indicate that most of the energetic and biosynthetic effort in mosquitoes is directed towards the initiation of an efficient digestive process, and this comes at the expense of other activities such as flight and ovary development. However, ovary development is quickly resumed as noted at 24 h PBM.

Blood meal digestion and yolk protein synthesis

Previous analyses of blood meal-induced changes in gene expression in *An. gambiae* were based on sequencing cDNA libraries constructed with RNA extracted from either NBF females or BF females at 24 h PBM, and these identified 168 (Holt *et al.*, 2002), or 435 (Ribeiro, 2003) gene transcripts to be significantly more or less transcribed in BF mosquitoes. The list of regulated transcripts at 24 h PBM determined in our study includes those previously described, but also is much larger, reflecting more sensitive detection limits of the techniques applied in our analyses. However, in spite of the different techniques, the expression profiles of 80% of regulated transcripts identified by those EST analyses are consistent with what is observed in microarray experiments (Marinotti *et al.*, 2005). A total of 6584 transcripts vary in concentration between the time mosquitoes ingest a blood meal and 24 h later (Fig. 2). While 3961 (~60%) are up-regulated, 2623 (~40%) are down-regulated at this physiological stage. Significant
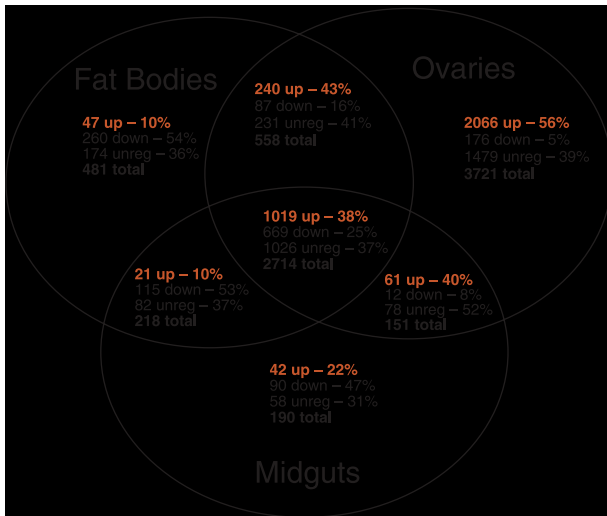
Figure 5. Three-way comparison of gene expression in fat bodies, ovaries and midguts of adult female *Anopheles gambiae* at 24 h post blood meal (PBM). Genes were considered as expressed in a tissue when its respective transcript was assigned as present by GCOS analysis in all three samples derived from that tissue. Up-, down- or no regulation where derived from the comparisons of transcript accumulation between nonblood-fed and 24 h PBM females performed with whole mosquitoes (http://www.angagepuci.bio.uci.edu/).

differences in BF females, 24 h PBM, reflect a continuation of the variations seen at 3 h PBM with a qualitative and quantitative decrease in transcripts associated with locomotion and response to environment stimuli, and present new trends such as the activation of secretory activity by the midgut cells (which results in the formation of the peritrophic matrix and accumulation of digestive enzymes in the lumen of the midgut), activation of fat body cells (responsible for the accumulation of lipid reserves and synthesis of vitellogenic proteins) and progress of oogenesis. The set of genes up- or down-regulated at 24 h PBM is therefore not identical to those regulated earlier at 3 h PBM. Several of the digestive enzymes expressed abundantly earlier during digestion are now repressed and replaced by different enzymes. This phenomenon is consistent with the two-phase blood digestion process described previously wherein early serine proteases, expressed prior to or immediately subsequent to a blood meal and related to the initiation of the digestion, generate signal molecules that in turn modulate mosquito-wide physiological changes, leading to the completion of digestion and egg production (Barillas-Mury *et al.*, 1995; Muller *et al.*, 1995). Vitellogenin, vitellogenic cathepsin B and other transcripts either not seen as up-regulated or regulated at low levels at the beginning of the gonotrophic cycle are up-regulated remarkably at 24 h PBM. The accumulation of large quantities of these transcripts and their translation result in massive yolk protein synthesis and deposition in

fast growing oocytes. These proteins are deposited in granules and serve as amino acid reserves for future embryonic development (Ahmed *et al.*, 2001; Raikhel *et al.*, 2002).

Spatial distribution of gene expression in gonotrophic females

The accumulation of specific transcripts in isolated fat bodies, midguts and ovaries of females at 24 h PBM was also determined (Fig. 5). This time point was selected for the extensive changes in gene expression occurring in vitellogenic mosquitoes, and the selected tissues are involved in intensive protein synthesis associated with blood digestion and egg formation as discussed above. We identified 2714 genes expressed in all three tissues and corresponding to housekeeping genes, and generated lists of genes expressed exclusively in fat bodies (481), ovaries (3721) or midguts (190) (Supplementary Material Tables S63–65). Vitellogenin, vitellogenic cathepsin B, hexamerin and lipophorin are proteins known to be expressed in the fat bodies of mosquitoes (Zakharkin *et al.*, 1997; Sun *et al.*, 2000; Raikhel, 2004) and this is confirmed by our analysis. Similarly, several serine proteases, aminopeptidases, perithophin, the microvillar protein G12 and other genes previously known to be expressed in midgut cells (Muller *et al.*, 1993; Vizioli *et al.*, 2001; Xu *et al.*, 2005), were confirmed by our results. The ovaries have the largest number of tissue specific transcripts. These components include a vast assortment of RNA species that support early embryonic development. For example, the dorsal/ventral and anterior/posterior axes of the future embryo are laid down during oogenesis (Goltsev *et al.*, 2004). The somatic follicular epithelium surrounding each egg chamber is also an important source of structural proteins for the eggshell. Analysis of other dissected tissues should provide an account of all genes expressed in the gonotrophic females and provide further support to the tissue specificity indicated by the partial analysis presented here.

Completion of the gonotrophic cycle

After an impressive change in the pattern of gene expression during the first and second days PBM, quantitative variations between the second and third days are less noticeable (Fig. 2B). However, important qualitative changes occur (Figs. 3 and 4). At 48 h PBM, those genes determined as strongly up-regulated at 24 h PBM, including vitellogenin, vitellogenic cathepsin B and several digestive enzymes, are down-regulated and return to lower levels similar to those seen in NBF females. However, other genes are up-regulated at this stage. Several odourant binding protein transcripts are up-regulated between 24 and 48 h PBM, and they may represent females regaining full capacity to respond to host odours and/or their ability to search for cues that will lead them to oviposition sites. A third group of genes regulated at 48 h PBM includes

those with sequence similarity to chorion genes of other insect species and have an expression pattern consistent with their function as components of the mosquito eggshell.

A new increase in the number of regulated transcripts is observed between 72 and 96 h PBM corresponding to oviposition and the return of the female to a resting or previtellogenic stage. Several of the transcripts seen as up-regulated at these latter time points represent residual transcripts of genes with strong expression earlier during the development. For example, vitellogenin is up-regulated more than 100-fold at 24 h after feeding but only two-fold at 72 h PBM. Other genes have their transcripts accumulated to the maximum level at 72 h PBM. This group includes those with sequence similarity to the *bicaudal*, *exuperantia*, *bicoid*, *hunchback*, *nanos* and *oskar* genes of *D. melanogaster*. These fruit fly maternally-expressed mRNAs are deposited in the oocytes and are involved with the determination of antero-posterior axis and segmentation of the developing embryos (St. Johnston & Nusslein-Volhard, 1992). Their orthologous genes in *An. gambiae* exhibit similar patterns of expression and are considered to be involved in the same biological processes (Goltsev *et al.*, 2004; Calvo *et al.*, 2005). The diminished amount of these transcripts at 96 h PBM is consistent with their proposed functions, reflecting the oviposition of the first cluster of eggs and a cessation of follicular development.

Ageing in adult mosquitoes

We compared patterns of transcript accumulation in mosquitoes at 15 days PBM with mosquitoes of the same age (18 days) that had never been fed on blood, with 3-day-old NBF mosquitoes, and with females at 96 h PBM. There is little variation in gene expression between 96 h PBM females and 15-day PBM females, and among these two samples and 18-day-old females that never took a blood meal. These comparisons show only three up-regulated transcripts between 96 h PBM and 15 days PBM samples corresponding to a putative infection-responsive short peptide, a putative chitinase expressed in response to bacteria and pathogen infection, and another similar to the *D. melanogaster* gene *CG16995* that has been annotated in the gene ontology database as a molecule related to the defence response to biotic stimuli. Therefore, these genes could represent the response of mosquitoes to environmental contamination (fungi and bacteria) instead of true ageing (senescence). The down-regulated genes correspond to a variety of components including mitochondrial proteins, salivary gland proteins, fat body-expressed molecules, and digestive enzymes. However, the down-regulation is in most cases relatively minor, with a more than 10-fold variation in transcript accumulation occurring for only 13 transcripts (Supplementary Material Tables S12 and S13).

Considerable variation was observed in comparisons of NBF females (third day after emergence) and 18-day-old adult NBF or BF females (Supplementary Material Tables S22 and S23). In this comparison, it is still evident that only 10 genes are up-regulated in older mosquitoes while a group of 768 genes have their expression levels decreased. In addition to ageing, these variations may reflect the nutritional status of the mosquitoes that have not been fed on a second blood meal.

In *D. melanogaster*, senescence is characterized as a dynamic process, with approximately 2% of transcripts changing in their representation with age (Zou *et al.*, 2000). However, when reproduction-associated female-specific changes in gene expression also are considered, this percentage increases to nearly 23% of the genome (Pletcher *et al.*, 2002). Changes in gene expression were analysed during the lives of mice and rhesus monkeys, indicating, respectively, that roughly 2 and 6% of the genes are regulated with age. According to our analyses, 5% of the mosquito genome has its expression modulated during the ageing process. Our comparison of nonvitellogenic female mosquitoes at 3 and 18 days after emergence was designed to eliminate most of the reproduction-related gene expression effects. Therefore our result (5% of the mosquito genome varying in expression in an age-dependent manner) agrees with the data reported previously for other organisms.

An important consideration for investigations of gene expression regulation during the ageing process is that diets imposing calorific restriction are accompanied by a slowing of the age-related changes in transcript levels in *D. melanogaster* (Pletcher *et al.*, 2002). A detailed comparison of the *An. gambiae* genes regulated during ageing and/or restricted nutritional status with those identified in *D. melanogaster* is yet to be performed.

Constitutive and abundant expression in adult mosquitoes

An interesting subset of *An. gambiae* genes is that represented by the most abundantly and constitutively expressed transcripts. These genes were identified by the sum of all signal values determined throughout mosquito development, creating a list from which the 200 genes showing the strongest signals and constitutive expression patterns in mosquitoes were analysed further (Fig. 6 and Supplementary Material Table S66). Forty-eight per cent of these genes correspond to transcripts related to protein synthesis and modification, with the majority encoding ribosomal proteins. Other transcripts related to protein synthesis, including translation initiation factors and elongation factors as well as proteins of the transcription machinery (RNA polymerase II) and RNA editing (splicing factor), are also found. Genes encoding mitochondrial proteins, including ADP/ATP translocase, ATP synthase and cytochrome *c* oxidase, are among those strongly expressed and correspond
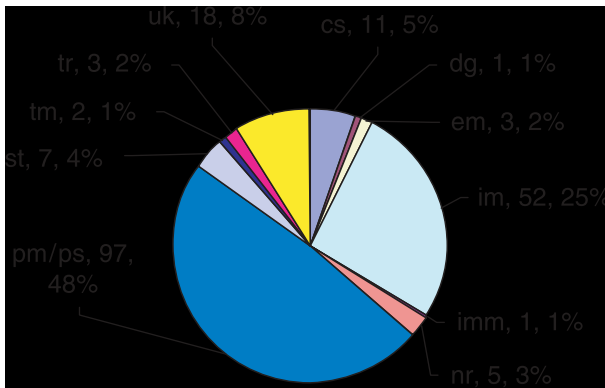
Figure 6. Functional distribution of 200 abundantly expressed transcripts of *Anopheles gambiae* mosquitoes. The sequences were classified according to their functions related to: cs, cytoskeleton (including muscle related proteins); dg, digestion; em, extracellular matrix; im, intermediate metabolism; imm, immunity; nr, nuclear regulation; pm/ps, protein synthesis and modification; 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. Details in Supplementary Material Table S64.

to the second most abundant group at 25% of the 200 selected genes. It is interesting to note that, despite variations of these mitochondrial products during the gonotrophic cycle, overall they remain among the most abundant products in mosquitoes. Actin, myosin and α-tubulin transcripts are also found in this group, and this is consistent with their ubiquitous expression and participation in the cytoskeletal organization of cells. A cecropin expressed abundantly in mosquitoes may reflect the continuous response of the mosquitoes to environmental pathogens. The *ferritin* gene is also expressed abundantly throughout adult life and there is no evidence of its regulation upon blood feeding. In contrast, ferritin is up-regulated after blood feeding in *Ae. aegypti* (Geiser *et al.*, 2003), therefore either *An. gambiae* responds differently to the load of iron ingested with the blood, or the induction of *ferritin* gene expression occurs shortly after the meal and the peak of expression was not detected within the sampling intervals used here. Arrestins and opsins are important and abundant components of the visual system of insects (Graf *et al.*, 1996) and are expressed continually at high levels. Polyubiquitins also are expressed abundantly in mosquitoes. These genes encode precursor molecules that are cleaved in the cytoplasm to generate ubiquitin monomers that are involved in numerous cellular functions, including chromatin structure modification, DNA repair, cell cycle modulation, protein degradation and stress response. The *polyubiquitin* gene of *An. gambiae* is expressed constitutively in larvae, pupae and adults, and shares high identity at the DNA sequence level to polyubiquitins of various organisms, ranging from 75.9% in *Saccharomyces cerevisiae* to 85.5% in

D. melanogaster (Beard *et al.*, 1996). Because of its strong and continuous expression, the *D. melanogaster polyubiquitin* gene promoter has been used in insect transgenesis experiments. Genetically transformed insects, including *An. albimanus,* carrying a chimeric polyubiquitin promoter-DsRed1 transgene express the marker protein throughout development (Handler & Harrell, 1999; Perera *et al.*, 2002; Allen *et al.*, 2004). Genes encoding members of a second group of ubiquitin molecules that fuse to ribosomal proteins are also among the strongly expressed genes in *An. gambiae*. These *ubiquitin fusion* genes are involved in ribosome biogenesis (Finley *et al.*, 1989). Interestingly, 14 genes abundantly expressed in *An. gambiae* are related to products with unknown functions. They have no significant match in BLAST searches to proteins with known functions (nonredundant database) or conserved domains [gene ontology (GO) and PFAM databases]. It will be important to determine the biological functions of these molecules.

Final comments

In conclusion, this study exploited a commercial DNA microarray platform to identify differentially expressed genes during the adult life of *An. gambiae.* The embryonic, larval and pupal stages are yet to be studied and the determination of gene expression profiles in whole insects at all these stages, plus the determination of expression patterns in specific organs and cells will provide a comprehensive knowledge of transcription in *An. gambiae*. Further studies are needed to define the relationships among these genes and their products that are involved in mosquito development and reproduction. Although much of this analytical work is descriptive, the output is rich in identifying many new gene products that may be associated with fundamental aspects of *An. gambiae* biology. Acknowledging that microarray derived results must be taken with caution, the database that we are providing, which includes gene annotations and expression patterns for 14 900 *An. gambiae* genes, is a valuable tool for researchers working in all fields of malaria vector biology.

Experimental procedures

Mosquitoes

The Pink-eye strain of *An. gambiae* (Githeko *et al.*, 1992) was maintained at 25 °C, 75–85% relative humidity and a 18/6 h light/dark cycle. Larvae were fed on finely powdered fish food (Tetramind, Tetra Werke, Germany) mixed 1 : 1 with yeast powder. Adults (males and females) were kept in cages with access *ad libitum* to raisins and water. For blood feeding, adult female mosquitoes were fed on mice anaesthetized with a mixture of ketamine and xylazine. Males and females were kept together in the cage until the blood meal. Blood-fed females were transferred to a separate cage and kept without males for the remaining

duration of the experiment. The transferred females were kept with access to raisins and water and were offered a cup with water as oviposition site at all times. Egg laying was observed between 72 and 96 h PBM. Three samples composed of five mosquitoes each were collected for each experimental time point and the RNA was immediately extracted.

Target preparation/processing for GeneChip analysis

Isolated total RNA samples were processed as recommended by Affymetrix, Inc. (Affymetrix GeneChip Expression Analysis Technical Manual, Affymetric, Inc., Santa Clara, CA, USA). In brief, total RNA was initially isolated using TRIzol Reagent (Gibco BRL Life Technologies, Rockville, MD, USA) and purified further by passing it through an RNeasy spin column (Qiagen, Chatsworth, CA, USA). Eluted total RNAs were quantified and a portion of the recovered material adjusted to a final concentration of 1 µg/µl. Three independent samples were evaluated to each time point and each experimental sample was compared to a common reference [nonblood-fed females, 3 days after emergence (NBF)]. All starting total RNA samples were quality-assessed prior to beginning target preparation/processing steps by resolving a small amount of each sample (typically 25–250 ng/well) on to a RNA Laboratory-On-A-Chip (Caliper Technologies, Mountain View, CA, USA) that was evaluated on an Agilent Bioanalyser 2100 (Agilent Technologies, Palo Alto, CA, USA). Single-stranded, then double-stranded cDNA was synthesized from the poly (A)+ mRNA present in the isolated total RNA (10 µg total RNA starting material each sample reaction) using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) and poly (T)-nucleotide primers that contained a sequence recognized by T7 RNA polymerase. A portion of the resulting ds-cDNA was used as a template to generate biotin-tagged cRNA from an *in vitro* transcription reaction, using the BioArray High-Yield RNA Transcript Labeling Kit (T7) (Enzo Diagnostics, Inc., Farmingdale, NY, USA). 15 µg of the resulting biotin-tagged cRNA were fragmented to strands of 35–200 bases in length following prescribed protocols (Affymetrix GeneChip Expression Analysis Technical Manual). Subsequently, 10 µg of this fragmented target cRNA was hybridized at 45 °C with rotation 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 software (version 1.1.1, Affymetrix, Inc.) using default values (scaling, target signal intensity = 500; normalization, all probe sets; parameters, all set at default values).

Statistical analysis and data display

The signal values generated by GCOS 1.1.1 software enabled us to perform a Bayesian *t*-test using a web-based statistical analysis package, Cyber-T at http://visitor.ics.uci.edu/genex/cybert/ (Long *et al.*, 2001), and generate a list of probe sets with a *P*-value of less than 0.001. To monitor the expression of genes over the different experimental time points, an expression profile and a K Means clustering were carried out using the software package GeneSpring (Silicon Genetics, Redwood City, CA, USA) according to the supplier's recommendations. Cluster and Tree View softwares (Eisen *et al.*, 1998; http://rana.lbl.gov/EisenSoftware.htm) were used for data display and editing.

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Supplementary Material

The following material is available for this article online:

Tables S1–23. Statistical analysis of sex-, oogenesis- and ageing-related variations of transcript accumulation in *Anopheles gambiae* mosquitoes. Expression values (columns D-I) were used as input to a Bayesian *t*-test at Cyber-T (http://visitor.ics.uci.edu/genex/cybert/). The Cyber-T output including *P*-values (column AD) and fold variations (column AF) are presented for each probe set. Known or putative functions of genes represented by each probe set can be retrieved using the hyperlinks of the tables to access http://www.angagepuci.bio.uci.edu/

Tables S24–62. A Welch ANOVA-based statistical analysis of gene expression during mosquito development with multiple testing correction, Benjamini and Hochberg False Discovery Rate, with a 0.001 *P*-value cutoff was performed. Genes regulated during mosquito development were identified and grouped according to similar patterns of expression using the K-Means clustering method and Genespring software. A graphical representation of the clustering is displayed in Fig. 4 and the hyperlinked lists of probe sets included in each cluster included here.

Tables S63–65. Hyperlinked Excel spreadsheets containing the lists of genes expressed exclusively in the fat bodies (63), ovaries (64) or midguts (65) of mosquitoes at 24 h post blood meal (PBM). Genes were considered as having tissue specific expression when their transcripts were assigned as present (GCOS analysis) in all three samples of that tissue and absent from the samples of the other two tissues analysed. Transcripts present in more than one tissue but with more than 90% of the total signal in one of them were also assigned as tissue specific.

Table S66. Abundantly and constitutively expressed *Anopheles gambiae* transcripts were identified by the sum of all signal values determined by microarray experiments throughout mosquito development. The 200 transcripts showing the strongest sum of signals and constitutive expression were analysed further. Functional assignments were derived from the Annotated Gene Chip Plasmodium/Anopheles genome array database. The identified transcripts were classified according to their functions related to: cs, cytoskeleton (including muscle related proteins); dg, digestion; em, extracellular matrix; im, intermediate metabolism; imm, immunity; nr, nuclear regulation; pm/ps, protein synthesis and modification; st, signal transduction; tm, transcriptional machinery; tr, transporters; uk, unknown. Numbers of blood-fed and nonblood-fed expressed sequence tags were derived from Ribeiro (2003).

Figure S1. Global patterns of gene expression during oogenesis and ageing in *Anopheles gambiae* adult females. Selected genes mentioned in the text that undergo regulation in expression following a blood meal. (A) serine proteases; (B) other digestive enzymes, including salivary components; (C) oogenesis-related transcripts; (D) environmental perception-related transcripts and (E) muscle, hormone and transporter transcripts. The arrow indicates sequential time points identical those in Fig. 3. Transcript IDs and additional annotation was obtained from the microarray database at http://www.angagepuci.bio.uci.edu/

Figure S2. Distribution of transcripts showing statistically significant differences in accumulation (*P*-value < 0.001) following a blood meal, when compared with nonblood-fed females. Absolute values of fold variation were considered and the transcripts grouped according to their levels of fold variation. A total of 9724 transcripts varied in accumulation at least one of the times examined. Of these, 5423 varied two-fold or more and 2625 at least three-fold. Only 126 of the regulated transcripts varied more than 100-fold after a blood meal.

This material is available as part of the online article from http://www.blackwell-synergy.com